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Aspergillus Flavus reprogrammed morphological and chemical attributes of *Solanum lycopersicum* through *SlGSH1* and *SlPCS1* genes modulation under heavy metal stress

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ABSTRACT

Cadmium (Cd) and chromium (Cr) in agricultural soils is becoming a serious threat in tomato. The complete removal of these heavy metals (HMs) from contaminated soil is challenging. However, the use of plant endophytes might be beneficial. The pragmatic role of endophytic fungi *Aspergillus flavus* (Ch-01) in tomato under Cd and Cr stress was, therefore, investigated. *A. flavus* enhanced growth attributes in tomato seedlings under Cd and Cr stress. Besides, it remediated Cd and Cr toxicity by reducing their translocation from the roots to the upper part of the tomato plants. In fact, *A. flavus* associated plants developed tolerance against Cd and Cr toxicity via the expression of *SlGSH1* and *SlPCS1* genes. Both genes helped in metal chelation and mitigation of Cd and Cr toxicity. From the results, it is concluded that *A. flavus* is a plant growth promoting and HMs alleviating endophyte, hence it might be used as a biofertilizer.

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Introduction

Biotic and abiotic stresses are the main reasons that are responsible for the low food production around the globe. Among the abiotic stresses, heavy metal toxicity has shown a negative correlation with crop yield. In fact, the key sources of heavy metal contamination in soils are technological activities (such as production of plastics, textiles, microelectronics, wood preservatives; mining, mine waste, smelting, tailings) and agrochemical applications (pesticide, synthetic fertilizers and sewage irrigation) (Ma et al. 2011). The crops cultivated on such soils have damaged water uptake, transpiration and photosynthesis, which results in low crop yield (Bibi et al. 2018; Ikram et al. 2018; Qadir et al. 2020). Cadmium (Cd) and chromium (Cr) are one of the important elements that falls in the heavy metal group. Both elements may enter the soil through industrial wastes, fertilizers and insecticides (Ishaq et al. 2020). High water solubility and mobility of Cd and Cr in the agricultural soils makes them easy to enter the roots of the crops and translocate to various edible parts, leading to heavy metal toxicity (Groppa et al. 2012; Bibi et al. 2018; Qadir et al. 2020). Such toxicity can deter crop yield by effecting leaf and root growth, and inhibiting enzymatic activities (Nematshahi et al. 2012). As Cd and Cr are difficult to be biodegraded that is why they are very persistent in nature. The removal or transformation of Cd and Cr to less harmful forms is, therefore, necessary to sustain agriculture (Amari et al. 2014). Certainly, plants have their own defense mechanisms (including stress-related genes) in order to regulate heavy metal toxicity. For example, overexpression of GSH1, GSH2, PCS1, and PCS2 are known to increase heavy metal tolerance by enhancing the glutathione (GSH) and phytochelatins (PCs) contents (Gasic and Korban 2007; Brunetti et al. 2011; Kühnlenz et al. 2014). Moreover, PCs are known as heavy metal-tolerant peptides, which can be encoded by PCS1 (AT5G44070) and PCS2 (AT1G03980). Both genes were observed to be strongly induced in *Arabidopsis thaliana* upon exposure to heavy metal stress (Kühnlenz et al. 2014). Besides the induction of stress-responsive genes, endophytes can also help the host plant species to withstand various stresses, like heavy metal, drought, high temperature, and salinity (Jan et al. 2019; Khushdil et al. 2019; Muhammad et al. 2019; Nusrat et al. 2019). In recent years, endophytes have attracted huge interest due to their role in host plant development and defense. Endophytes can contribute to plant hormones, enzymes, enhance nitrogen fixation and nutrient supply, and can act as biocontrol agents (Hamayun et al. 2017; Bilal et al. 2018; Kang et al. 2019; Singh et al. 2020; Ismail et al. 2020a). In addition to the plant growth-promoting potential, the endophytes are capable of heavy metal chelation and/or sequestration in contaminated soils (Zahoor et al. 2017; Qadir et al. 2020). Amongst the endophytes, various Aspergillus species (*Aspergillus japonicas, Aspergillus violaceofuscus*, *Aspergillus niger*, and *Aspergillus flavus*) have attracted plant biologists to explore their strong mutualistic associations with the host plants (Ismail et al. 2018; Ismail et al. 2019; Ismail et al. 2020b; Ismail et al. 2020c). Various species of Aspergillus have also shown their ability to secret sufficient amounts of secondary metabolites, including butenolides, alkaloids, terpenoids, cytochalasins, phenalenones,

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ρ-terphenyls, xanthones, sterols, diphenyl ether, and anthraquinone derivatives with diverse biological (El-hawary et al. 2020).

Quite recently, it has been noticed that the main crops of the Mardan region have been significantly affected by the HMs due to fast-growing industrialization (Amin and Ahmad 2015). The study was further confirmed by Hussain et al. (2013), who showed that the people of Mardan and nearby areas are exposed to HMs through consumption of contaminated vegetables, especially *S. lycopersicum*. Keeping the potential role of endophytes in agriculture, and health of the Mardan community in view, we investigated the role of endophytic fungi (*A. flavus*) in countervailing Cd and Cr stress in *S. lycopersicum*. To the best of our knowledge, the Cd and Cr stress-alleviating potential of *A. flavus* has not been investigated till now. Hence, we explored the effect of *A. flavus* on physiological, biochemical parameters and gene expression of growing *S. lycopersicum*.

Material and methods

Chemicals and reagents were obtained from Merk (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany) and Fluka (Buchs, Switzerland). Molecular biological products were obtained from Qiagen (Hilden, Germany) and BIORON GmbH (Römerberg Germany). For RT–PCR analysis T100™ Thermal Cycler – Bio-Rad (USA) was used.

Isolation and culturing of endophytic fungi

For the isolation of endophytic fungi, *Capsicum annum* L. (chilli) was selected. Chilli plants were collected from three different sites of district Mardan. Plants were uprooted, enveloped in plastic bags and moved to PMI (Plant-Microbes-Interaction) laboratory, Department of Botany, Abdul Wali Khan University Mardan. The selected parts of the plant were washed under tap water to remove dirt. The dirt-free plant parts were surface sterilized by dipping them into 70% ethanol for 10 min and then rinsed with sterile distilled water to remove traces of ethanol. The intact plant parts were then checked for any contamination by using potato dextrose agar media (PDA). The non-contaminated sterilized plant parts, that is, leaves, stems and roots were cut into small pieces (0.5–1 cm) and kept on plates of autoclaved Hagem minimal medium (0.5% glucose, 0.05% KH_2-PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% NH_4Cl, 0.1% $FeCl_3$, 80 ppm streptomycin and 1.5% agar; pH 5.6 ± 0.2) at the rate of 5 pieces/dish. Different colonies of endophytes with different colors were observed in each plate after seven days of incubation in Hagem minimal medium. Each strain was picked and sub-cultured on PDA plates for obtaining their respective pure cultures. The purified strains were then stored in the refrigerator at 4°C till further use. The purified strains were added to the Czapek broth (50 ml) in 250 ml conical flasks. The flasks were incubated in a shaking incubator at 27°C and 140 rpm for 10–14 days to get fungal secondary metabolites. Czapek broth was composed of 1% glucose, 1% peptone, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.001% $FeSO_4{\cdot}7H_2O$; pH 7.3 ± 0.2.

Determination of fungal growth in HMs containing PDA medium

A total of 27 strains of fungal endophytes were isolated from *C. annum*. The strains were then re-cultured on PDA containing salts of heavy metals ($CdCl_2$, K_2CrO_4) in two different concentrations (100 and 500 µg/ml), respectively. The strains that entirely failed to develop on the PDA media supplemented with heavy metals were discarded, whereas the strains that showed growth were kept for further use.

Determination of fungal growth in HMs containing Czapek medium

Out of the total isolates, 12 resistant strains (labelled as Ch-01 to Ch-12) showed growth on HMs contaminated PDA medium and were selected for preliminary screening. Heavy metal's resistance of the endophytic fungal strains was further evaluated in terms of growth rate in heavy metal contaminated Czapek medium. The Czapek medium containing Cd or Cr at two different concentrations (100 and 500 µg/ml) in 250 ml flasks was used to test the tolerance level of the selected endophytes. The growth rate was noted after keeping the flasks in shaking incubator at 30°C and 120 rpm for seven days. A control experiment comprised of endophyte in the HMs free Czapek medium ran side by side for comparison. Fungal fresh biomass/flask of each culture was weighed with the help of electrical balance. The strain Ch-01 performed best under 100 and 500 µg/ml of Cd or Cr stress. The biomass and culture filtrate (CF) of the Ch-01 was then stored in the refrigerator at 4°C till further use.

Screening of fungal endophytes CF on seedlings of *Oryza sativa*

Growth promotion or inhibition potential of Ch-01 was observed by applying fungal CF to *O. sativa* seedlings under control as well as heavy metal stress conditions. For this purpose, healthy, mature, pathogen-free rice seeds of variety Kainat were used. Seeds were surface sterilized as described earlier and then soaked in distilled water for germination in the petri plates. The petri plates containing soaking seeds were incubated at 35°C for 72 h. Uniform sized seedlings were chosen and carefully transferred to the plastic pots (6 seedlings/pot) containing 30 ml of water–agar medium (0.8%). The pots were moved to the growth chamber for two weeks (Khan et al. 2011). Te conditions of the growth chamber were: relative humidity 70%; day/night cycle: (14 h – 28°C ± 0.3; 10 h – 25°C ± 0.3). The outline of the experiment was as follows:

Control 1 = Distilled water treated seedlings.

Control 2 = Czapek nutrient media treated (1 ml/pot) seedlings.

Fungal CF = Fungal CF (1 ml) was applied to the tip of each seedling at the 2-leaf stage.

Cd1 = 100 µg/ml Cd treated seedlings.

Cd2 = 500 µg/ml Cd treated seedlings.

Cr1 = 100 µg/ml Cr treated seedlings.

Cr2 = 500 µg/ml Cr treated seedlings.

Cd1+fungal CF = 100 µg/ml Cd stress with fungal CF (1 ml) was applied to the tip of each seedling at the 2-leaf stage.

Cd2+fungal CF = 500 µg/ml Cd stress with Fungal CF (1 ml) was applied to the tip of each seedling at the 2-leaf stage.

Cr1+fungal CF = 100 µg/ml Cr stress with fungal CF (1 ml) was applied to the tip of each seedling at the 2-leaf stage.

Cr2+fungal CF = 500 µg/ml Cr stress with fungal CF (1 ml) was applied to the tip of each seedling at the 2-leaf stage.

For each treatment four replicates were used. After seven days of fungal CF application, the effect of the fungal CF and HMs stress was determined in terms of root and shoot lengths and fresh and dry weights of the rice seedlings.

Quantification of HMs content by atomic absorption spectrophotometer

Endogenous content of Cd and Cr was determined in rice samples by the ICE 3000 atomic absorption spectrophotometer (Thermo Scientific, USA). For this purpose, the powdered rice samples were dried for 72 h at 70°C. For acid digestion, oven-dried samples (0.5 g) were taken in 25 ml beakers. To the samples, 4 ml of nitric acid (HNO_3) and 1 ml of perchloric acid ($HClO_4$) were added and the samples were left on bench top for an overnight incubation. On the next day, the samples were placed in the water bath for an hour to evaporate acids and the concentrated samples were filtered. Finally, the filtered samples were diluted to a final volume of 25 ml with distilled water. Samples were then carried to the Central Resource Laboratory (CRL), University of Peshawar and tested for the presence of heavy metals (Cd and Cr) on the atomic absorption spectrophotometer. Measurements of the metals were conducted in an acetylene/air flame, and the operational conditions were set according to the manufacturer's guidelines.

Preservation of best growth promoting and HMs resistant strain

Ch-01 fungal strain with the best rice growth promotion potential was transferred to a 15 ml falcon tube containing glycerol (50%) under sterile conditions for future use and identification of the fungal species. The properly sealed glycerol stocks were kept in −80°C freezer as well as 4°C refrigerator for further use (Stein et al. 2008).

Extraction of DNA from the selected strain

DNA extraction from the selected strain was carried out in accord with the well-established protocol of Khan et al. (2008). The extracted DNA was then amplified by PCR. Quantity of the extracted DNA and its purity were measured by Termo Scientifc Nano Drop spectrophotometer at 260 nm.

Identification of the selected strain

The selected endophytic fungal strain was identified by amplifying their ITS region of 18S rDNA with universal primers, ITS-1 (5′-TCC GTA GGT GAA CCT GCGG-3′), and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Lee Taylor and Bruns 1999). The PCR reaction was performed in a 30 µl reaction mixture with 20 ng of genomic DNA as a template using EF-Taq (SolGent, Korea) as follows: Taq polymerase activation at 95°C for 2 min, 35 cycles of 95°C for 1 min, 55 and 72°C for 1 min each, terminating with a 10-min step at 72°C. The PCR products were then loaded onto an agarose gel along with DNA markers (DNA ladder) and subjected to electrophoresis for 30 min. The gel was formed using 0.01 g/ml ethidium bromide stain and studied under UV lamp. The same primers ITS-1 (5′-TCC GTA GGT GAA CCT GCGG-3′), and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to sequence the clone PCR fragment as described earlier (Rokhbakhsh-Zamin et al. 2011). By joining the reverse and forward reads using code codon aligner, the consensus sequence was created (version 7.2.1, Codon code corporations). The final sequence was used as a BLAST query in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) to search the closest matches in GenBank database. The closest matches were retrieved and analyzed to build a phylogenetic tree on MEGA 7 (version 7.0.18).

Bioassays on S. lycopersicum

Bioassay experiment was performed on seeds of *S. lycopersicum* cv. Rio grande, provided by National Agricultural Research Centre (NARC) Islamabad. The seeds were brought to the PMI lab and surface sterilized with 70% ethanol followed by rinsing thrice with sterilized distilled water. The sand was supplied to the PMI laboratory by the Mardan plant nursery, which was free from any heavy metal contamination. The sand was autoclaved at 121°C for 20 min to kill all microbes. For seed germination, cool-sterilized sand was shifted to plastic pots (250 g sand per pot). The pots were inoculated with fungal mycelium (2 g/100 g of sand) before seeds culturing (8 seeds/ pot). Pot experiment was conducted in triplicate with an experimental outline as;

Pot 1: Control1 (irrigated with DW)
Pot 2: Control2 (Czapek broth treated, i.e. 2 ml/100)
Pot 3; Fresh biomass of fungi (2 g/100 g)
Pot 4: Cd treated (100 µg/g)
Pot 5: Cd treated (500 µg/g)
Pot 6: Cr treated (100 µg/g)
Pot 7: Cr treated (500 µg/g)
Pot 8: Cd treated (100 µg/g) + Czapek broth (2 ml/100 g)
Pot 9: Cd treated (500 µg/g) + Czapek broth (2 ml/100 g)
Pot 10: Cr treated (100 µg/g) + Czapek broth (2 ml/100 g)
Pot 11: Cr treated (500 µg/g) + Czapek broth (2 ml/100 g)
Pot 12: Cd treated (100 µg/g) + Fresh biomass of fungi (2 g/100 g)
Pot 13: Cd treated (500 µg/g) + Fresh biomass of fungi (2 g/100 g)
Pot 14: Cr treated (100 µg/g) + Fresh biomass of fungi (2 g/100 g)
Pot 15: Cr treated (500 µg/g) + Fresh biomass of fungi (2 g/100 g).

The pots were kept in a controlled-environment with a long photoperiod (16 h light/8 h dark) at 23 ± 1°C. After three weeks from seed germination, Cd and Cr stress (500 µg/g of sand) were given in two steps with an interval of a week. After every three days till harvesting, distilled water was provided regularly. Plants were harvested after two months from seed germination. For all the samples, root and shoot length were calculated by a ruler, whereas

fresh and dry weights were recorded by an electrical balance and total chlorophyll content was determined using a chlorophyll meter (SPAD). The HMs content was determined via atomic absorption spectrophotometer (PerkinElmer 108 Lambda 35 UV/Vis Spectrophotometer) by well-established method of Zahoor et al. (2017). Part of the samples were also treated with liquid nitrogen and stored at −80°C for the determination of phytohormones and secondary metabolites.

Quantification of total soluble sugar content

Total soluble sugar content in fresh leaves extract of *S. lycopersicum* has been determined with slight modifications in the method of Mohammadkhani and Heidari (2008). Fresh leaves (0.5 g) were ground by a mortar and pestle. Then distilled water (5 ml) was added and the mixture was centrifuged at 4000 rpm for 5 min. In separate test tubes, 0.1 ml of plant samples were transferred and added 1 ml phenol (80%) to each. The samples were incubated for 10 min at room temperature. After incubation, 5 ml con. H_2SO_4 were added to eaSch sample and the samples were left on the bench top for one hour. The optical density of the samples was measured at a wavelength of 485 nm using a spectrophotometer (PerkinElmer Lambda 25 double beam spectrophotometer).

Determination of proteins content

For the determination of total soluble proteins in plant samples, the method of Lowry et al. (1951) was used with minor changes. Four types of reagents were made, Reagent A (2 g of Na_2CO_3, 0.4 g of NaOH, 1 g of $KNaC_4H_4O_6{\cdot}4H_2O$, 100 ml DW), Reagent B (0.5 g of $CuSO_4{\cdot}5H_2O$, 100 ml DW), Reagent C (50 ml of reagent A, 1 ml of reagent B), Reagent D (1:1 Solution of folin-phenol reagent + DW).

Leaves of *S. lycopersicum* (1 g/sample) were ground in phosphate buffer (1 ml) using mortar and pestle. The sample was centrifuged at 3000 rpm for 10 min. To the sample supernatant (0.1 ml) DW was added to set the volume 1 ml in a new test tube. Reagent C (1 ml) was added and the mixture was properly stirred for 10 min. Reagent D (0.1 mL) was finally added to the mixture and the mixture was left for 30 min. The optical density was determined at a wavelength of 650 nm. Folin reagent was used as blank.

Quantification of phytohormones and secondary metabolites in plant material

Determination of IAA

IAA was quantified using Salkowski reagent as described by the Benizri et al. (1998). Plant material (0.5 g) was crushed by mortar and pestle in 5 ml of distilled water. The solution was centrifuged at 10,000 rpm for 15 min. The collected supernatant (1 ml) from the sample solution was mixed with 2 ml of Salkowski reagent (Salkowski reagent = 1 ml of 0.5 M $FeCl_3$ + 50 ml of 35% $HClO_4$) in a test tube. The sample mixture was incubated for 30 min in the dark at room temperature. The optical density was taken at 540 nm using UV/VIZ spectrophotometer. Salkowski reagent (4 ml) was used as control.

Analysis of proline

Proline content was determined in fresh leaves of *S. lycopersicum* according to the procedure of Bates et al. (1973). Plant leaves (0.2 g) were crushed in 5 ml of 3% aqueous sulphosalicylic acid. The resultant solution was then centrifuged at 4000 rpm for 5 min. The supernatant from the sample solution was taken in a separate 10 ml test tube. A 2 ml of acid ninhydrin reagent (6 M of 20 ml H_3PO_4, 30 ml of CH_3COOH, 1.25 g of ninhydrin) was added to each sample. The mixture was heated for 60 min in a water bath. Toluene (4 ml) was applied to each sample after cooling. Layers of toluene were then separated with a dropper. OD was taken at 520 nm using UV/VIZ spectrophotometer. Toulene was used as a blank.

Determination of flavonoids

Flavonoids in samples were determined using the method of Khatiwora et al. (2010). Fresh tissues of *S. lycopersicum* (0.5 g) were crushed in 5 ml ethanol (80%) with the help of mortar and pestle. The crushed sample was transferred to the shaking incubator for 24 h at 120 rpm. After shaking, samples were centrifuged for 15 min at 25°C and 10,000 rpm. Supernatant (0.5 ml) was taken in 10 ml test tube and added 0.1 ml potassium acetate, 0.1 ml $AlCl_3$ (10%) and 4.3 ml of 80% methanol. The contents of the tubewere shaken vigorously and left for incubation at room temperature. After 30 min of incubation, the absorbance was measured at 415 nm using spectrophotometer.

Estimation of phenols

Total phenolics in leaves of plant samples were determined with the same method described by Khatiwora et al. (2010). Fresh tissues of *S. lycopersicum* (0.5 g) were crushed in 10 ml of methanol. The sample extract (2 ml) was added to 1 ml of distilled water to get a final volume of 3 ml. A 50% of Folin Ciocalteau reagent (0.5 ml) and 20% of Na_2CO_3 (2 ml) was added to the diluted extract. After the appearance of a blue color, the mixture solution was heated for 1 min, then cooled and the absorbance was measured at 650 nm.

Molecular study for gene-expression analysis in *S. lycopersicum*

In silico analysis for protein homology of γ-glutamylcysteine synthetase1 and phytochelatin synthase1

Orthologue screening for *γ-glutamylcysteine synthetase1* and *phytochelatin synthase1* encoding genes was carried out using plant genome database 'Phytozome' based on amino acid sequence similarity (Goodstein et al. 2012).

Plant material and growth conditions

For assessing molecular mechanism, another experiment on seeds of *S. lycopersicum* was carried out with the same procedure as described earlier. Plants were harvested after two months from seed germination and their fresh leaves were processed for total RNA extraction on the spot.

RNA preparation and cDNA synthesis

From *S. lycopersicum* seedlings, total RNA was extracted using the GeneJET Plant RNA Purification Kit (Thermo Scientific™) following the manufacturer's instructions. For digestion of contaminating genomic DNA, the DNase treatment was performed using RNase-free DNase obtained from TURBO DNase Kit; Ambion (Cambridgeshire, UK), during the isolation step. Using the RevertAid First Strand cDNA Synthesis Kit by Invitrogen (Karlsruhe, Germany), approximately 2 µg of total RNA was reverse transcribed.

Oligonucleotide sequence designing and synthesis

Gene-expression analysis of heavy metal stress-resistant genes *phytochelatin synthase1 (SlPSC1)* and *γ-glutamylcysteine synthetase1/glutathione* synthetase1 *(SlGSH1)* in *S. lycopersicum* was performed by primers designed through primer 3.0 (Untergasser et al. 2012). *ACTIN2 (Sl11g005330)* worked as an internal control and primer sequences were chosen from Klay et al. (2014). Primers were synthesized from Bio Basic (Korea). Primer list is mentioned in Supplementary Table 1.

Statistical analysis

All the experiments were done in triplicates. ANOVA was applied to analyze the data. The means that were significantly different from each other were further analyzed by DMRT (Duncan Multiple Range Test) at $p < .05$, via SPSS-20 (SPSS Inch., Chicago, IL, USA).

Results

Isolation of HMs resistant fungal isolates

A total of 27 strains were isolated from the *C. annum* (chilli), but only 12 strains (labelled as Ch-01 to Ch-12) showed resistance to heavy metal (Cd and Cr) stress (Results not shown).

Effect of HMs containing Czapek medium on growth of fungal isolates

After exposing to heavy metal (Cd and Cr) stress, the growth rate of Ch-02 and Ch-08 was very low, Ch-03 and Ch-05 grew moderately, Ch-06 and Ch-11 grew well, whereas the strain Ch-01 performed outstandingly (results not shown). In the stress-free conditions, the fresh biomass of the strain Ch-01 was 15.73 g, where a loss of 10.5% was observed after exposing the Ch-01 strain to 500 µg/ml of the Cr (Figure 1).

Effect of Ch-01 CF on the growth of O. sativa *seedlings under elevated Cd and Cr*

For further screening CF of endophytic Ch-01 was applied to *O. sativa* seedlings at two-leaf stage under elevated Cd and Cr. After harvesting, the effect of the fungal extract under HMs stress was determined by measuring the growth attributes of rice seedlings. Rice seedlings treated with fungal isolates Ch-01 showed maximum growth in a stress free as well as under elevated HMs as compared to the control seedlings (control1 = DW, control2 = Czapek treated). Maximum shoot length (18.7 cm) was observed in Ch-01 seedlings, whereas the shoot length in DW treated seedling was 13.7 cm and Czapek treated seedling was 14.4 cm. The decline in shoot length at elevated HMs (Cr 500 µg/ml) was also very low in Ch-01 treated seedlings (3.3%) as compared to DW (17%) and Czapek (15.2%) treated seedlings. Similarly, an increase in root length, fresh and dry weight of Ch-01 associated rice seedlings were noted in Cd and Cr stress-free environment. Moreover, the presence of Cd and Cr in the medium merely affected the growth parameters (root length, fresh and dry weight) of Ch-01 associated rice seedlings (Supplementary Table 2).

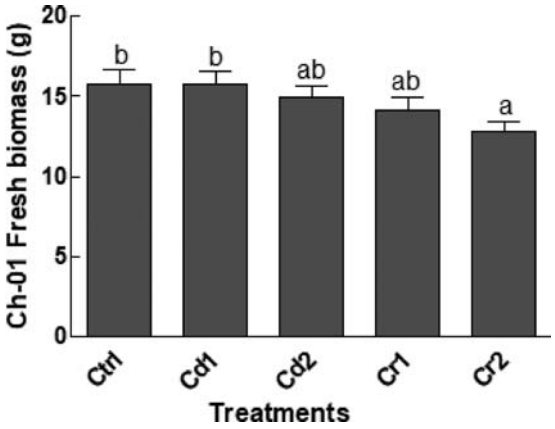

Figure 1. Ch-01 fresh biomass obtained in Czapek broth media containing Cd and Cr in different concentrations. Czapek broth media was placed in a shaking incubator (28°C, 120 rpm) for one week.

Influence of Ch-01 fungal CF on the accumulation of Cd and Cr in O. sativa *seedlings*

The effect of Ch-01 fungal CF on the Cd and Cr uptake and accumulation by rice seedlings was assayed (Supplementary Figurea 1(A and B)). The results revealed a significant ($p < .05$) reduction in HMs concentration was notice in seedlings treated with CF of Ch-01 as compared to CF free seedlings. A decrease of 70.67% and 70.44% in Cd accumulation, and 70.24% and 68.5% in Cr accumulation were found in seedlings that were treated with CF of Ch-01 in comparison to the seedlings that were not treated with CF of Ch-01 (Supplementary Figures 1(A and B)).

Identification of ch-01 strain

DNA sequence analysis was carried out for the molecular identification of selected endophytic fungi. The results revealed that the Ch-01 strain exhibited higher levels of ITS (18 S rDNA) sequence identity (98%) to *A. flavus*. When thirteen closely related sequences were subjected to phylogenetic analysis, the sequence of Ch-01 formed 98% clad with the sequences of *A. flavus* (Figure 2). The strain was thus identified and named as *A. flavus*. The sequence was submitted under accession No. MT462229 to NCBI GenBank.

Effect of endophyte *A. flavus* on growth attributes of *S. lycopersicum* under Cd and Cr stress

A significant ($p < 0.05$) increase in all growth parameters was observed in *S. lycopersicum* seedlings treated with the

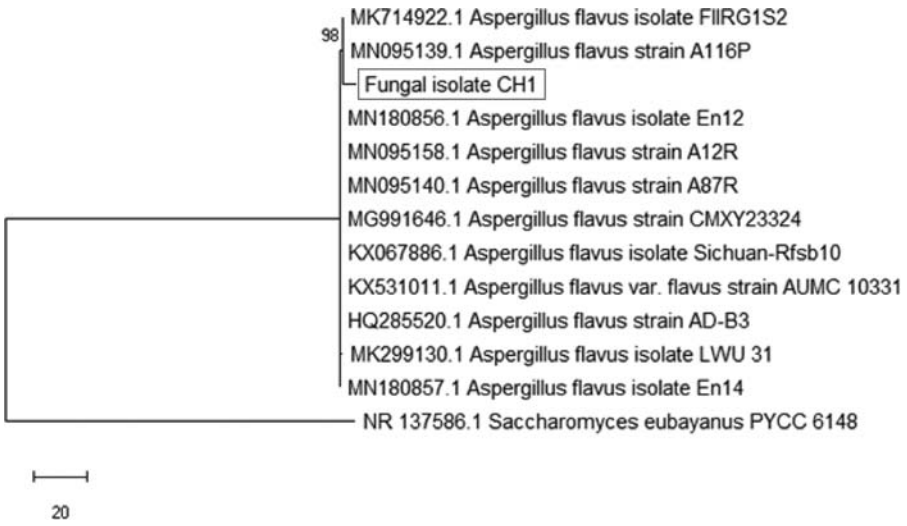

Figure 2. Maximum Parsimony analysis of Fungal Isolate Ch-1 (May be identified as *Aspergillus flavus*, as our isolate forms 98% clad with sequences of *A. flavus*).

biomass of *A. flavus* (Table 1). An increase of 16.13% was noted in shoot length of the endophyte associated *S. lycopersicum* seedlings as compared to non-inoculated seedlings. Also, with the application of Cd (100 and 500 mg/kg) and Cr (100 µg/ml and 500 µg/ml) showed declination in the shoot lengths of *S. lycopersicum* seedlings. However, *A. flavus* associated *S. lycopersicum* showed a less pronounced effect on the shoot lengths. Total chlorophyll contents of *S. lycopersicum* seedlings from DW and Czapek treatments were significantly lower as compared to the *A. flavus* associated seedlings under stress and stress free conditions. Moreover, results of root length, fresh and dry weights of *S. lycopersicum* were also influenced by the application of *A. flavus* under high levels of Cd and Cr (Table 1).

Influence of A. flavus *on Cd and Cr uptake and accumulation in leaf and root part of* S. lycopersicum

A. flavus decreased the toxicity of Cd and Cr in *S. lycopersicum* by decreasing their uptake and accumulation in the aerial parts of the seedlings (Figure 3). A significant reduction in Cd and Cr concentrations was found in leaves of *S. lycopersicum* associated with *A. flavus* as compared to the *A. flavus*-free seedlings. After the application of Cd (100 mg/kg, 500 mg/kg) and Cr (100 mg/kg, 500 mg/kg), *S. lycopersicum* seedlings took up HMs via roots and accumulated them in the upper parts of the plants, i.e. leaves. However, the amounts of Cd and Cr were significantly low in *S. lycopersicum* leaves after association with *A. flavus*. A decline of 68.4% and 70% in Cd concentrations at 100 and 500 mg/kg levels were observed in *A. flavus* associated *S. lycopersicum* leaves as compared to the *A. flavus*-free seedlings (Figure 3A). Similarly, *A. flavus* inoculated *S. lycopersicum* leaves accumulated 71.5% and 69.8% less Cr at 100 and 500 mg/kg levels as compared to the non-inoculated leaves (Figure 3 (B)). Contrary to these findings, HMs was more concentrated in *A. flavus* associated *S. lycopersicum* roots as compared to the *A. flavus*-free seedlings (Figure 3(C and D)).

Effect of A. flavus *and HMs on nutritive value of* S. lycopersicum

A. flavus inoculated *S. lycopersicum* seedlings had significantly ($p < .05$) higher level of soluble sugars compared to the control seedlings. After Cd and Cr stress, the sugars level of the *S. lycopersicum* seedlings declined sharply. However, this drop in sugar level was much

Table 1. Effect of fungal endophyte *A. flavus* on growth attributes of *S. lycopersicum* under elevated Cd and Cr stress.

Treatments		Total chlorophyll content (SPAD)	Shoot length (cm)	Root length (cm)	Fresh weight (gm)	Dry weight (gm)
Ctrl	DW	35.3 ± 0.02^{cg}	$13 \pm 1.1^{a-d}$	4.5 ± 0.1^{fg}	0.81 ± 0.01^{de}	0.2 ± 0.001^{fg}
	Czpk	36.0 ± 5.2^{dg}	$13.7 \pm 0.8^{a-d}$	4.6 ± 0.12^{fg}	0.85 ± 0.01^{e}	0.21 ± 0.01^{g}
	Ch-01	37.8 ± 0.06^{fg}	18.7 ± 6.1^{d}	5.8 ± 0.17^{h}	1.17 ± 0.01^{h}	0.28 ± 0.006^{i}
Cd_1	DW	$33.9 \pm 0.02^{b-g}$	11.8 ± 0.6^{abc}	4 ± 0.29^{cde}	0.74 ± 0.01^{c}	0.18 ± 0.002^{de}
	Czpk	$33.5 \pm 0.1^{a-f}$	$12.6 \pm 0.7^{a-d}$	4.3 ± 0.1^{def}	0.79 ± 0.01^{d}	0.2 ± 0.003^{ef}
	Ch-01	38.5 ± 0.12^{g}	17.4 ± 0.2^{cd}	5.7 ± 0.06^{h}	1.1 ± 0.06^{g}	0.27 ± 0.002^{i}
Cd_2	DW	$32.7 \pm 0.03^{a-e}$	10.7 ± 0.1^{ab}	3.1 ± 0.58^{ab}	0.71 ± 0.001^{bc}	0.18 ± 0.001^{cd}
	Czpk	$31.8 \pm 0.02^{a-d}$	10.9 ± 0.1^{ab}	3.4 ± 0.1^{abc}	0.68 ± 0.01^{b}	0.17 ± 0.01^{bcd}
	Ch-01	36.7 ± 0.03^{efg}	15.8 ± 0.2^{bcd}	4.7 ± 0.05^{g}	0.99 ± 0.001^{f}	0.25 ± 0.01^{h}
Cr_1	DW	31.3 ± 0.02^{abc}	10 ± 1.2^{ab}	3.6 ± 0.05^{bcd}	0.68 ± 0.005^{b}	0.16 ± 0.01^{b}
	Czpk	$32.1 \pm 0.01^{a-d}$	$12.7 \pm 0.1^{a-d}$	3.8 ± 0.1^{cde}	$0 .79 \pm 0.01^{d}$	0.2 ± 0.001^{ef}
	Ch-01	$32.1 \pm 1.2^{a-d}$	17.1 ± 0.6^{cd}	5.6 ± 0.17^{h}	1.1 ± 0.001^{g}	0.27 ± 0.003^{i}
Cr_2	DW	29.2 ± 0.03^{a}	9 ± 0.6^{a}	3.2 ± 0.12^{ab}	0.56 ± 0.01^{a}	0.14 ± 0.005^{a}
	Czpk	29.9 ± 0.01^{ab}	10.7 ± 0.03^{ab}	2.9 ± 0.03^{a}	0.67 ± 0.001^{b}	0.17 ± 0.006^{bc}
	Ch-01	$34.7 \pm 0.06^{c-g}$	15.7 ± 0.1^{bcd}	4.6 ± 0.03^{fg}	0.98 ± 0.01^{f}	0.25 ± 0.001^{h}

Notes: D.W: Distilled water; Czpk: Czapek broth medium; Ctrl = control (0 µg/mL Cd or Cr); Cd_1 = cadmium (100 µg/mL); Cd_2 = cadmium (500 µg/mL); Cr_1 = chromium (100 µg/mL); Cr_2 = chromium (500 µg/mL). Each value is Mean ± SD of three replicates with standard error. Different letters are significantly different ($p < .05$) as assessed by Duncan's Multiple Range Test (DMRT).

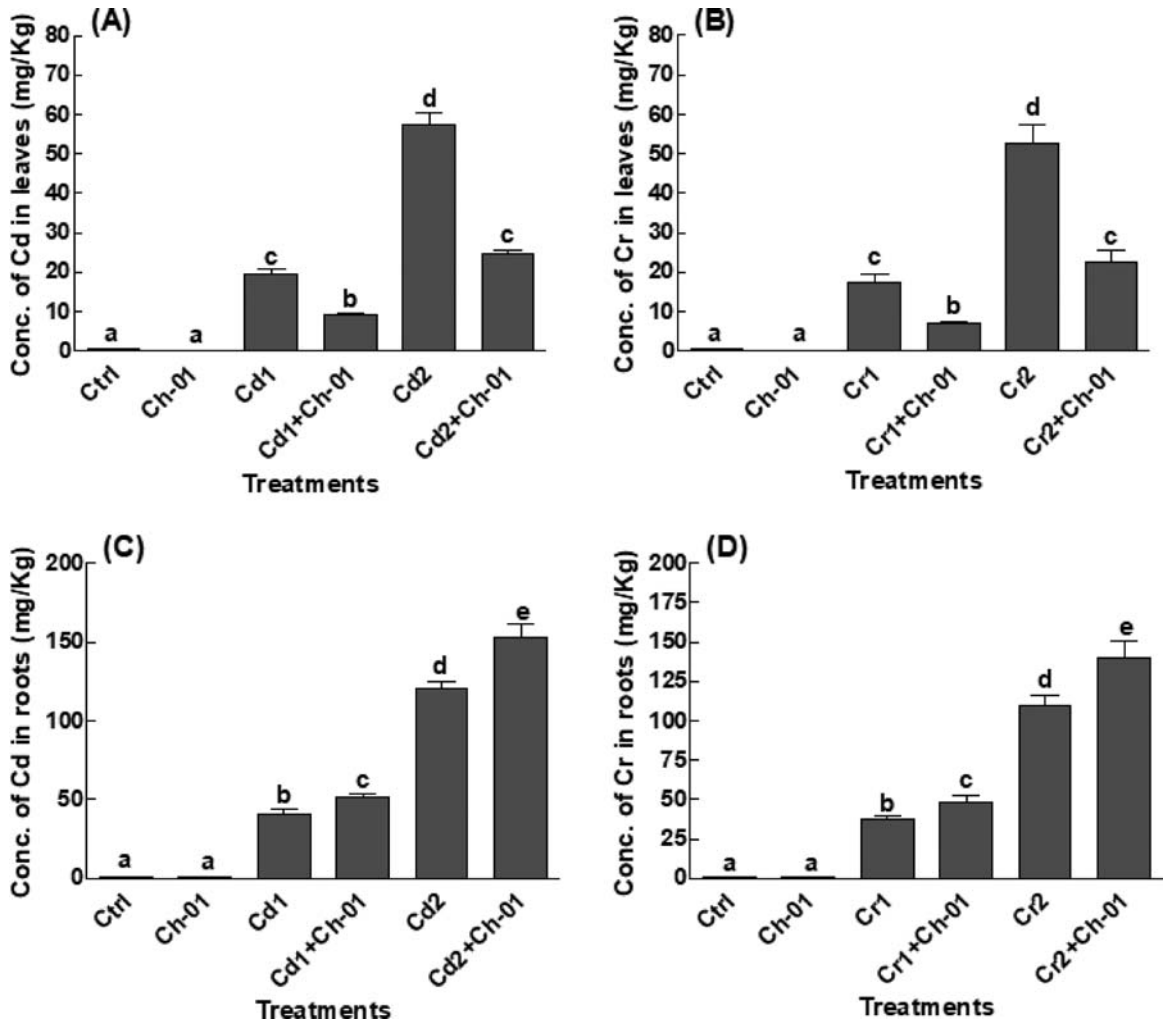

Figure 3. Effect of endophytic fungi Ch-01 (*A. flavus*) on endogenous Cd and Cr content under elevated cadmium Cd1 (100 µg/g), Cd2 (500 µg/g) and chromium Cr1 (100 µg/g), Cr2 (500 µg/g) stress. (A): Cd, Cr concentration in leaves of *S. lycopersicum* plant; (B): Cd, Cr concentration in roots of *S. lycopersicum* plant; Co: control; F: fungi. Data were statistically analyzed using DMRT at $p < .05$. Different letters are significantly different while error bars show standard error.

lower in *A. flavus* inoculated seedlings as compared to the control seedlings (Figure 4(A)). Parallel results have been noticed for total protein contents in *A. flavus* associated *S. lycopersicum* seedlings as compared to *A. flavus* free seedlings (Figure 4(B)).

Effect of A. flavus *and HMs on secondary metabolites of* S. lycopersicum

The levels of secondary metabolites in Cd and Cr treated *S. lycopersicum* seedlings significantly ($p < .05$) increased. However, the levels of secondary metabolites in Cd and

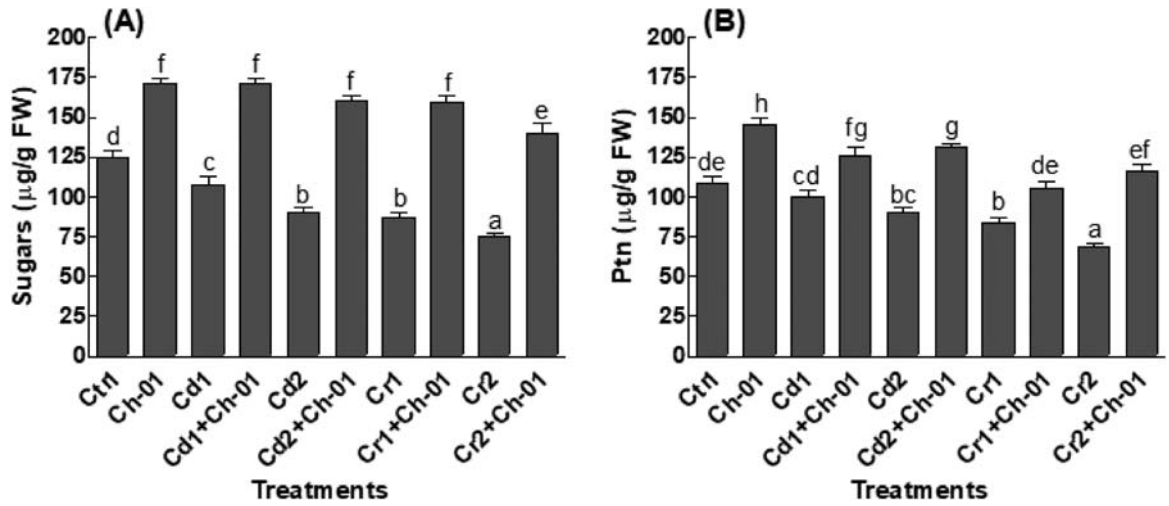

Figure 4. (A) Effect of *A. flavus* on concentration of soluble sugar under elevated Cd1 (100 µg/g), Cd2 (500 µg/g) and Cr1 (100 µg/g), Cr2 (500 µg/g) stress. (B) Effect of *A. flavus* on total proteins content under elevated Cd1 (100 µg/g), Cd2 (500 µg/g) and Cr1 (100 µg/g), Cr2 (500 µg/g) stress. Co: control. Data were statistically analyzed using DMRT at $p < .05$. Different letters are significantly different while error bars show standard error.

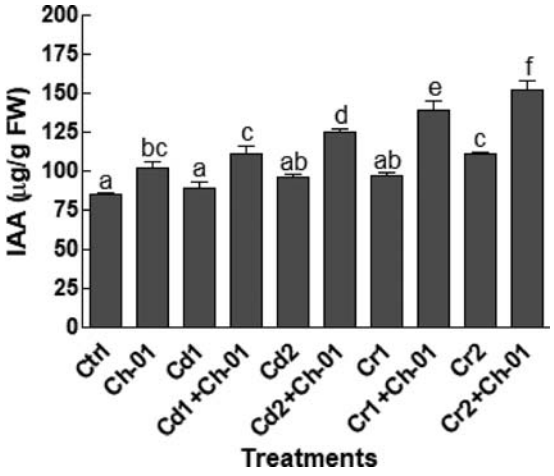

Figure 5. Effect of *A. flavus* on IAA content under elevated Cd1 (100 µg/g), Cd2 (500 µg/g), and Cr1 (100 µg/g), Cr2 (500 µg/g) stress. Co: control. Data were statistically analyzed using DMRT at $p < .05$. Different letters are significantly different while error bars show standard error.

Cr treated *A. flavus* associated seedlings were higher as compared to *A. flavus* untreated seedlings. Moreover, after exposure to Cd and Cr stress, the IAA level of the *A. flavus* associated *S. lycopersicum* seedlings were significantly ($p < .05$) boosted as compared to *A. flavus* free seedlings (Figure 5). The highest level of IAA (152.9 µg/g) was found in *A. flavus* inoculated *S. lycopersicum* seedlings exposed to 500 mg/kg of Cr. Likewise, high proline contents (15.2 µg/g) were observed in *S. lycopersicum* seedlings associated with *A. flavus* under 500 mg/kg of Cr stress (Figure 6(A)). A similar trend was observed for the flavonoids contents (Figure 6(B)). Also, a significant rise in phenolics (143.5 µg/g) was noted in *A. flavus* inoculated *S. lycopersicum* seedlings compared to *A. flavus* free seedlings under 500 mg/kg of Cr stress (Figure 6(C)).

Genes responsible for HMs detoxification and chelation mechanism

In silico identification of γ-glutamylcysteine synthetase1 and phytochelatin synthase1 orthologues

In silico analysis revealed that *S. lycopersicum* harbors an orthologue of *γ-glutamylcysteine synthetase1* encoded by *Solyc08g081010.2.1 (SlGSH1)* with 89.3% protein sequence homology and an orthologue of *phytochelatin synthase1* encoded by *Solyc09g072620.2.1 (SlPCS1)* with 82.9% protein sequence homology (Table 2).

Influence of endophytic fungi, A. flavus *inoculation on the gene-expression pattern in* S. lycopersicum *plants under elevated Cd and Cr*

Gene-expression pattern under elevated Cd and Cr showed a modulation in gene expression for *SlGSH1* and *SlPCS1* in *S. lycopersicum*, following the inoculation with Ch-01 (*A. flavus*) under control and Cd (500 µg/g) and Cr (500 µg/g) stress conditions. As an internal control, *Actin2* was used. *SlGSH1* and *SlPCS1* in *S. lycopersicum* were up regulated upon inoculation with *A. flavus* in the presence of Cd and Cr compared to non-inoculated and control plants (Figure 7). A working model is presented in Figure 8 to illustrate the possible role of *A. niger* in *S. lycopersicum* tolerance Cd and Cr stress.

Discussion

Heavy metals (HMs) are natural components of the earth crust, but their biochemical balance and geochemical cycles have been disturbed by an exponential increase in industrial, agricultural, domestic and technical activities (Topalidis et al. 2017). The accumulation of these toxic metals in plant might result from growing crops in HMs polluted soils (Sabir et al. 2014). The accumulation of HMs to toxic levels in plants may lead to low crop yield, which is alarming to the growing population of the world. In such circumstances, phytoremediation (plants employed for the elimination of HMs from the soil) and bioremediation (reclaiming contaminated soil with living organisms) are reliable and cost-effective techniques as compared to the traditional approaches (Bibi et al. 2018; Qadir et al. 2020). This situation, however, could be efficiently alleviated by exploring the plant-microbe partnership. Such partnership is not only essential for the survival of both partners, but it can play a vital role in agriculture, such as reduction of HMs toxicity in plants (Bibi et al. 2018; Qadir et al. 2020). Our results in table-1 showed that the isolated *A. flavus* is a growth promoter. After association with S. *lycopersicum* seedlings, *A. flavus* relieved the toxicity of cadmium and chromium. Also, *A. flavus* significantly reduced the adverse effects of Cr and Cd toxicity in *S. lycopersicum* through physiological, bio-chemical and by molecular adaptative strategies. The results also revealed that the uptake and accumulation of Cd and Cr were significantly low in leaves of *A. flavus* associated *S. lycopersicum* seedlings than in *A. flavus* free seedlings. This shows that *A. flavus* might helped the *S. lycopersicum* seedlings to avoid translocation of the HMs to the leaves and fruits of the plant species as observed by Bibi et al. (2018). It might also be possible that the HMs were retained by host plants roots, which inhibited the translocation of HMs to host plant shoots (Li et al. 2012). As roots are the primary site of the plants that are in direct contact with the soil, therefore, the toxic metals can accumulate here at first. Shahabivand et al. (2016) predicted about 3–4% of the total Cd can be translocated from the sunflower roots to the cells of shoots. We also observed higher amounts of HMs in endophyte associated roots of *S. lycopersicum* as compared to endophyte-free roots exposed to different concentrations (100 and 500 mg/kg) of Cd and Cr. Our results are consistent with the previous findings, where an increased accumulation of Cd in roots and decreased accumulation in leaves were observed in *P. indica* associated sunflower plants (Shahabivand et al. 2017). It might be possible that endophytes helped the host plants in the accumulation of HMs in root tissues than in shoot through extracellular or intracellular absorption mechanisms (Li et al. 2017). Also, huge accumulation of HMs in host plant roots might be attributed to Casparian strips of the root endodermis that might block the apoplastic pathways thus restricting the roots to translocate the HMs (Luo et al. 2014). Endophytic fungi may also be capable of locking metals by various mechanisms (including metal binding to extracellular polysaccharides, adsorption by cells, disposal to cell organelles such as vacuole, or by

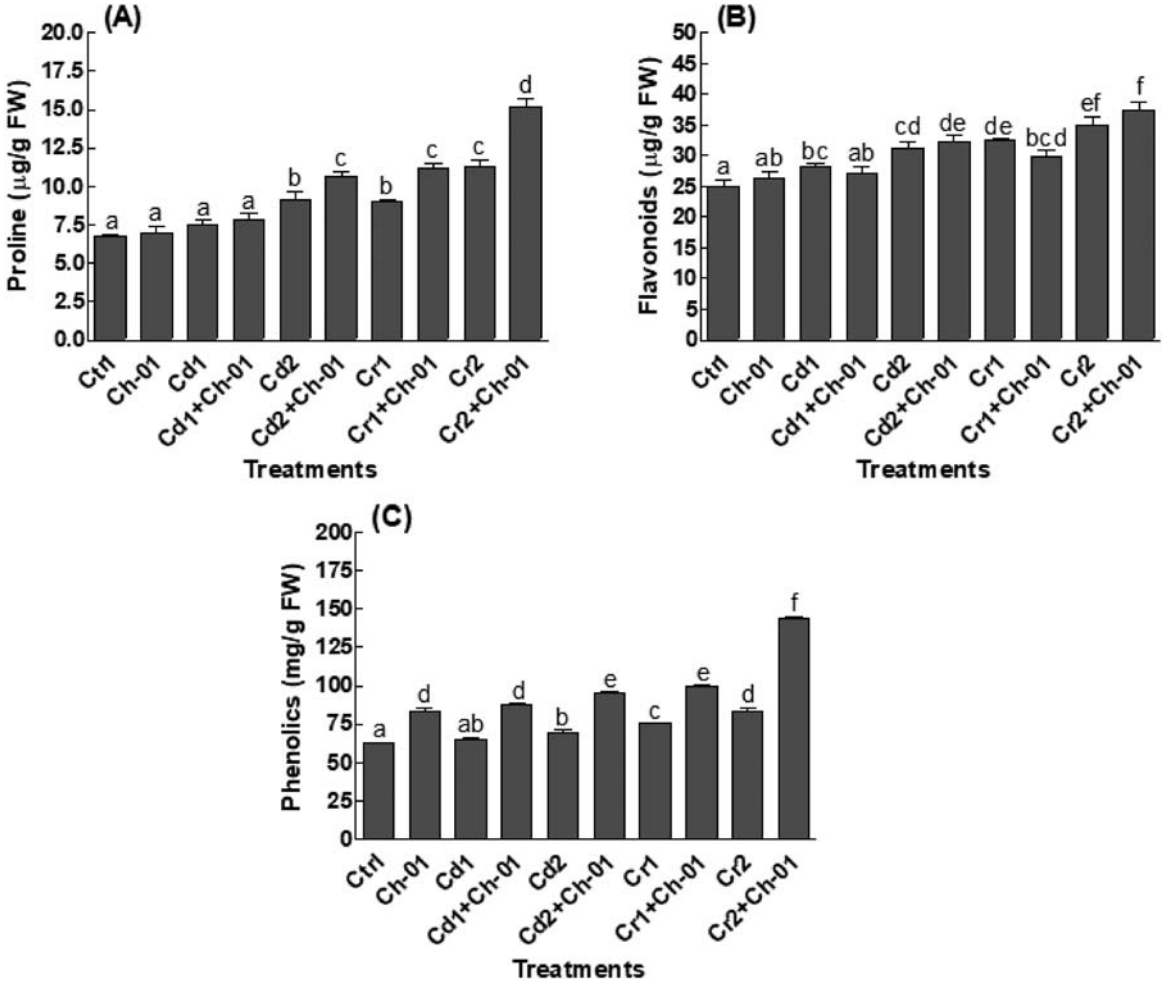

Figure 6. (A) Effect of *A. flavus* on Proline content under elevated Cd1 (100 µg/g), Cd2 (500 µg/g) and Cr1 (100 µg/g), Cr2 (500 µg/g) stress. Co: control. (B) Effect of *A. flavus* on flavonoids content under elevated Cd1 (100 µg/g), Cd2 (500 µg/g) and Cr1 (100 µg/g), and Cr2 (500 µg/g) stress. Co: control. (C) Effect of *A. flavus* on phenolics content under elevated Cd1 (100 µg/g), Cd2 (500 µg/g) and Cr1 (100 µg/g), Cr2 (500 µg/g) stress. Co: control. Data was statistically analyzed using DMRT at $p < .05$. Different letters are significantly different while error bars show standard error.

intracellular phytochelatin and metallothionin sequestration) (Bibi et al. 2018).

Our results showed a significant production of IAA, flavonoids, phenols, proline, soluble sugars and proteins by *S. lycopersicum* in the presence of *A. flavus*. The amounts of secondary metabolites increased with increasing level of Cd and Cr to minimize the adverse effects of HMs toxicity. Our results are parallel to previous research which showed that endophytes alleviates HMs toxicity and their adverse effects by enhancing the concentration of phytohormones

Table. 2. Orthologues for γ-glutamylcysteine synthetase 1 (GSH1) and Phytochelatin synthase 1 (PCS1) encoding gene.

γ-glutamylcysteine synthetase 1 (GSH1)				
Gene Info Identifier	Source Organism	AA Identity (%)	CDS Length	AA Length
AT4G23100.1	*Arabidopsis thaliana*	100	1569	522
Solyc08g081010.2.1	*Solanum ycopersicum*	89.30	1572	523
Bol014965	*Brassica oleracea capitata*	94.10	1545	514
Gorai.006G209400.5	*Gossypium raimondii*	90.20	1581	526
Manes.02G022200.1	*Manihot esculenta*	89.50	1572	523
Potri.001G104500.1	*Populus trichocarpa*	89.50	1581	526
Ciclev10019701m	*Citrus clementina*	87.90	1581	526
Lus10035496	*Linum usitatissimum*	87.70	1575	524
mrna32478.1	*Fragaria vesca*	89.10	1551	516
Glyma.05G207600.3	*Glycine max*	84.30	1515	504
Cucsa.303150.2	*Cucumis sativus*	87.50	1578	525
DCAR_004633	*Daucus carota*	73.00	1332	443
Brara.C04655.1	*Brassica rapa*	93.50	1554	517
30190.m011214	*Ricinus communis*	90.00	1581	526
Phytochelatin synthase 1 (PCS1)				
AT5G44070.1	*Arabidopsis thaliana*	100	1458	485
Bol012856	*Brassica oleracea*	96.9	1458	485
Brara.F03767.1	*Brassica rapa*	96.7	1458	485
Solyc09g072620.2.1	*Solanum lycopersicum*	82.9	1638	545
Prupe.8G219900.2	*Prunus persica*	80.6	1509	502
Carubv10026307m	*Capsella rubella*	93.0	1458	485
Thhalv10003193m	*Eutrema salsugineum*	86.6	1476	491

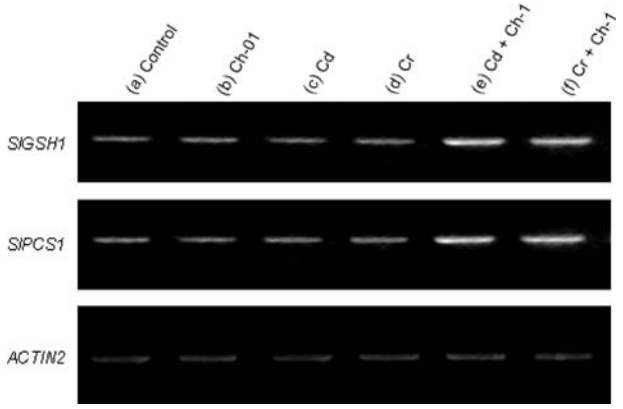

Figure 7. Effect of *A. flavus* (Ch-01) inoculation on gene-expression pattern in *S. lycopersicum* under elevated Cd and Cr. Upregulation is observed in gene expression for *SlGSH1* and *SlPCS1* in comparison to *ACTIN2* (an internal control, house-keeping gene) following the inoculation with *A. flavus* in *S. lycopersicum* plants upon supplementation with Cd (500 µg/g) and Cr (500 µg/g), compared with metal treated *S. lycopersicum* plants, endophyte-inoculated *S. lycopersicum* plants and control plants. Lanes correspond to the respective descriptions as, (a) Control, (b) Ch-01, (c) Cd, (d) Cr, (e) Cd + Ch-01, and (f) Cr + Ch-01.

and secondary metabolites, such as IAA, proline, flavonoids and phenolics (Zahoor et al. 2017; Bibi et al. 2018; Qadir et al. 2020). Sugar levels decreased gradually with the application of Cd and Cr stress. This may be due to the lowering of photosynthesis or increasing respiration rate. However, we noted a less pronounced effect of Cd and Cr stress on sugar contents in *A. flavus* associated *S. lycopersicum* seedlings. Likewise, the concentration of proteins in endophyte inoculated *S. lycopersicum* was also considerably higher compared to endophyte free plants under HMs stress as well as in stress free conditions. It is believed that elevated concentrations of HMs can directly affect the stability of the proteins, thus affecting protein final turnover. Endophytes inoculation might play an important role in preventing the inhibition of protein synthesis by increasing assimilation of the plant nitrate (Khan and Lee 2013).

A. flavus also improved the defense mechanism of host plant *S. lycopersicum* against HMs stress. The improvement in the defense mechanism of the *A. flavus* associated *S. lycopersicum* seedlings might be due to the synthesis of stress-related proteins as well as signalling molecules to

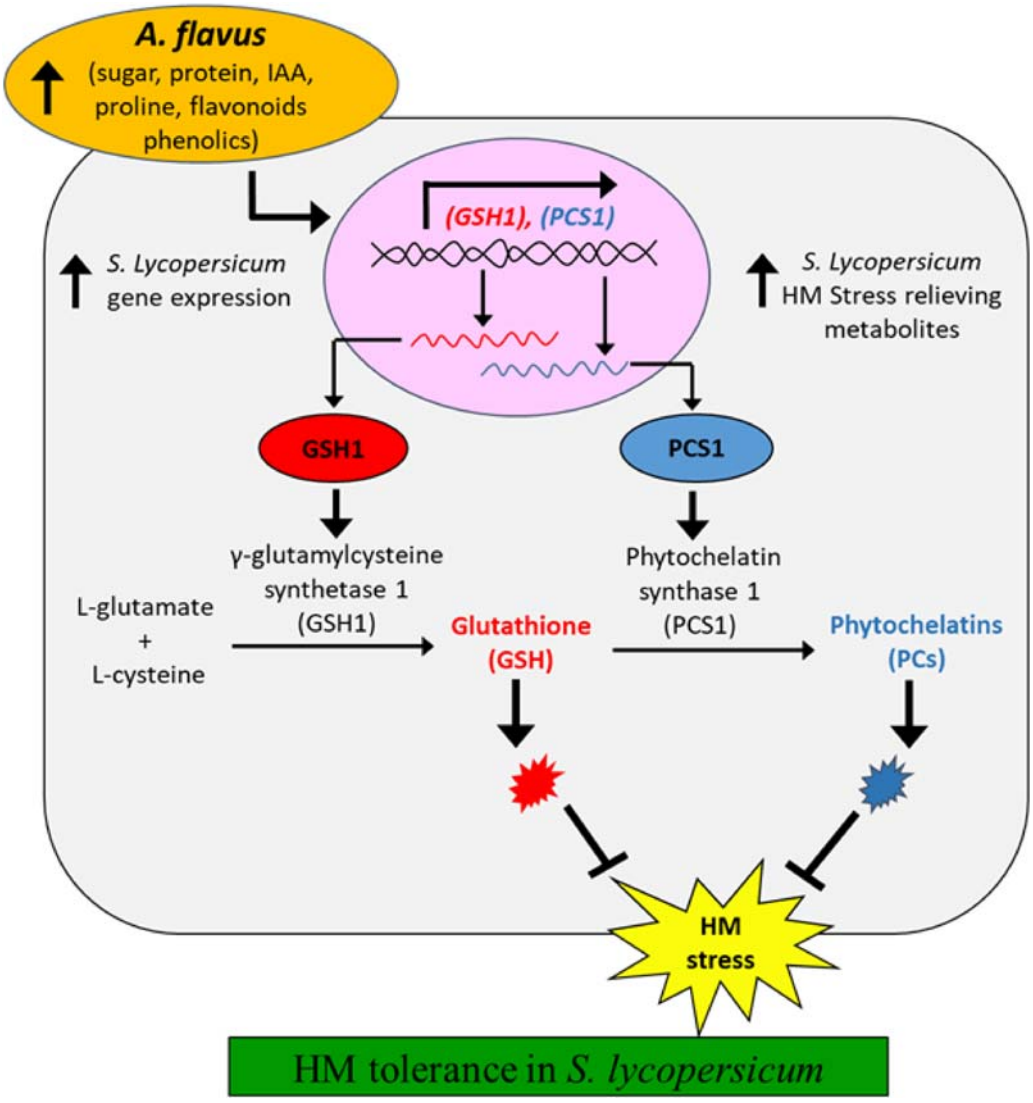

Figure 8: A working model of the role of A. flavus in S. Lycopersicum responses to heavy metal stress. Cd and Cr stress inhibits the normal growth of plants. Application of metal-resisting endophytic A. flavus Ch-01 not only augmented S. Lycopersicum growth under metals toxicity, but also positively influenced S. Lycopersicum root resistance to metal stress. Ch-01 remediated metal toxicity by secreting IAA and beneficial metabolites under metals induced-stress that initiated HM tolerance response and host growth via modulating gene expression of metal chelation and stress mitigation-related genes such as GSH1 and PCS1, thus, reducing metals accumulation and distribution in host tissues, leading to increased HM tolerance.

induce the gene expression of hosts concerning the defense mechanism. One of the critical components of defense mechanism is the transcription factors (TFs). TFs involved in signalling networks are responsible for the regulation of defense response through modulation of the expression of various genes responsible for stress-tolerance in plants. A recent report showed that WRKY12 directly binds to the W-box in the promoter of *GSH1*, but not in the promoter of *GSH2*, *PCS1*, and *PCS2*. Therefore, one of the major gene regulatory-cascade for Cd tolerance is comprised of *WRKY12*-GSH1. The *WRKY12*-GSH1 controls the synthesis of GSH and PCs, where *WRKY12* acts as a negative regulator for *GHS1* gene expression and Cd-tolerance (Han et al. 2019). Genes are responsible for the mechanism of metal chelation in plants, which is a major component of defense mechanisms against HMs stress. The genes *GSH1, GSH2, PCS1* and *PCS2* are recognized for their role in the biosynthesis of GSH and PC (Gasic and Korban 2007; Brunetti et al. 2011; Kühnlenz et al. 2014). Phytochelatin synthase (PCS) is a protease-like enzyme that catalyzes the production of metal-chelating peptides, the phytochelatins, from glutathione (GSH) (Rauser 1995). In *Arabidopsis thaliana*, PCSs are encoded by two genes, *PCS1 (AT5G44070)* and *PCS2 (AT1G03980),* which are highly up-regulated upon Cd stress (Kühnlenz et al. 2014). It is understood that GSH and PCs have important roles in heavy metal detoxification. In the current study, the upregulation of *SlPCS1* gene is indicating higher phytochelatin synthase1 enzyme production and activity for metal detoxification in *A. flavus* inoculated plants under heavy metal stress. Semi-quantitative RT–PCR analysis showed a higher relative expression of *SlGSH1* and *SlPCS* in endophyte-inoculated *S. lycopersicum* plants under elevated Cd and Cr stress. Certainly, *SlGSH1* and *SlPCS1* are the most relevant genes responsible for the mitigation of Cd and Cr stress in *S. lycopersicum*. These results signify the first report demonstrating endophytes *A. flavus* as a promising candidate to remediate HMs from contaminated soil and *S. lycopersicum*, encouraging plant growth resulting in safer food production for human feeding.

Conclusion

The current study reports that the endophytic fungus Ch-01 (*A. Flavors)* isolate from chilli is not only a plant growth promoter under HMs stress, but it is also capable of reducing toxicity of Cd and Cr and their adverse effects in *S. lycopersicum*. Moreover, *A. flavus* boosted growth under Cd and Cr toxicity by adapting the plant physiological, biochemical and molecular strategies. From the results, it is concluded that *A. flavus* can serve as a potential candidate to be used as a biofertilizer in Cd and Cr contaminated soils.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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