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# AUXIN PRODUCTION AND ANTIFUNGAL ACTIVITY OF BACILLUS SPP. PROMOTING THE GROWTH OF HELIANTHUS ANNUUS L.

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**Abstract**

The rhizobacterial strains were evaluated for auxin production and their effectiveness in improving the growth of Helianthus annuus L. Colorimetric analysis of bacterial auxin showed that MS-1, MS-6, and MS-2 produced 51, 32, and 21.5 µg/mL of auxin respectively at 1000 µg/mL concentration of L-tryptophan. Fourier Transform Infrared Spectroscopy (FTIR) was also performed to detect bacterial auxin production. The ability of bacterial isolates to produce ammonia and hydrogen cyanide was tested. All the strains produced ammonia. In the hydrogen cyanide test, except the MS-10 strain all gave positive results. The biocontrol potential of bacterial strains was also evaluated by performing antifungal assay against Fusarium oxysporum. The strains MS-6 and MS-10 showed significant antifungal activity against F. oxysporum. The pot trials were performed by inoculating the seeds of H. annuus in single and mixed cultures under natural conditions. This inoculation greatly improved the plant growth by increasing the shoot length, fresh and dry weight. The strain MS-6 increased the shoot length by 33.6% when compared with the control. Compared to the control, MS-2 elevated the fresh and dry weight by 58% and 77% respectively. Results showed that rhizobacterial strains have the potential to enhance plant growth and control fungal pathogens in laboratory trials.

**Keywords:** Plant growth-promoting rhizobacteria, Auxin production, FTIR analysis, Antifungal activity, Plant-microbe interactions

## Introduction

The terrestrial environment consists of an important component known as soil. It is a rich source of various organic and inorganic compounds that play an integral role in maintaining biodiversity (Adhikari and Hartemink, 2016). It has been found that soil acts as a habitat for about 25% of diverse living organisms on Earth. Bacteria, fungi, protists, nematodes, and mites form a lively soil community. These living organisms ensure that the soil is fertile and healthy enough to grow plants (Yang et al., 2018).

The region located in the vicinity of plant roots is referred to as the rhizosphere. It is about 2 mm away from the roots of plant (Dotaniya and Meena, 2021). It is mainly inhabited by different bacterial species because of the secretion of root exudates. The exudates secreted by the root system comprise of amino acids, sugars, organic acids, enzymes, and various growth factors. These exudates act as microbial attractants and because of this reason rhizosphere is generally termed a central point of the microbial population (Mohanty et al., 2021).

The bacteria that are naturally found in the rhizosphere of the plants are known as plant growth-promoting rhizobacteria (PGPR). Plant health-promoting rhizobacteria (PHPR) and nodule-promoting rhizobacteria (NPR) are some other names of PGPR (Raj et al., 2020). PGPRs are categorized into two main types on the basis of their location. Extracellular PGPR for instance Pseudomonas and Bacillus are free-living in nature which means they are located on the exterior layer of root hair. Intracellular PGPRs such as Rhizobia and Frankia are symbiotic which means they are located in the root nodules (Sharma et al., 2017).

PGPR are beneficial bacteria that promote plant growth via direct and indirect strategies. These strategies function in a combined way and thus positively impact the growth and yield of plants. Direct strategies include nitrogen fixation, siderophores production, phytohormones production, and solubilization of nutrients such as phosphorus and potassium (Jeyanthi and Kanimozhi, 2018). Indirect strategies include the production of antibiotics, hydrogen cyanide, degradative enzymes, exopolysaccharides, and induced systemic resistance (Etesami and AdI, 2020).

Indole-3-acetic acid (IAA) is the type of auxin extensively produced by bacteria. It has been researched that about 80% of the rhizospheric bacteria produce IAA. Tryptophan acts as a precursor which stimulates the synthesis of IAA. Even a minor concentration of tryptophan is responsible for the production of IAA (Jha and Saraf, 2015). IAA plays its role in the establishment of an extensive root

system. Root system liberates large amounts of exudates and attracts a large population of microbes in the rhizosphere. This fascinating strategy leads to the greater production of IAA. The consumption of nutrients by an extensive root system boosts plant growth (Ahemad and Kibret, 2014). It has been investigated that Lysinibacillus is responsible for increasing the yield of corn crops by the synthesis of IAA (Guerra et al., 2023).

Soil is widely populated by the members of the genus Bacillus. These gram-positive rods are capable of producing spores thus they can survive unfavorable conditions for an extensive period. Bacilli are well-recognized PGPR because they up-regulate the plant growth by the synthesis of plant hormones. They indirectly promote plant growth by the production of induced systemic resistance and bacteriocins. They release certain secondary metabolites such as antifungal compounds that protect the plants against fungal diseases (Sansinenea, 2019). Govindasamay et al. (2011) reported the antifungal activity of Bacillus isolated from the rhizosphere of sorghum against Curvularia lunata.

Sunflower (H. annuus L.) is an economically important oil crop. It is grown across the globe because it can be easily adapted to a wide variety of environmental conditions. It is involved in the mass production of vegetable oil. The seeds of sunflower can be used as confectionaries and livestock feed. Sunflower oil possesses potential roles in the health, energy, and industrial sectors (Rauf et al., 2020).

The present study was oriented to identify the bacterial strains isolated from the rhizospheric soil of mulberry plant (Morus nigra L.) by morphological and biochemical analysis. The growth-promoting potential of the bacterial strains was evaluated by the auxin production, ammonia, and hydrogen cyanide production test. The production of auxin was identified through spectrophotometry and FTIR. The ability of bacterial strains to show antagonistic behavior against fungi was evaluated using a dual culture plate assay. The practical applications of these findings were evaluated by conducting the pot trials using H. annuus L. in natural conditions.

## Materials and Methods

**Sample collection and isolation of rhizobacteria:** One gram of rhizospheric soil samples were collected from M. nigra L. located at different sites at the University of the Punjab. The soil sample was serially diluted and spread over L-agar plates. A total of 10 isolated bacterial colonies were selected after the spread plate method. The bacterial colonies were named from MS-1 to MS-10 and purified using the quadrant streaking technique. The purified bacterial strains were maintained on L-agar plates.

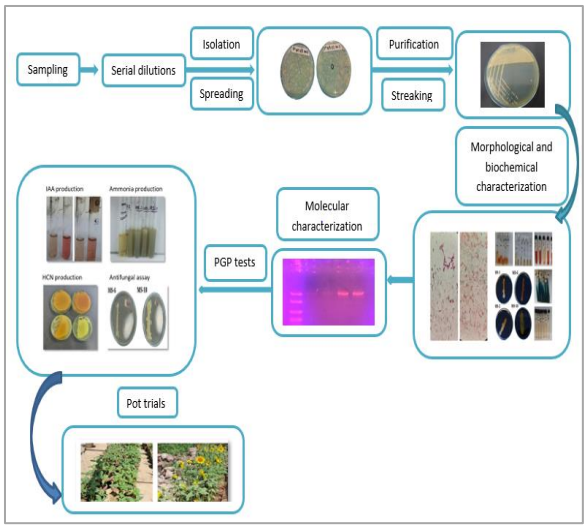


**Fig.1** Flowchart representation of the experiments used to detect the characteristics of isolates obtained from the rhizospheric soil of M. nigraL.

**Characterization of bacterial strains:** The bacterial strains were morphologically characterized using differential staining techniques i.e. gram and endospore staining. The strains were biochemically characterized using different tests such as catalase, oxidase, MR-VP (Methyl red- Voges Proskauer), nitrate reduction, urease, citrate utilization, and starch hydrolysis test. The morphological and biochemical identification was carried out by following the procedures mentioned in the laboratory manual for Microbiology by Cappuccino-Sherman.

**16S rRNA gene sequencing:** The strains were molecularly characterized using 16S rRNA gene sequencing. The bacterial isolates were allowed to grow overnight and their complete genomic DNA was extracted using FavorPrep TM Tissue Genomic DNA Extraction Mini Kit. 16S rNA gene was selected for magnification by Polymerase Chain Reaction (PCR). PCR was executed using forward primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1522r (5′-AAGGAGGTGATCCA (AG)CCGCA-3′). The products obtained by PCR were purified using Thermo scientific GeneJET Gel Extraction Kit. The purified products were finally sequenced using forward primer.

**Estimation of bacterial auxin production:** The quantity of auxin was estimated by inoculating the bacterial strains in 20 mL L-broth supplemented with

0 and 1000 µg/mL of L-tryptophan. The experiment was conducted in replicates. The strains were cultivated at 37°C for 72 hours on a shaker operated at 120 rev/min. The bacterial suspensions were centrifuged after 72 hours to attain the stationary phase cultures. Two mL of Salkowski reagent was added into the falcon tube containing 1 mL supernatant. The tubes were kept in the dark for about 30 minutes for the development of pink to red color. The intensity of color was detected by measuring the absorbance of the bacterial suspensions at 535 nm. The quantity of auxin produced by bacterial strains was obtained using a standard curve.

**FTIR analysis for bacterial auxin:** The colorimetric evaluation of bacterial auxin was validated using FTIR. The bacterial strains were grown in 20 mL L-broth containing 1000 µg/mL of L-tryptophan. The strains were cultivated at 37°C for 72 hours on a shaker rotating at 120 rev/min. The supernatant (pH 2.5-3) obtained after centrifugation was added in equal amount of ethyl acetate and shaken vigorously. After 24 hours, the ethyl acetate layer was drawn out and shifted to the beaker placed in a water bath set at 60°C. The purified auxin extracts were restored in 2 mL methanol after the complete vaporization of ethyl acetate. The auxin extracts and standard IAA were subjected to FTIR.

**Antifungal assay:** A dual culture plate assay was performed to detect the antifungal activity of rhizobacterial strains. At a distance of 2 mm from one end of the plate, the strains were streaked on potato dextrose agar. The bacteria were allowed to grow overnight at 37°C. After 24 hours, a plug of F. oxysporum was positioned at a distance of 2 mm from the other end of the plate. The fungus was allowed to grow at 28°C for 5-7 days. A plate with only fungal growth was used for comparison. The zone of inhibition was observed after 7 days.

**Pot trials under normal conditions:** The bacterial suspensions adjusted to 107 colony forming units (CFU) were prepared by introducing fresh bacterial colonies in 10 mL autoclaved distilled water. This absorbance of suspensions was regulated according to McFarland standard 2. The seeds of H. annuus L. were surface sterilized using 0.1% mercuric chloride solution. The seeds were dipped in the solution for 2 minutes and then autoclaved distilled water was used to eliminate any remains of mercuric chloride. Sterile seeds were submerged in the bacterial suspensions for about 30 minutes. The seeds submerged in sterile deionized water were used for comparison. A total of 30 pots were arranged for the pot trial involving both single and mixed culture inoculations. Ten seeds were sown in each pot and the pots were watered regularly during the trial. Growth parameters such as shoot length, fresh and dry weight were recorded after harvesting.

**Statistical analysis:** The data obtained for the production of bacterial auxin and the parameters of plant growth was subjected to ANOVA by using SPSS Version 28. The mean values were separated using Duncan's multiple range test ($p \leq 0.05$).

**Production of ammonia:** The bacterial strains were grown in 10 mL peptone water. 1 mL of Nesseler's reagent was added to the bacterial cultures after incubation. The development of yellowish to brown color was observed.

**Production of hydrogen cyanide:** The bacterial colonies were streaked on nutrient agar plates containing glycine. The sterile filter papers were soaked in a solution containing sodium carbonate and picric acid. The filter sheets were positioned on the agar surface and the plates were incubated. The change in color of the filter paper was observed.

## Results

**Characterization of bacterial strains:** All the strains were Gram-positive. All the strains were spore formers except MS-7, MS-8, and MS-9. Biochemical characterization was subjected to MS-1, MS-2, MS-5, MS-6, and MS-10 strains. All these strains were positive for catalase, oxidase, methyl red, and citrate utilization tests. The strains were negative for the Voges-Proskauer and urease test. In case of starch hydrolysis test, MS-5, MS-6, and MS-10 were capable of degrading starch as zone of inhibition was observed. MS-1 and MS-2 strains showed negative results as they were not capable of degrading starch.

**16S rRNA gene sequencing:** Molecular characterization was subjected to MS-6 and MS-10 strains. 16S rRNA gene sequences of these strains showed the highest percent identity with Bacillus sp. and Bacillus safensis respectively.

**Estimation of bacterial auxin production:** All of the ten rhizobacterial isolates developed a pink to red color with the addition of Salkowski reagent. This color development pointed towards the fact that all strains were capable to produce auxin. At 0 µg/mL concentration of L-tryptophan, MS-2, MS-10, and MS-6 produced 11, 9 and 8 µg/mL of auxin respectively. At 1000 µg/mL concentration of L-tryptophan, significant levels of auxin were produced by MS-1 (51 µg/mL), MS-6 (32 µg/mL), and MS-2

(21.5 µg/mL). The results indicated that bacteria synthesized a greater amount of auxin in the medium containing L-tryptophan as compared to the medium without L-tryptophan.

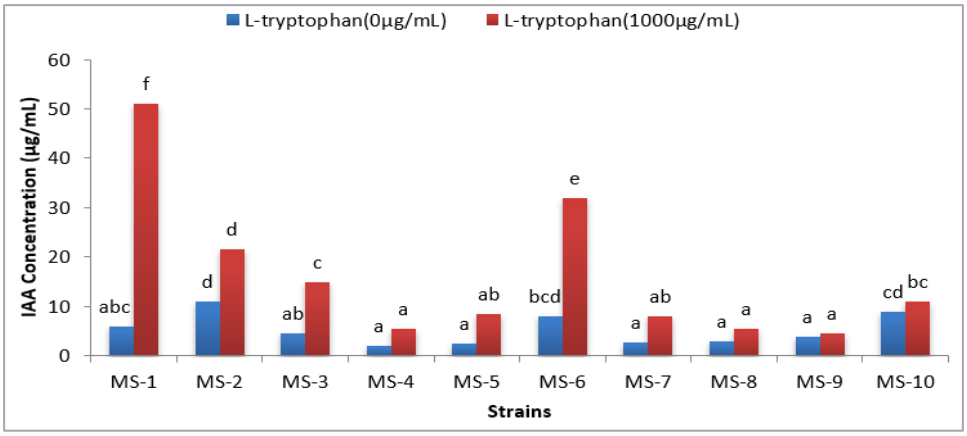


**Fig.2** In-vitro auxin production by bacterial strains at different concentrations of L-tryptophan. Colored bars represent the mean of 2 replicates.

**FTIR analysis of bacterial auxin:** The spectral analysis of IAA derived from the auxin-producing isolates, namely MS-6 and MS-10, was carried out together with synthetic IAA. For the strains, MS-6 and MS-10, a characteristic peak of the OH group was obtained at 3317.63cm-1. The C-N stretch was observed at 1021.46 cm-1. The characteristic aromatic ring peak appeared between 1425 and 1500 cm-1, representing (C=C). The carboxyl peak appeared between 2850 and 2950 cm-1, which typically represents COOH. All these peaks closely matched the IAA standard.

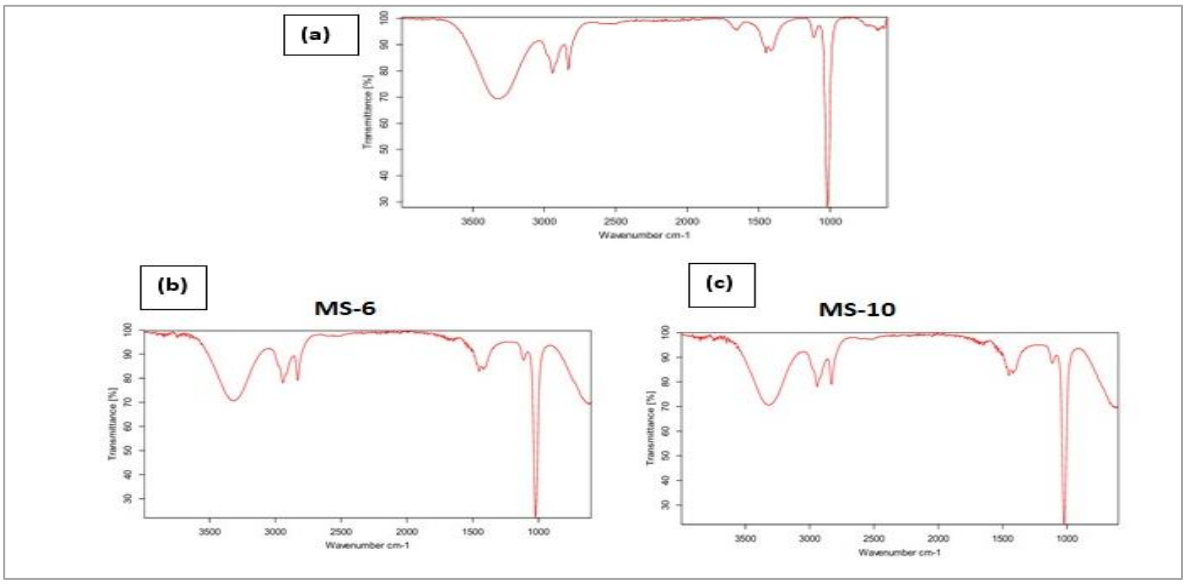


**Fig.3** Spectra obtained through FTIR analysis. (a) Standard IAA (b) IAA extracted from MS-6 strain (c) IAA extracted from MS-10 strain

**Ammonia and HCN production:** MS-1, MS-2, MS-6 and MS-10 strains were tested positive for ammonia production as they indicated brown color on the incorporation of Nesseler's reagent. In HCN production test, MS-1, M2-2, and MS-6 strains showed positive results as the color of the filter sheet

appeared brown after incubation. The intense brown color was observed in case of MS-2 strain. The negative result was seen for MS-10 strain as the yellow color of filter sheet remained the same.

**Antifungal assay:** MS-6 and MS-10 strains showed significant antifungal activity against F. oxysporum. The fungal zones of inhibition were clearly visible in the case of both strains.

**Pot trials under normal conditions:** Single and mixed cultures were used for the pot trials. MS-1, MS-2, MS-6, and MS-10 strains were used as single cultures. Mix 1 (MS-1+6), Mix 2 (MS-1+10), Mix 3 (MS-2+6), and Mix 4 (MS-2+10) were used as mixed cultures. The shoot length and shoot fresh weight were recorded after harvesting the plants. The plants were dried at 80°C for 24 hours for the measurement of dry weight. A significant increase in shoot length was observed for MS-6 (33.6%), Mix 2 (29.1%) and MS-2 (19.4%) when compared with the control. In comparison with the control, the highest response for fresh weight was recorded by MS-2 (58.1%), Mix 2 (47.7%) and MS-10 (25.4%). The highest level of dry weight was recorded in the case of MS-2 (77%), Mix 2 (64.9%), and Mix 4 (32.2%) when compared with the control.

**Table.1** Effect of inoculations of rhizobacterial strains on shoot length, shoot fresh and dry weight of H. annuus L. under wire house conditions

| ***Strains*** | ***Shoot Length (cm)*** | ***Fresh Weight (g)*** | ***Dry Weight (g)*** |
|---|---|---|---|
| Control | 41.25±1.25(ab) | 7.63±0.57(ab) | 3.48±0.25(bcd) |
| MS-1 | 47.12±2.91(abcd) | 7.64±1.48(ab) | 4.01±0.81(abcd) |
| MS-2 | 49.25±3.91(bcd) | 12.06±2.84(b) | 6.16±1.50(d) |
| MS-6 | 55.12±4.10(d) | 8.98±1.12(ab) | 4.21±0.70(abcd) |
| MS-10 | 48.12±1.27(bcd) | 9.57±1.70(b) | 4.97±0.94(bcd) |
| Mix 1 | 47.25±1.03(abcd) | 7.39±0.80(ab) | 2.96±0.23(ab) |
| Mix 2 | 53.25±2.65(cd) | 11.27±1.38(b) | 5.74±1.04(cd) |
| Mix 3 | 39.00±1.48(a) | 4.46±0.61(a) | 2.12±0.18(a) |
| Mix 4 | 45.00±3.00(abc) | 9.48±1.00(b) | 4.60±0.71(abcd) |

"Mean and ± S.E of eight replicates, different letter indicates significance difference among mean value using Duncan's multiple range test ($p \leq 0.05$).

**Discussion**

The present study was carried out to assess the growth potential of rhizobacterial strains isolated from M. nigra L. The strains were identified using morphological and biochemical tests. The strains were Gram-positive and spore formers. It means that they possess thick layer of peptidoglycan in their cell walls and are capable to bear stressed conditions. Toyota (2015) declared spore-forming bacteria as potential growth promoters in contrast to non-spore-forming bacteria.

PGPR are well known for improving the growth and yield of plants by the production of various substances such as lytic enzymes, siderophores, antibiotics and phytohormones (Tanveer and Ali, 2022). IAA is a widely studied and well-known phytohormone responsible for various functions such as differentiation of cells, formation of vascular tissues along with side roots and apical dominance (Iqbal et al., 2016). In the current study, all the ten bacterial strains were tested for the production of auxin utilizing the techniques of colorimetric and FTIR analysis. The spectra obtained by FTIR analysis

of pure auxin extracts of MS-6 and MS-10 strains were similar to the spectra of manufactured IAA standards in the current study. In case of in-vitro auxin production, the strains displayed variable values of IAA at 0 and 1000 µg/mL concentrations of L-tryptophan. The significant levels of auxin were produced by MS-1 (51 µg/mL) and MS-6 (32 µg/mL) at 1000 µg/mL concentration of L-tryptophan.

Yilmaz et al. (2022) published the results for auxin production by the PGPR strain. He found out that the strain produced 0.76 µg/mL of auxin in the medium without L-tryptophan whereas; it produced 6.37 µg/mL of auxin in the medium with L-tryptophan. Kumar et al. (2021) reported that certain Rhizobium strains generate a greater amount of auxin in the medium containing tryptophan. The same pattern of results was seen in the existing research. The strains produced higher levels of auxin in the medium supplemented with L-tryptophan as compared to the medium without L-tryptophan. For instance, MS-6 produced 8 µg/mL of auxin in the medium without L-tryptophan whereas; it produced 32 µg/mL of auxin in the medium with L-tryptophan.

Ammonia production is a vital plant growth-enhancing trait exhibited by PGPR. Nitrogen is a necessary element required by the plants. PGPR fulfills this requirement by accumulating and supplying nitrogen to the plants thus positively impacting the plant growth (Arora et al., 2013). Hyder et al. (2020) isolated the bacterial strains from the rhizospheric soil sample of chilli pepper and evaluated the ammonia-producing ability of these strains. Except for one, all the strains were capable of generating ammonia. Kumari et al. (2018) reported the promising results of ammonia production by the rhizobacterial strains obtained from mung beans. A similar trend of results was seen in the existing research. All the strains produced ammonia when grown in peptone water.

One of the most important indirect mechanisms of PGPR is cyanogenesis i.e. the production of toxic compound HCN. This agent protects the plants from weeds and thus plants can get adequate amount of nutrients and space (Ahmad et al., 2008). The present study evaluated the ability of bacterial strains to produce this toxic metabolite HCN. The majority of the strains tested positive for this experiment. Agustiyani et al. (2022) reported that out of 15, 8 rhizobacterial strains tested positive for HCN production.

Sunflower is known to be susceptible to the fungal attack caused by F. oxysporum. PGPRs such as Bacillus play their protective role in the production of antifungal compounds and thus improve the growth of plants (Hernández-Morales et al., 2018). Khan et al. (2018) declared B. subtilis as a potential antifungal agent against F. oxysporum. A research conducted by Akintokun and Taiwo (2016) revealed that B.subtilis and P.aeruginosa were capable enough to protect tomato plant from the fungal attack. In the current study, MS-6 and MS-10 strains showed a visible zone of inhibition in response to F. oxysporum.

The pot trials in the present research project showed that both single and mixed culture inoculations on the seeds of H. annuus L. improved the plant growth. MS-6 strain enhanced the shoot length by 33.6% when compared with the control. MS-2 strain improved the fresh and dry weight by 58.1% and 77% respectively on comparison with the control. Singh et al. (2019) published that rhizobacterial inoculations enhanced the vegetative features of sunflower from 0.25 to 0.8 times when compared with the control. Patel et al. (2023) declared that Bacillus sp. enhanced the length of shoot and root along with the quantity of leaves of groundnut and chickpea. Bacillus is a well-recognized rhizobacterial strain that promotes the plant growth and yield by the production of auxin and antifungal compounds (Saxena et al., 2020).

## Conclusion

The results of this study suggested that there is a strong relationship between the concentration of L-tryptophan and bacterial auxin production. Bacillus sp. i.e. MS-6 and MS-10 can be used as biocontrol agents as they exhibit significant antifungal activity. The single and mixed culture inoculations enhanced the growth parameters of H. annuus L. that suggest their potential as biofertilizers. Application of Bacillus sp. is an excellent approach to tackle the adverse effects of chemical fertilizers and for the development of a sustainable agricultural system.

## Contribution of authors:

Sadia Malik conducted the experiments, collected the data and prepared the first draft of the manuscript. Basharat Ali provided the experimental layout and thoroughly analyzed the manuscript.

## Conflicts of interest:

There are no conflicts of interest among the authors.

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