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ULTRAVIOLET INDUCED ENDOPHYTIC *ASPERGILLUS TAMARI* SK12 REVEALED FASTER GROWTH AND MODULATION OF PHYSIOCHEMICAL ATTRIBUTES OF OKRA UNDER COPPER STRESS

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Abstract

In today's world, the most serious environmental and biological problem is the contamination of agricultural lands by the heavy metals. Copper is an essential element for the normal growth of plants but its concentration can build up in soil to a level that can adversely affect plant growth. Endophytic fungi associate with plants and improve their fitness to tolerate environmental stresses. In this work, a plant growth promoting and copper tolerant endophytic fungus, *Aspergillus tamari* SK12, isolated from plants in contaminated areas was exposed to ultraviolet irradiation for various durations. The UV irradiated variant (UIV) showed superior growth in the absence and presence of Cu than the wild type strain. Higher biomass produced by the UIV was accompanied by greater quantities of IAA, SA, Phenols and flavonoids, enabling this strain to tolerate Cu more efficiently. Fungal capabilities to alleviate Cu stress in *Abelmoschus esculentus. When okra seedlings were exposed to 750 ppm of Cu, root and shoot growth were reduced to 64% and 30.4% of the control. Both the wild type and UIV endophytes were beneficial for the growth of okra seedlings where growth remained higher than the control even in the presence of 750 ppm of Cu.* The mutant strain effectively colonized roots of seedlings and absorption of copper from soil to root was also cut down to 50% of the wild SK12. The UIV colonized seedlings also kept ROS levels in normal range with the help of peroxidase enzyme and the non-enzymatic antioxidants i.e. Phenols and flavonoids. The UIV inoculated also accumulated up to higher levels of IAA and SA than control and wild strain. These Hormones increases ROS scavenging by increasing antioxidant activities and protect seedlings against Cu stress by reducing lipid peroxidation and the malondialdehyde levels. All these features enabled the endophyte (UIV) associated seedlings to grow normally even in the presence of 750ppm of Cu. We concluded that UIV strain had the ability to alleviate Cu stress in okra seedlings in multiple way; from reducing its uptake to improved ROS scavenging system through enhanced antioxidant activities and modulation of IAA and SA.

Key words: Bioremediation; Endophytic fungi; Heavy metal stress; Phytohormones; ROS scavenging; UV-induced mutation.

Introduction

Urbanization and industrialization have significantly contributed to uplift the living standards in human society. However, several devastating impact have been associated with these advantages. Pollution of soil, water and air is the most significant drawback of modern living, which is harmful to life on earth. One of the major forms of pollution is heavy metal contamination of soil and water. A metallic element which has high density (4.5g/cm3) referred as heavy metals. These metals include Arsenic (As), Platinum, Copper (Cu), Chromium (Cr), Zinc (Zn), Iron (Fe), Cobalt (Co), Nickel (Ni), and Cadmium (Cd) etc. For the normal growth of plants and animals some heavy metals are very essential (Wintz *et al.,* 2002). Certain heavy metals are very important for plant growth at low concentration and at high concentration these metals become toxic, suppressing plant growth. Vehicle emissions, coal burning wastes industrial wastes and other activities are the main sources of these heavy metals. Inorganic fertilizers, fungicides, antibiotics and phosphate fertilizers also have a moderate amount of heavy metals depending upon their sources (Sandeep *et al.,* 2019).

One of the heavy metal is copper (Cu) in which serves as plant nutrient and is needed for normal metabolism. Cu is crucial for electron transport, respiration in mitochondria, oxidative stress responses and signaling of hormones etc. (Wang *et al.,* 2004). This metal also plays an essential role in oxidative phosphorylation. Copper is an important heavy metal that is needed for the plant growth, playing a very important role in carbon dioxide assimilation (Marques *et al.,* 2018). Cu is also the main constituent of proteins like plastocyanin and electron transport chain (Chen *et al.,* 2019). Also, Cu is a part of many enzymes which play an essential role in respiration, photosynthesis and in woody plants they also form lignin (Li *et al.*, 2023). However, beyond optimal level, Cu can be very toxic to plants and can cause reduced seed germination, lower iron availability and low shoot vigorous. High level of copper concentration effects the plant growth and cause cytotoxicity (Saleem *et al.,* 2020). The reduced biomass in plants is the common indicator of high Cu stress (Nazir *et al.,* 2019).

To reclaim soil contaminated by excessive amount of Cu, several remedies have been suggested i.e., by physical-mechanical, chemical and biological systems depending on soil properties. Bioremediation uses living organism including microbes to reduce Cu contamination (Ali *et al.,* 2022). Living organisms may perform several activities to reduce the toxicity of Cu such as bioaccumulation and biotransformation etc. Different techniques of bioremediation include nutrients, leachate collection, and aeration and treatment bed systems. The sindrows type of bioremediation is used to increase microbial degradation activities. Another method of bioremediation is the bioreactor, which transforms reactants into specific products. Different operational modes for bioreactors exist, such as fed-batch, batch, and sequencing batch. The fact that this approach of bioremediation can regulate process parameters in a bioreactor suggests that a biological reaction is taking place (Mohan *et al.,* 2006). But the best and widely accepted way is phytoremediation. Phytoremediation generally refers to the use of microbes and plants in order to reduce the harmful effects of contaminants in the environment. The natural ability of plants are used in order to absorb or accumulate and degrade other substances, either organic or inorganic (Du *et al*., 2024). For the reduction of contaminants in the soil, one of the most efficient ways is phytoremediation which is eco-friendly. For the accumulation of metals with higher concentrations, plants are used (McIntyre, 2003). Metals can be detoxified with the help of microorganisms. In metabolic reactions they are involved in the processes of enzymatic reactions of metals. Many microorganisms such as bacteria have the ability of oxidative coupling of aromatics and alcohols with the reduction of manganese and iron. The process of bioremediation is performed by several microorganisms such as bacteria, algae and fungi in order to produce dimethyl selenide or trimethylamine, that are volatile derivatives (Sudame, 2003). Bio precipitation is a technique which is generally uses by sulfate reducing bacteria. For the conversion of sulfate to hydrogen sulfide, the process of bio precipitation is used in order to react HMs making them non-soluble (Diels *et al.,* 2002). Many remediation processes have been discovered in order to avoid hazardous effects on living organisms including plants and human beings. Money has been spent in high amount by using conventional processes to recover HMs polluted sites (Sheng *et al.,* 2012). For the extraction of these heavy metals from the lands and soil, the process of phytoremediation are used (Turan and Esringu, 2007). And it has been noted that phytoremediation is a safer and easiest way as compared to conventional methods (Fischerová *et al.,* 2006). As studied above, that these HMs are not degrade but only change from one state into another state. Due to the change in HMs from one state into other, the heavy metals become less toxic, more volatile and more or less water soluble (Alkorta *et al.,* 2004). Among various organism used for bioremediation, endophytic fungi are the least explored yet having great potential to minimize Cu toxicity and improve host plant growth (Gadd & Pan, 2016).

Endophytic fungi are silent plant partners inhabiting plant tissue but remain invisible, metabolically active though. Endophytes are known to modulate host physiology and improve their fitness to tolerate biotic and abiotic stress. Endophytic fungi are known to tolerate heavy metals and alleviate their toxicity in host plant. Random mutagenesis may be performed on endophytic fungi to induce permanent changes in their genome and metabolism. Though this technique may induce both positive and negative changes and to select the desired mutant among different variants may also be a challenge, the technique is easy to apply and don't require expertise in the field of molecular biology lost (Najafi *et al.,* 2014). Use of ultraviolet radiation for random mutagenesis has been in use since decades (Narayanan & Sakthivel, 2010). The primary and secondary metabolites content of the microbes can be enhanced by mutating the microbes (Parekh *et al.,* 2000). Although UV portion of the light is mostly absorbed by the ozone layer however artificial UV light can be generated in the labs using the UV lamps. It is known that the activity of fungi and their cell replication rate can be reduced using the UV irradiation as UV irradiation bring genomic changes in the fungi by inducing the formation of pyrimidine dimers (Bintsis *et al.,* 2000). However microbes have the ability to repair their DNA damaged by the UV irradiation (Sinha & Häder, 2002). Researches show that most of the mutated microbes contains a special protein i-e DNA photolyases that have the ability to restore microbes to their original undamaged state (Brettel & Byrdin, 2010).

The present study was designed to check the potential impact of UV irradiation on the Cu tolerance potential of isolated endophytic fungi. The aim was to generate a mutant that can promote *Abelmoschus esculentus* L. growth even in the presence of excess amount of copper. The key parameters chosen to assess in the mutant were growth improvement and enhancement of the release of IAA, SA, flavonoids and phenols which are key factors adding to the metal alleviation potential of endophytes.

Materials and Methods

Isolation of endophytic fungi: Healthy plants growing on copper contaminated soil were uprooted and transported to PMI lab, Department of Botany, AWKUM. For fungal isolation different parts of the plants were washed and then sterilized with 70% ethanol and sodium hypochlorite for 1 min and 3 minutes respectively. The plant parts were then washed with double distilled water for two min (Stone *et al.,* 2004). After this, plants parts were inoculated on hagum media plates for seven days in the laminar flow hood for fungal growth. Agar plates were also inoculated with the imprints of the sterilized surface of the plant segment to demonstrate the efficiency of the surface sterilization protocol. To get pure colonies the isolated endophytes were plated on PDA (Potato Dextrose Agar) plates (Jan *et al*., 2022).

Screening of isolated strains for heavy metal ($CuSO_4.7H_2O$) tolerance: The isolated strains were cultured in Czapek dox broth having known concentrations i.e., 500 ppm, 1000 ppm, 1500ppm and 2000 ppm of $CuSO_4.7H_2O$. Growth of strains was monitored by weighing fungal biomass. The strains capable of growth in Cu containing media were selected for further study.

Screening endophytes for copper stress alleviation in *Abelmoschus esculentus:* Selected endophytes were screened for copper stress alleviation in *Abelmoschus esculentus* L. Seeds were obtained from Agricultural Research Institute Tarnab, Peshawar, and Khyber Pakhtunkhwa. Uniform seeds were allowed to germinate in pots containing sterile soil having fungal biomass, 500 ppm or 750 ppm of Cu individually or in binary combination with fungal biomass. Each treatment had 3 replicates. The pots were placed in growth chamber kept under following conditions, i.e., 50– 80% relative humidity, 14:10 light/dark photoperiod, and temperature, 30°C for 14 days. The growth parameters were noted after 14 days of germination. Strains capable of promoting growth of the host seedlings in the presence of Cu were selected.

Molecular identification of fungal isolate SK12: DNA were extracted from freeze-dried mycelium in order to carry out molecular identification (Li *et al.,* 2015). In 500 µl buffer (sodium-dodecyl sulfate) SDS, 0.5 M Tris-HCL and 0.1 M NaC l, about 200 of fungal biomass were ground, vortexed for 10-15Sec at pH 8. For 30 minutes, the mixture was heated at 65 C°. After that the mixture was centrifuged for 5 minutes at 11000×g. Equal volume of supernatants and phenol chloroform-isoamyl, alcohol was mixed and centrifuged for five minutes at 10000×g. Chloroform-isoamyl were mixed with aqueous layer (24:1) and centrifuged for 5 min at 10000×g. Equal volume of absolute ethanol was mixed with separated supernatant followed by incubation for 60 min at 4ºC and then centrifuged for 10 min at 14000xg for DNA precipitation. DNA amplification was done with ITIS and 4 regions via polymerase chain reaction. Reaction mixture consist 7 µL of double distilled water, 1 µL of each primer, 1 µL of DNA sample and 10 µL PCR master mix in mixture. Initial denaturation for 2 min at 95ºC was done for PCR amplification, followed by annealing for 1 min at 55ºC and extension at 72ºC for 5 min. Total 35 cycles were performed. The gel purified PCR fragment was subjected to sequencing reaction through sequencing service-proving agency.

Morphology of the fungal strains: To investigate the morphology of SK12 and SK12 (45), slide culturing technique were used (Agu & Chidozie, 2021). A slab of PDA (potato dextrose sugar) was mounted on the slide under sterilized condition. The fungal spores were inoculated from all four sides of the block which was then covered by the cover slip in order to avoid surface growth. Slides were incubated for 3 days in sterile Petri plates at 30˚C. The endophytes were stained after incubation with lacto phenol cotton blue dye and were examined at 40X using a light microscope.

Ultra violet treatment: The isolated endophytes were subjected to ultra Violet (UV) treatment for inducing random mutation in their genome. For this purpose, spore suspension (1×10^7 spores/mL) of the selected endophytic fungi were prepared by scraping spores from the colony surface and suspending them in sterilized distilled water. The spore suspension was put in autoclaved Petri dishes which were then be exposed to UV-C light (254nm) for different time period i-e, for 15 minutes, 30 minutes and 45 minutes. UV exposed spores were grown on PDA plates and the colonies were observed for altered phenotypes. The mutants were screened as described above and the mutant having significantly higher potential for Cu tolerance and it alleviation in the host seedlings was selected.

Characterization of endophytic fungi (wild types and mutants): Using the colorimetric methods, the amount of phytohormones like auxins and salicylic acid (SA) were monitored. Total phenols, flavonoids, anti-oxidant enzymes and metal chelators were also assessed in the endophytes (Dwibedi & Saxena, 2020). In order to quantify the amount of phytoharmones fungal filtrate were obtained by filtering fungi grown in the Czapek broth.

Indole Acetic Acid (IAA): In order to find out the amount of IAA in fungi culture filtrate Salkowski reagent was used as described previously (Hussain & Hasnain, 2011). The colored complex formed because of reaction between IAA and Salkowski reagent was monitored at 540nm via spectrophotometer (PerkinElmer Lamda 25 double beam spectrophotometer). Standard curve produced by taking OD of different IAA concentrations replacing the extract.

Salicylic acid (SA): For the determination of SA amount in FCF, the method of (Warrier *et al.,* 2013) was used. We added 1% of freshly prepared ferric chloride to 0.1 mL of FCF to make a final volume of 3mL. At 540nm absorbance were recorded. Standard curve was produced by taking OD of different SA (10, 20, 30, 40, 60, 80 and 100 µg/mL) concentrations replacing the fungal culture filtrate (Jan *et al*., 2022).

Flavonoids: To find out the amount of total flavonoids in the culture filtrate of the selected fungus, $ALCl_3$ method was followed (El Far & Taie, 2009). Reaction mixture had 4.8 mL methanol (80%), potassium acetate 100µL (10%), 100µL $AlCl_3$ (10%), and 0.5mL of FCF. The mixture was incubated in room temperature for 30 minutes. After shaking vigorously, absorbance of the sample was recorded at 415 nm. Standard curve was produced by using known concentrations of quercitin (15µg-500µg).

Phenolic content: For the determination of phenol content in FCF a modified method of (Ainsworth & Gillespie, 2007) was used. 200µL of FCF were mixed with 2.8 ml of double distilled water and 0.5 mL of Folin-ciocalteu reagent (1:1) were added to the mixture. At room temperature the mixture were incubated for four minutes. After incubation, 2 mL of 20% sodium carbonate were added to the mixture and heated for 1 minute. At 650 nm absorbance were recorded after cooling. Standard curve was produced by using known concentration of catechol. The quantity of phenol were calculated from the standard curve in the selected sample and expressed in mg of equivalent catechol/g.

Alleviation of Cu stress in *A. esculentus:* The endophytes (wild type and mutant) were used as inoculant for Cu stressed *A. esculentus*. For this purpose, *A. esculentus* seeds were grown in plats pots containing autoclaved garden soil. The following treatments were applied to the plants: control (no metal, no fungus), 500 ppm of $CuSO_4.7H_2O$, wild type fungus, mutant fungus, wild type or mutant fungus + 500 ppm of $CuSO_4.7H_2O$. The plants were harvested after 45 days and different parameters were assessed.

Root colonization: *After harvest, Abelmoschus esculentus* roots inoculated with endophytic fungi were stained with lactophenol cotton blue to visualize colonization of endophytic fungi in the host tissues (Pal *et al.,* 2020). Briefly, a few drops of lactophenol cotton blue were added on to the root sections positioned on glass slide which were allowed to stand for 2 to 3 min at 30°C. Using 70% ethanol the excess dye was removed from the tissues and the stained root section was then washed with distilled water. Root sections were observed under light microscope to visualize fungal hyphae in them.

Modulation of plant metabolites and phytohormones under stress

Total flavonoids and indole acetic acid: A 0.5 gm of seedling was crushed in 5 mL 80% ethanol in order to quantify the total flavonoids and left overnight in incubator. Centrifugation was done for 15 minutes at 7500 rcf. In 25 mL Falcon tubes the supernatant were stored at 4C. $AlCl_3$ method was used for the quantification of flavonoids as described above (El Far & Taie, 2009).

Salicylic acid: 0.5 gm of seedling was crushed in 5 mL 80% ethanol in order to extract salicylic acid from the seedlings and left overnight in incubator. Centrifugation was done for 15 minutes at 7500 rcf. Supernatant were stored at 4C in 25 mL Falcon tubes. The method of (Warrier *et al.,* 2013) was used in order to estimate the salicylic acid in extract as described above in fungal section.

Total phenol content: 0.5 gm of seedling were homogenized in 5 mL of ethanol in order to extract total phenolic contents from the seedlings. The mixture was incubated for 3 hours at 40 C temperature. At room temperature centrifugation were done for 10 minutes at 7500 rcf. In 5 mL distilled water the supernatant of extract were re-dissolved and stored at 4°C. The method of (Ainsworth & Gillespie, 2007) was used in order to measure the total phenolic contents in the extract as described earlier in fungal section.

Total sugar: 100µL of extract were taken in order to estimate the total sugar contents in the samples. Extract were added to 1 ml of 80% phenol. At room temperature the sample were incubated for 10 minutes (Khan & Naqvi, 2012). After incubation 5 mL of concentrated sulphuric acid was added to the mixture followed by re-incubation for 1h. At 485nm absorbance were recorded against the blank. Standard curve was produced by using known concentrations of glucose.

Antioxidant enzyme activities in *Abelmoschus esculentus* under Cu stress

Peroxidase activity: For dehydrogenation the guaiacol were used as a substrate in order to estimate the Peroxidase activity. 0.1g of the plant sample was grounded in 3ml of 0.1 M phosphate buffer (PH 7.0) using pistil and mortar for the purpose of enzyme extraction. The mixture was then centrifuged for 15 minutes at 18000g. Reaction mixture consists 0.05ml of 20mM guaiacol, 0.1ml of enzyme extract and 0.1 M phosphate buffer (3 mL) at pH 7, 0.03 ml of 12.3 mM hydrogen peroxide (Gore *et al.,* 2017). POD activity was calculated after shaking the reaction mixture by the following formula. The change in optical density by 0.1 (t) was recorded at 436 nm.

$$\text{Enzyme activity} = (500/\Delta t) \times (1/1000) \times (TV/VU) \times (1/f\ wt))$$

In the given formula;
Δt = time change in minute,
TV = total volume of extract
VU = Volume used (Sousa *et al.,* 2012)
f wt = fresh weight of the leaf tissue in (g).

DAB staining protocol: 3,3′-Di-aminobenzidine (DAB) was used in order to visualize the accumulation of total radical oxygen species (ROS) in the leaves, following the methodology of (Daudi & O'Brien, 2012). In DAB solution the leaves were dipped followed by continuous heating in water bath for about 3 hours to infiltrate the leaves with bleaching solution,–made by mixing ethanol, acetic acid and glycerol at the ratio of 3:1:1. In order to remove the chlorophyll contents the leaves were then boiled in ethanol by using water bath. The stained leaves were then visualized using light microscope.

1, 1-diphenylhydrazyl assay: Using the method of (Meng *et al.,* 2016), the 1, 1-diphenylhydrazyl (DPPH) radical scavenging activity in the samples were monitored. 1 ml methanol was mixed with 0.1gm homogenized plant leaves and equal volume of DPPH solution (0.04mg/ml). The samples were then incubated in the darkness for about 30 minutes. After incubation the absorbance of the samples were recorded through spectrophotometer at 517nm against the blank.

Statistical analysis: Statistical analysis included ANOVA and Duncan multiple range test (p = 0.05). The Mentioned procedures were performed to find statistical significance of the data using SPSS for windows ver 16.0.

Results

Isolation of endophytic fungi and their plant growth promoting triats: We isolated ten strains of endophytic fungi form different parts of *Portulaca oleracea*. After establishing their axenic cultures, all the isolates were inoculated on Abelmoschus esculentus in order to assess their plant growth promoting potential under Cu stress. Among the isolated strains SK12 was selected due to its ability to promote growth of Abelmoschus esculentus and alleviate copper stress (Fig. 1). In the presence of 500 and 750 ppm Cu stress, SK12 associate seedlings have the ability had 70% and 10% increase in shoot and root growth respectively, as compared to the control seedlings.

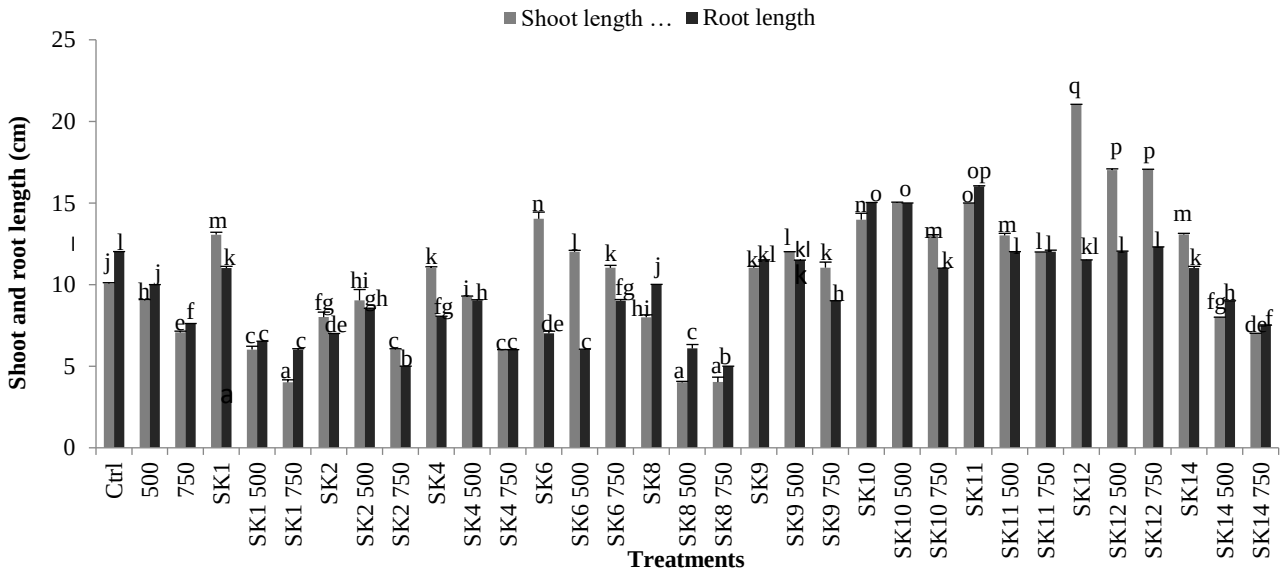

Fig. 1. Effect of different concentration of copper and endophytic fungi on the growth attributes of *Abelmoschus esculentus*. The seedlings were allowed to grow for 14 days in plastic pots containing autoclaved soil spiked with 500 and 750 ppm of copper. Significance among treatments is shown by labeling means values (three replicates) with different letters ($p<0.05$).

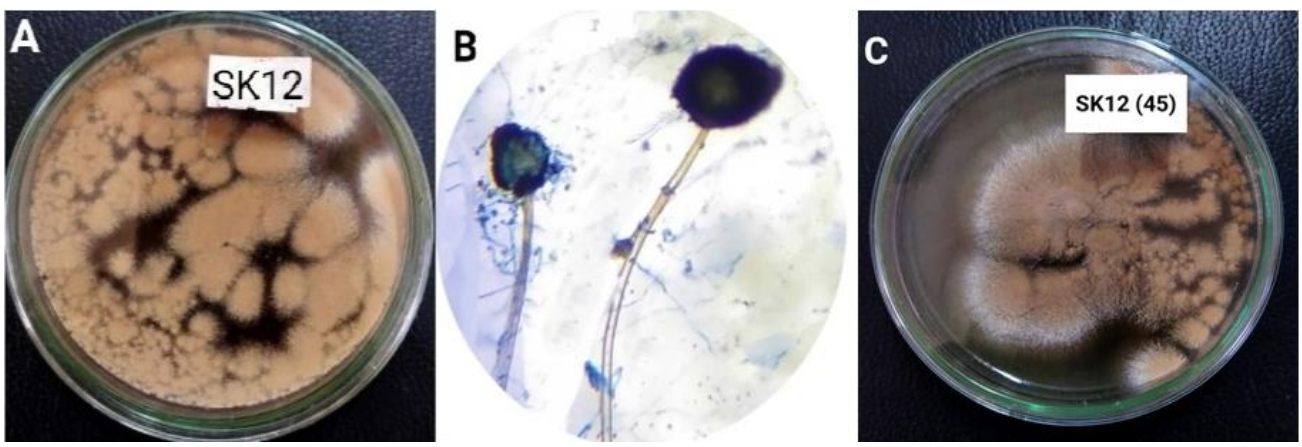

Fig. 2. Pure colony of the isolated endophytic fungus *Aspergillus tamari* SK12 on PDA (a), their conidiophores along with conidia visible under light microscope at 40X magnification (b) and colony of mutant *Aspergillus tamari* SK12 (45).

Preliminary identification: On the basis of morphological characteristics, the preliminary identification of fungal endophyte was carried out. Identification of fungal isolate and their mutant were done at least up to genus level based on texture, hyphae color, morphology of colony and spores (Fig. 2A, 2B and 2C).

Identification of fungal strain SK12: For the identification of fungal strains, ITS rDNA sequence was matched to the fungal ITS sequences in NCBI GenBank by using BLAST. The sequence showed high degree similarity excellent query cover with the ITS sequence of *Aspergillus tamari*. When subjected to phylogenetic analysis, the strain was clustered with *Aspergillus tamari* (Fig. 3). Hence, the isolate was labeled as *Aspergillus tamari* SK12.

Screening for copper tolerance: The Isolate SK12 and its mutant SK12 (45) were grown in the presence of different concentrations of Cu added in Czapek broth. UV exposure not only induced the isolate to grow faster and produce significantly higher biomass but also modulated its growth characteristics and the mutant produced beads of larger diameter than the wild strain (Fig. 1). There was a gradual decrease in fungal biomass with increasing concentration of copper from 0 to 2000 ppm (Fig. 4). However, at each copper level, growth of the UIV was greater than the wild type strain (WTS). Even at 2000 ppm growth of UIV was 42% greater than the WTS.

Qauntification of plant metabolites and phytohormones in fungal culture filtrate: The best mechanism to ensure the endophytes survival in abiotic stress is to change the pattern of metabolites production and microbial phytohormones. SK12 was found to produce Indole Acetic Acid both under metal stress and normal conditions. The UIV of SK12 released 7% greater quantity of IAA than the WTS (Fig. 5A). IAA production was significantly increased in culture filtrate of WTS and UIV with increase in the level of Cu stress. Both the strains released their highest amount of IAA (95 µg/mL by UIV and 78 µg/mL by WTS) in culture containing 2000 µg/mL of Cu. Release of IAA was always higher in UIV than WTS no matter what concentration of Cu was chosen.

Fig. 3. Phylogenetic association depicted by a neighbor-joining tree reflecting ITS rDNA sequence homology of *Aspergillus tamari* SK12 with closely related sequences obtained from NCBI GenBank.

The isolate SK12 also had the ability to release 34 µg/mL flavonoids and its quantity was similar in the cultures of WTS and UIV. Presence of Cu in the culture media enhanced the amount of released flavonoids in both the cultures. However, UIV released greater flavonoids than the WTS when Cu stress was applied. The only exception was seen in the culture having 1000 µg/mL of this heavy metal, where both the cultures released equal quantity of flavonoids. In the media containing 2000 µg/mL Cu, the UIV released 11% greater flavonoids compared to the WTS and its own Cu free culture (Fig. 5B).

SK12 was found to produce Salicylic acid both under metal stress and normal conditions. The UIV of SK12 released 12% greater quantity of Salicylic acid than the WTS (Fig. 6C). Salicylic acid production was significantly increased in culture filtrate of WTS and UIV with increase in the level of Cu stress. Both the strains released their highest amount of Salicylic acid (540 µg/mL by UIV and 473 µg/mL by WTS) in culture containing 2000 µg/mL of Cu (Fig. 5C). Release of Salicylic acid was consistently higher in UIV than WTS, regardless of the chosen concentration of Cu.

The production of phenolic contents was also significantly increased in culture filtrate of wild type strain and UIV with the increase in the level of Cu stress. Both the strains released their highest amount of Phenolic contents (1477 µg/mL by UIV and 1437 µg/mL by WTS) in culture containing 2000 µg/mL of Cu (Fig. 5D).

Role of mutated endophytic fungi in alleviating metal stress: Under heavy metal stress the mutated fungal endophyte significantly reduced the toxic effects and promoted the growth of *Abelmoschus esculentus compared to the wild endophyte plants and uninoculated plants*. Due to increase in copper concentration, the root and shoot length showed significant reduction (Fig. 6A and 6B). Addition of 750 ppm Cu had significant effect on shoot length of the seedlings, reducing it to 69.6% of the control. On the contrary, inoculation of seedlings with both SK12 and its mutant (UIV) led to an enhancement in shoot length, with the most significant increase observed case of UIV (Fig. 6A and 6B). SK12 and UIV colonized seedlings were impacted by exposure to varying levels of Cu (500 and 750 ppm), yet their shoot length remained greater than that of the non-endophytes counterparts. The stressor also affected root length, reducing it to 18% and 36% of the control in seedlings exposed to 500 and 750 ppm of Cu, respectively (Fig. 6B). On the other hand, the influence of SK12 and its mutant (UIV) on root length was positive, enhancing it by 14% and 12.5% compared to the control, respectively. Exposure of SK12 and its mutant infected seedlings to the selected concentrations of Cu led to an obvious reduction in root length. However, in UIV colonized seedlings, this parameter exceeded that of the control. Biomass of the seedlings was influence in similar manner to that of shoots and root length (Fig. 6C and 6D). For instance, both levels of Cu were detrimental to fresh and dry weights of the seedlings. The SK12 and its mutant associated seedlings had higher fresh and dry biomass compared to the control. When the UIV colonized seedlings were exposed to selected concentrations of Cu, both their fresh and dry biomass remained higher than that of the control.

Inoculation of SK12 modulates metabolites of *Abelmoschus esculentus under stress:* Total flavonoids contents were assessed in inoculated and uninoculated plants. In stress condition, the total flavonoids contents in the plants were increased compared to control plants (Fig. 7A). Inoculation of seedlings with SK12 and its mutant also increased the levels of flavonoids. When endophytes colonized seedlings were exposed to the stress, contents of flavonoids were further enhanced compared to control and the seedlings that received individual treatments. The leaves of UIV infected seedlings treated with Cu had the highest levels of flavonoids.

Exposure to Cu led to a significant drop in the total soluble sugar contents, with the magnitude of this decrease amplifying with the higher levels of the stressor (Fig. 7B). On the contrary, the SK12 inoculated seedlings had significantly higher soluble sugar contents. While Cu exposure did reduce soluble sugars in SK12 inoculated seedlings, the actual amount persisted higher compared to non-endophyte seedlings subjected to the stressor. Interestingly, exposure to both the chosen concentrations of Cu led to an improved in soluble sugar contents in UIV associated seedlings. While phenolic contents dropped with the addition of 500 ppm of Cu compared to the control, 750 ppm did not significantly affect this parameter (Fig. 7C). Both the SK12 and its mutant led to enhancement in the phenolic contents, although the increase was more pronounced in case of SK12. While exposure of both SK12 and UIV inoculated seedlings to Cu imposed a drop in their phenolic contents, Sk12 associated seedlings maintained higher levels compared to control. In contrast, the concentration of phenolics in UIV infected seedlings dropped to the lowest levels.

Salicylic acid levels were enhanced by all the treatments compared to the control (Fig. 7D). Exposure of endophytes associated seedlings to Cu synergistically impacted the endogenous levels of SA. The highest concentration of SA was recorded in Cu exposed UIV infected seedlings. Endogenous IAA levels showed a significant decrease in Cu treated seedlings (Fig. 7E). While inoculation with SK12 led to an increase in the IAA levels, exposure to Cu decreased the IAA. In case of UIV colonization, IAA levels enhanced, and Cu exposure further magnified this increase to the highest levels observed during current study.

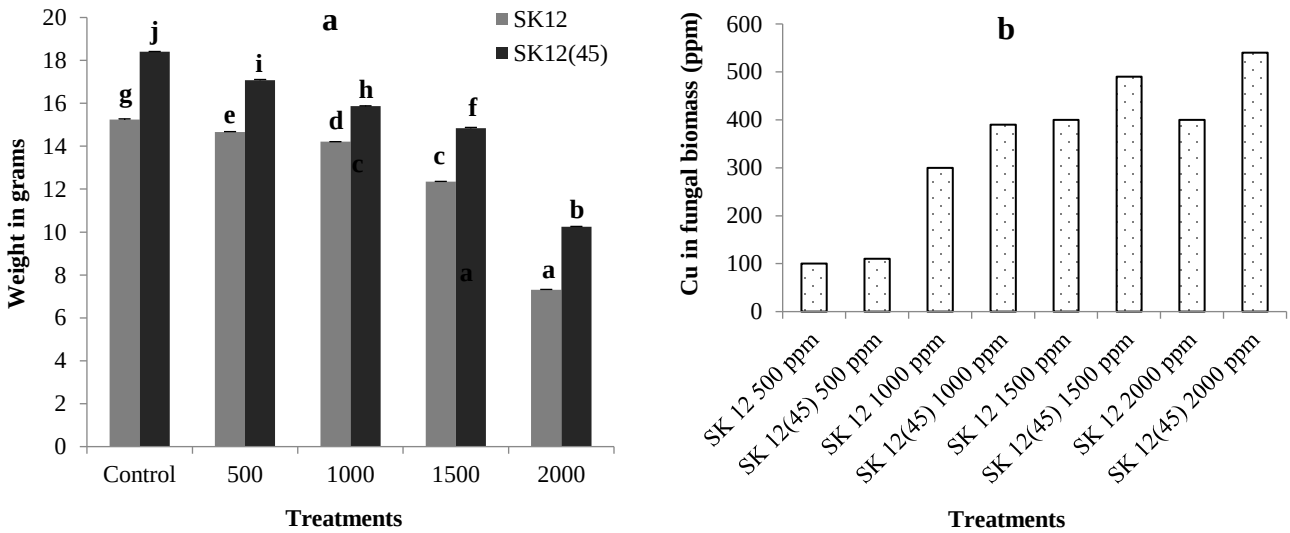

Fig. 4. Growth (Biomass production) of *Aspergillus tamari* (A) and accumulation of copper in fungal biomass (B). The endophyte *A. tamari* (SK12) and its mutant (SK12(45) were grown in Czapek dox broth having different concentrations of copper for 5 days at 30°C in a shaking incubator (130 rpm). Significance among treatments is shown by labeling means values (three replicates) with different letters ($p<0.05$).

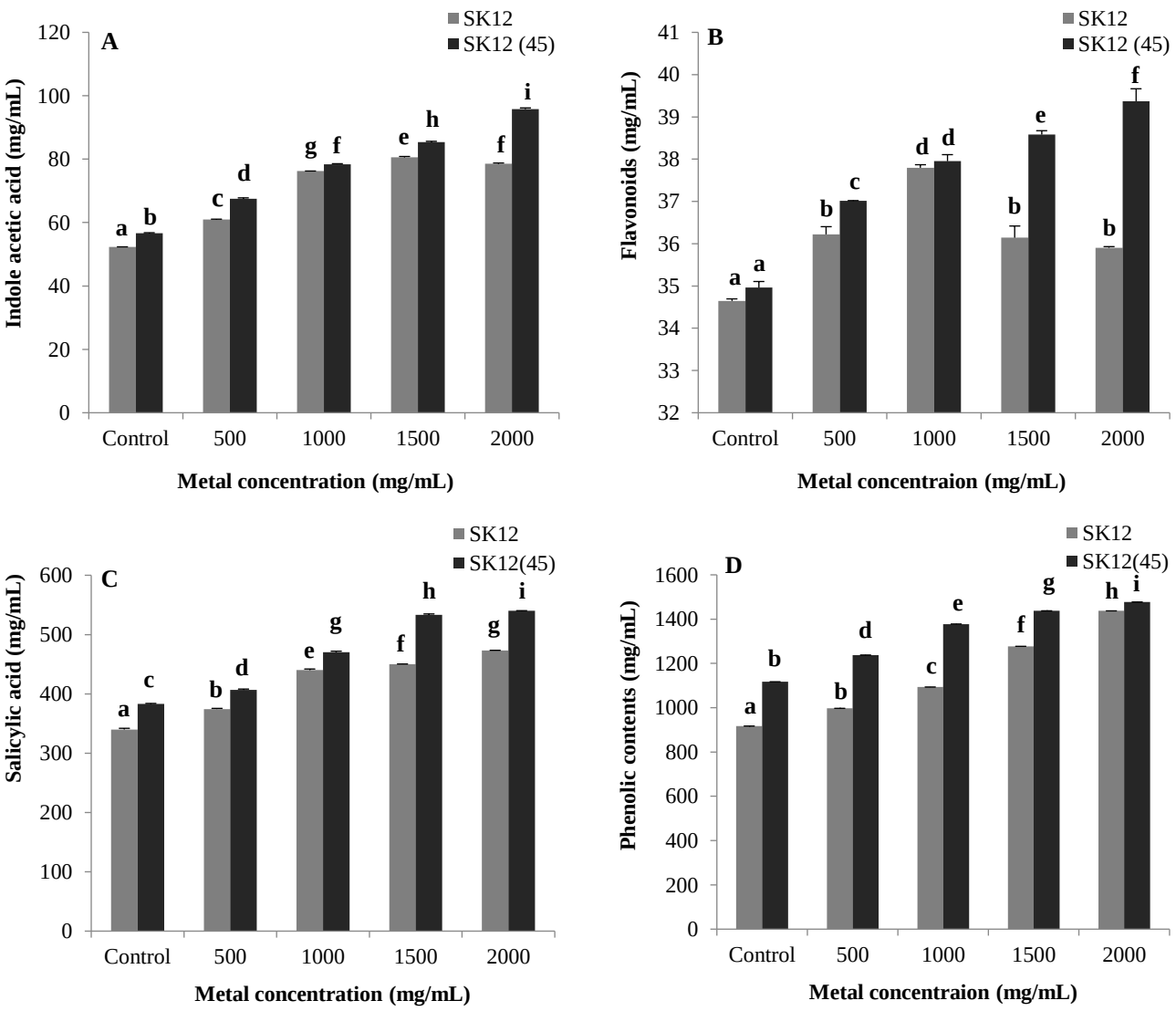

Fig. 5. Effect of different copper concentrations on exogenous concentration of (a) Indole Acetic Acid (b) Flavonoids (c) Salicylic Acid and (d) Phenolics in the cultural supernatant of wild type (SK12) and mutant (SK12(45) *Aspergillus tamari*. Significance among treatments is shown by labeling means values (three replicates) with different letters ($p<0.05$).

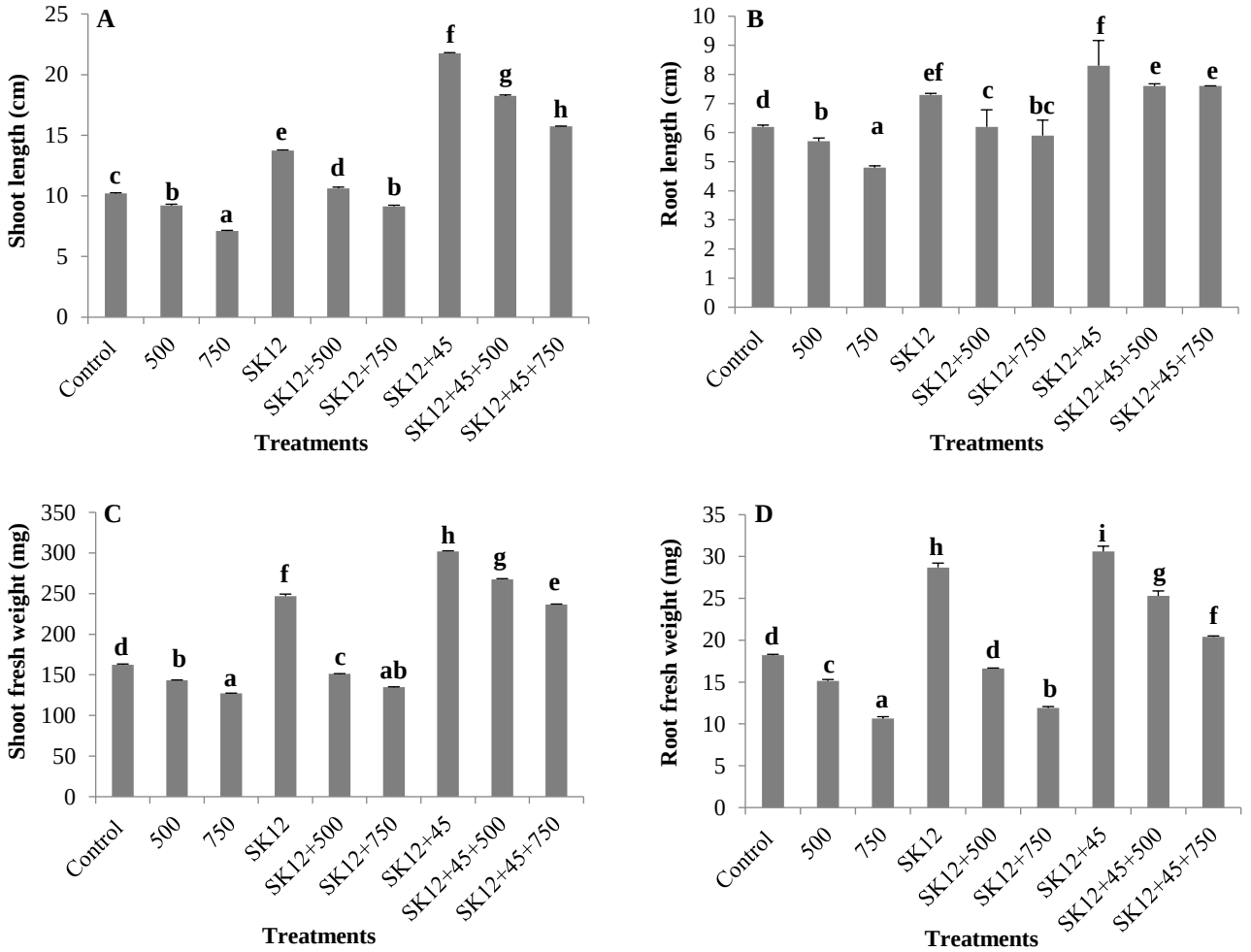

Fig. 6. Mitigation of copper stress in okra by *Aspergillus Tamarii* SK12 and its mutant SK12 (45). The seedlings were grown in soil inoculated with the fungal strains and after a growth of 45 days, Shoot length (A) Root length (B) Shoot fresh weight (C) and Root fresh weight (D) were recorded. Significance among treatments is shown by labeling means values (three replicates) with different letters ($p<0.05$). labels; 500, 500 ppm of Cu; 750, 750 ppm of Cu; SK12, endophytic fungus *Aspergillus tamari*; SK12+500, *Aspergillus tamari* and 500 ppm of Cu; SK12+750 *Aspergillus tamari* and 750 ppm of Cu

Copper in soil and seedlings parts: Un-inoculated okra seedlings accumulated higher levels of Cu than the endophyte associated seedlings, when grown in soil containing 500 or 750 ppm Cu (Fig. 8). Nearly 30% of the copper was accumulated in both the root and shoot of seedlings exposed to Cu ac a concentration of 500 ppm, 2/3 of which was in the roots. Increasing Cu level in the soil enhanced its accumulation in the seedlings, reaching close to 40% of the total Cu added in the soil. Endophyte associated seedlings had significantly lower quantity of Cu than the un-inoculated seedlings. Association with the mutant strain further reduced uptake and translocation of Cu from soil to root and the aerial parts (Fig. 8).

SK12 regulates antioxidant enzyme system in *Abelmoschus esculentus*: Effect of both Sk12 and its mutant (UIV) was studied on the ROS scavenging (peroxidase and DPPH assay) system of A. *esculentus.* In the presence of 500 ppm of Cu, DPPH free radical scavenging activity was enhanced by 16 % than the control (Fig. 9A). When concentration was increased to 750 ppm, DPPH free radical scavenging activity was reduced to 94% of the control. The free radical scavenging activity in the WTS and UIV colonized was higher than the respective controls. The UIV inoculated seedlings showed the highest antioxidant potential among various treatment groups. Activity of peroxidase was reduced to 86 and 95% of the control in seedlings exposed to 500 ppm and 750 ppm respectively (Fig. 9B). Both the wild and mutant strains enhanced peroxidase activity in the presence as well absence of Cu. Highest peroxidase was observed in UIV inoculated seedlings exposed to 750 ppm of Cu.

3,3′-Diaminobenzidine (DAB) stain assay: In the leaves of okra seedlings, brown spots formed by DAB stain, were observed in response to Cu exposure, revealing the presence of H_2O_2 (Fig. 10). Intensity and area of brown spot increased with increase in the concentration Cu applied in the soil. SK12 inoculated plant leaves alleviated the Cu stress and decrease the H_2O_2 production, which was determined by noticing their spotless tissues after the DAB stain treatment.

Root colonization: Under a light microscope, root colonization potential of the SK12 was examined by observing the lactophenol cotton blue stained root sections (Fig. 11). It was detected that SK12 has effectively colonized in the plant supplemented with endophytic fungal biomasses.

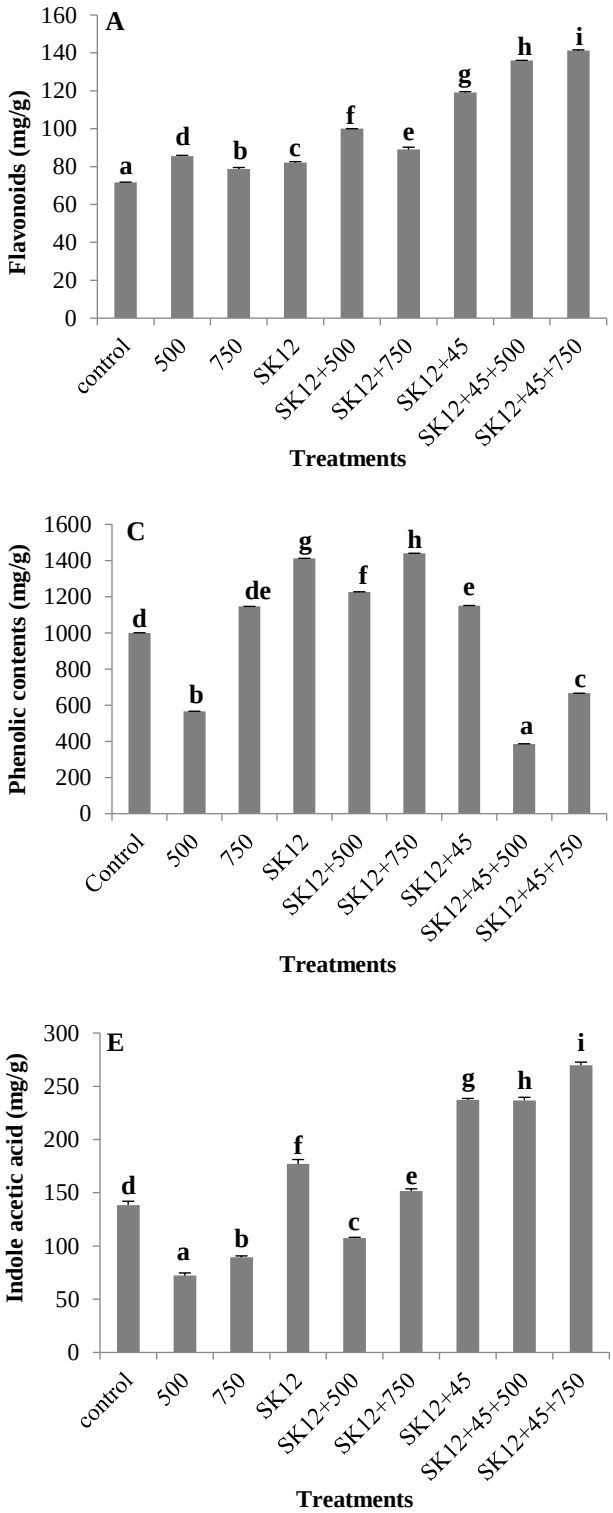

Fig. 7. Determination of (a) Flavonoids, (b) Soluble sugar (c) Phenolic contents (d) Salicylic Acid and (e) IAA in the leaves of in okra seedlings inoculates with wild type (SK12) and mutant SK12(45) *Aspergillus Tamarii* in the presence or absence of various concentration of Cu (i.e., 500 ppm and 750 ppm). Leaves of the seedlings grown for 45 days were used to determine the phytohormones. Bars show meant along with standard error for three replicates. Significant difference among treatment is depicted by different letters ($p<0.05$; using ANOVA and Duncan test). labels; 500, 500 ppm of Cu; 750, 750 ppm of Cu; SK12, endophytic fungus *Aspergillus tamari*; SK12+500, *Aspergillus tamari* and 500 ppm of Cu; SK12+750 *Aspergillus tamari* and 750 ppm of Cu.

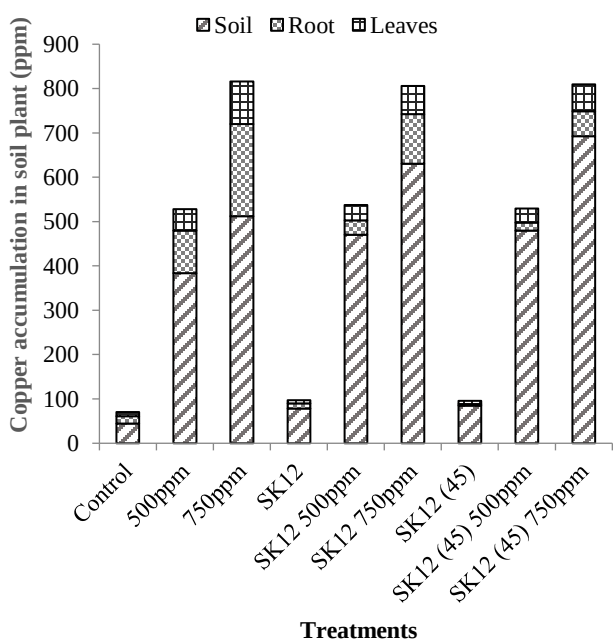

Fig. 8. Effect of wild type (SK12) and mutant (SK12 (45) *Aspergillus tamari* on copper uptake and its translocation to the aerial parts of okra seedlings. Amount of copper present in soil, root and leaves is shown in percentage. labels; 500, 500 ppm of Cu; 750, 750 ppm of Cu; SK12, endophytic fungus *Aspergillus tamari*; SK12+500, *Aspergillus tamari* and 500 ppm of Cu; SK12+750 *Aspergillus tamari* and 750 ppm of Cu.

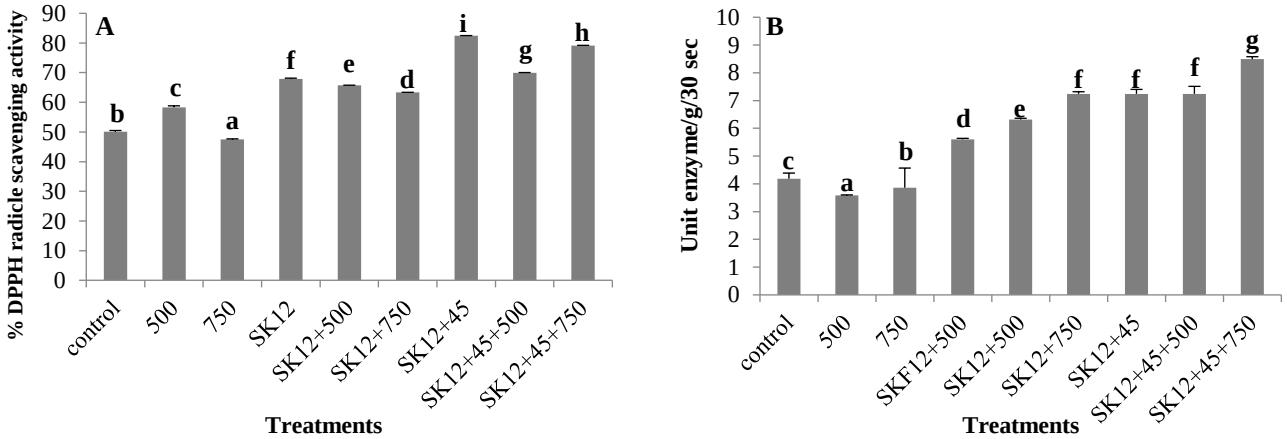

Fig. 9. Effect of Cu stress and inoculation of wild type (SK12) and mutant (SK12(45) *Aspergillus tamari* on A) DPPH in host plants B) Peroxidase. Significance among treatments is shown by labeling means values (three replicates) with different letters ($p<0.05$). labels; 500, 500 ppm of Cu; 750, 750 ppm of Cu; SK12, endophytic fungus *Aspergillus tamari*; SK12+500, *Aspergillus tamari* and 500 ppm of Cu; SK12+750 *Aspergillus tamari* and 750 ppm of Cu.

Discussion

Then endophytes isolated from *Portulaca oleracea*- had variable potential to grow in the presence of 2000- ppm of Cu. This heavy metal is a part of the nutrients supplement utilized by plant and associated microbes for normal growth and development. However, excessive supply of Cu becomes toxic for plants, which is associated with ROS burst. It has been suggested that 20 ppm is permissible in soil used for crop cultivation (Kumar *et al.,* 2021). Based on the Cu tolerance and plant growth promotion SK12 strain was selected for detailed investigation. Its identity was confirmed as *Aspergillus tamari*. This fungus has previously been reported to exist as endophyte and can tolerate Cu (Bhaskar *et al.,* 2020). To improve its ability to resist Cu, culture of A. tamari SK12 were exposed to UV light for various durations and among the 9 ex-irradiant, 1 strain was found to have higher growth in the presence of various concentrations of Cu. For instance, growth of the UIV was 30% greater than the WTS in media containing 2000 ppm of Cu. However, the metal tolerance index of the UIV remained comparable to the wild strain. This observation led to the conclusion that UV radiation had affected he growth related genes and the genes involved in metal tolerance have not been affected. Greater biomass production by UIV was associated with greater release of metabolites like IAA, SA, phenols and flavonoids, under control as well as metal exposure conditions. Beside its role in growth, IAA has been known for its role to interact with metals forming complexes and its indole ring has excellent antioxidant potential, interfering with peroxidation of lipids and proteins (Garcia-Caparros *et al.,* 2021). Salicylic acid is also known as stress response tool, playing a role to reduce the impact of stress in biological systems (Divi *et al.,* 2010). Metal chelation and antioxidant potential of phenols and flavonoids also make the UIV suitable for growth in metal contaminated environment and ideal bioremediating agent (Wang *et al*., 2020). Ultraviolet radiation has ubiquitous role in enhancing the process of evolution due to its ability to induce broad spectrum mutations with high speed. It is because of this potential, that UV radiation can be used to mutate organisms for desired features (Wargent & Jordan., 2013). Previous studies have shown the significance of UR radiation to produce desirable mutant endophytic fungi (Zaki *et al.,* 2021). When the mutant was further analyzed, it had significantly higher quantities of phenols, flavonoids, IAA and SA in its culture. These metabolites are effective assets against Cu stress as they counter the effect of the heavy metal at several fronts. For instance, IAA and SA improve antioxidant production, keeping the ROS level under control. They also, reduce lipid peroxidation and melonaldehyde concentration, which go up in the presence of excessive amount of Cu (El-Tayeb *et al.,* 2006).

Seedlings of *Abelmoschus esculentus* were exposed to different Concentrations of copper resulted in the reduction of root length, shoot length, fresh weight and dry weight. However, co-cultivating the seedlings with wild and UV irradiated variant (UIV) of *Aspergillus tamari* improved root length, shoot length, fresh weight and dry weight. When further investigated, the UIV had more pronounced impact on the growth of host seedlings and even in the presence of elevated levels of Cu the UIV associated seedlings had growth higher than the wild strain as well as control seedlings. This may be attributed to the enhanced growth of the UIV and making higher quantities of beneficial metabolites to the host seedlings exposed to Cu stress. The high growth phenotype had significantly greater levels of SA and IAA. This increase in the endogenous pool of plant growth hormones is one of the mechanisms for plants survival in abiotic stresses (Xiong *et al*., 2022). Increased IAA helps the plants to fight against the heavy metals by regulating the oxidative stress via inducing ROS detoxification enzymes (Paponov *et al.,* 2008). IAA dependent ROS management is done by increasing the activities of antioxidant enzymes. In our study we found that peroxidase was significantly higher in seedlings colonized by the wild and UIV strains. Mutation had enabled the *A. tamari* to enhance the activity of peroxidase of the host seedlings to a level higher than the wild strain. The DPPH assay also indicated that the host defense against ROS was quite strong in the presence UIV. Another reason for strong defense against ROS was the high accumulation of SA in the endophytes colonized seedlings. It has been known from previous studies that SA reduces $OH^{\cdot}$ generation during Cu exposure (Yang *et al*., 2024). This may either be achieved by metal chelation by SA or directly scavenging the radicals. Major site of damage in the presence of heavy metals is cell membrane of the plant cell. Membrane damage is done by peroxidation of its lipids when ROS consumes H^{+} of the cell membrane. As a result of SA induced drop in $OH^{\cdot}$ levels and ROS scavenging triggered by IAA, lipid peroxidation significantly drops (El-Tayeb *et al.,* 2006). These changes in the ROS generation and scavenging enable the seedlings to cope with Cu stress and grow optimally.

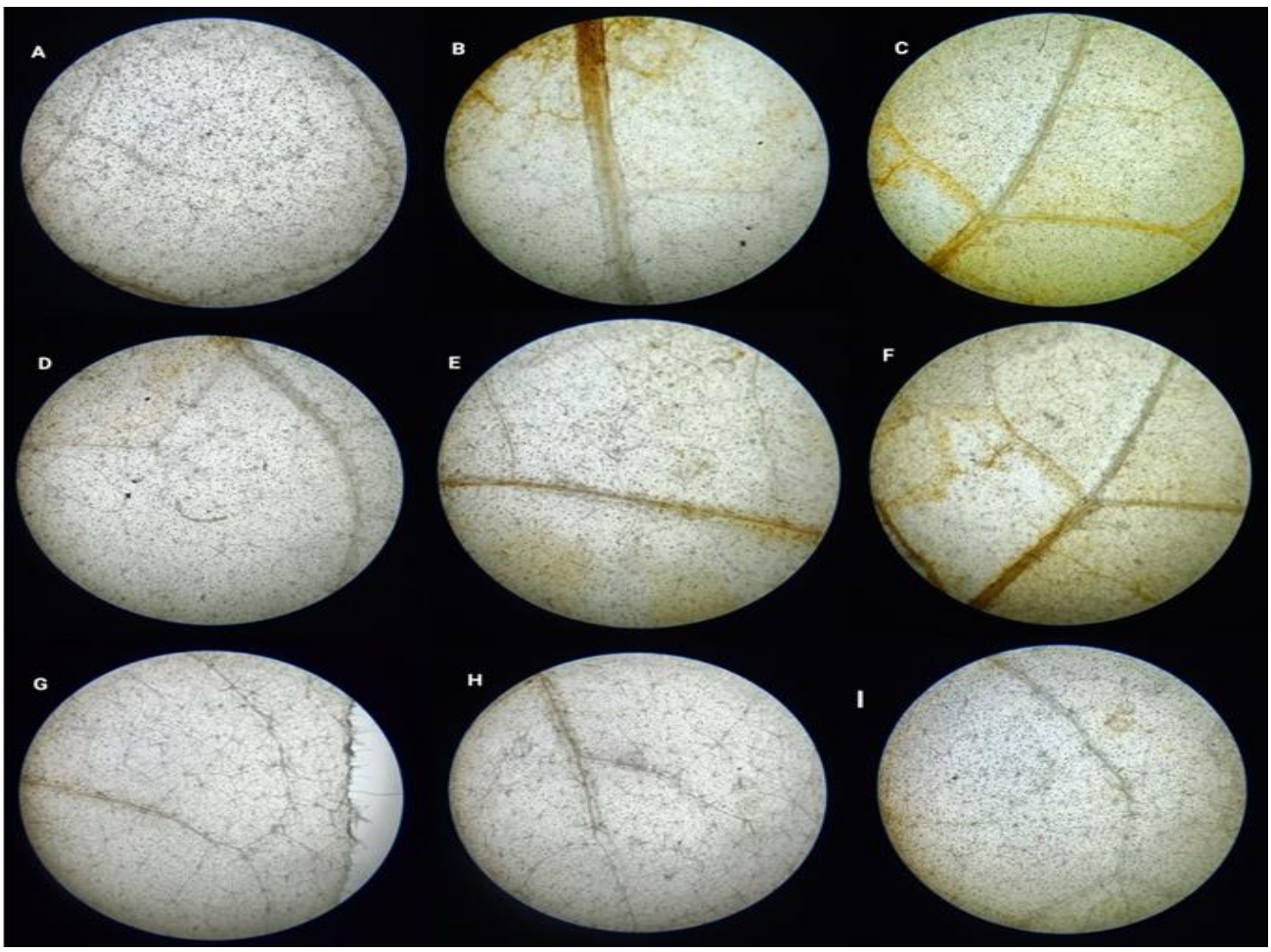

Fig. 10. Accumulation of ROS, depicted by brown spots produced by DAB stain, in the leaves of okra seedlings **A)** control, **B)** 500 ppm Cu, **C)** 750 ppm Cu, **D)** SK12**, E)** SK12+500 ppm**, F)** SK12+750 ppm, **G)** SK12 (45), **H)** SK12 (45) + 500 ppm Cu and **I)** SK12 (45) + 500 ppm Cu.

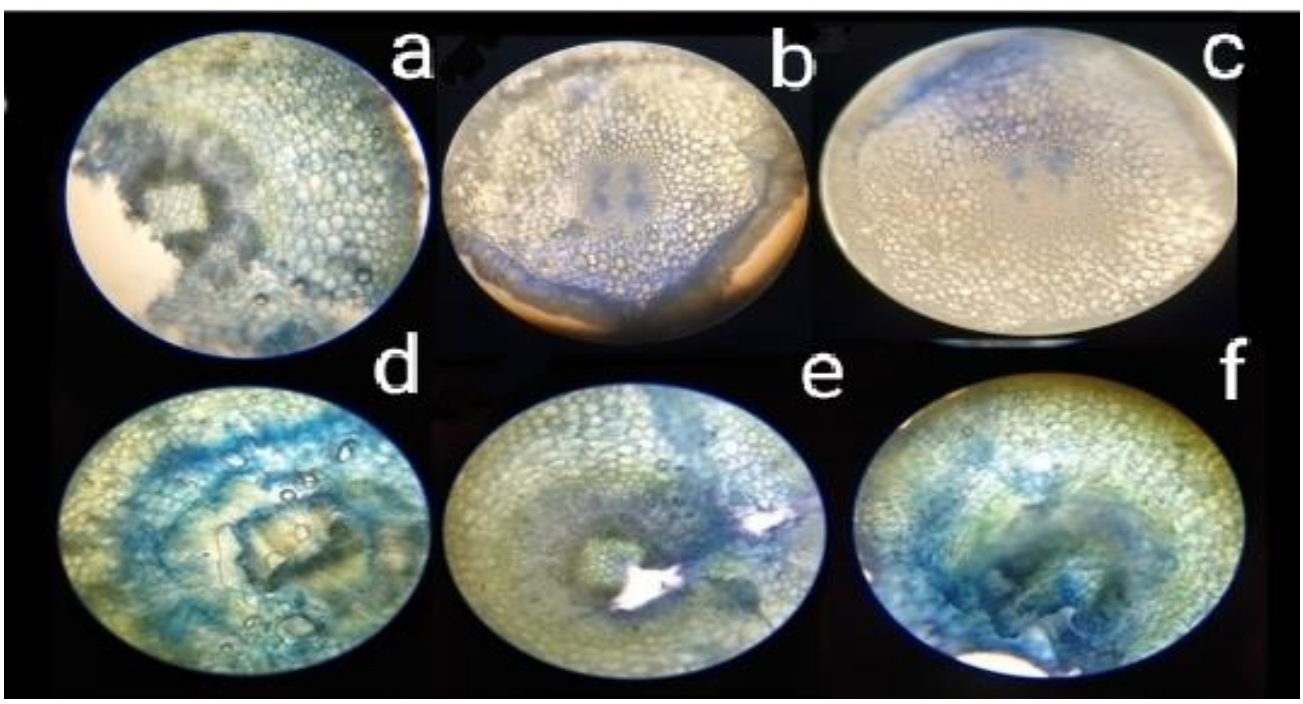

Fig. 11. Root colonization assay in okra seedlings A) SK12, B) SK12 + 500 ppm, C) SK12 + 750 ppm, d) SK12 + 45, E) SK12 (45) + 500 ppm and F) SK12 (45) + 750 ppm. Fungal mycelia were stained in the roots using phenyl cotton blue dye and transverse sections of roots were observed under light microscope (40X magnification).

In case of phenols with the increasing concentration of heavy metals the production of phenol by the plants were also increased. As compared to plants grown under normal condition, the plants grown under abiotic stressed produces higher concentration of phenols (Jańczak-Pieniążek *et al.*, 2022). In case of UV exposed SK12, the production of phenolic contents by the decreased were gradually decreased as increasing copper concentration. In case of flavonoids, plant treated with wild and UIV fungus showed a significant increase in flavonoids under metal stress. In higher concentration of metal stresses UV irritated SK12 have the ability to resist heavy metals (Husna *et al.,* 2021). Flavonoids are a well-known secondary metabolite synthesized by the plant. Their most important role in plant is the protective mechanism under stress. Flavonoids constitute an important component of an antioxidant system in living organisms which is active in smoothing the damaging effect of a number of environmental stresses. They are secondary metabolites to protect the plants and increase plant microbe interaction (Bonacina *et al.,* 2023). In case of soluble sugar, plants grown under normal condition, the soluble sugar contents were gradually decreases with the increasing concentration of copper, interestingly, okra seedlings inoculated with wild SK12 and UV exposed SK12 showed an increasing trend as increased metal uptake. In metabolic events of plants, sugar plays an active role and also participate in regulating genes which is involved in the photosynthesis and metabolism (Wang *et al.,* 2023).

Beside all the above mentioned modification for ROS management and metal chelating compounds, Cu uptake and translocation to the aerial parts was also reduced significantly by A. tamari (both wild and UIV strains). For instance, in the UIV colonized seedlings, Cu uptake was only 50% and 26.92% of the wild strain and control seedlings respectively. Translocation of the metal was also reduced to 65.6% and 62.5% of the wild and control seedlings. Previous studies have shown that root endophyte *Clethra barbinervis* enhances nutrients uptake and decreases heavy metal uptake by the host plants (Yamaji *et al.,* 2016). Endophytes may reduce Cu uptake and their translocation to the shoot parts under excessive Cu conditions (Khan & Lee, 2013).

Conclusion

It is concluded from the current study that *Aspergillus tamarii* have the ability to alleviate heavy metals when exposed to UV light. UV irradiated *Aspergillus tamarii* also enhanced the antioxidant system. *Aspergillus tamarii* to UV irradiation increase the bio-remediating competences of *Aspergillus tamarii* strain SK12.

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