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Exploring the supremacy of *Bacillus* species among sunflower endophytes as a promising bio stimulating tool

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Contribution	Bashir, S. designed the study and wrote the research paper, S. Hasnain & A. Iqbal assisted in the analysis of the quantum yield of plants.

Compromising food production due to the adverse effects of frequently utilized agrochemicals is one of the alarming issues of the world to be resolved quickly. However, the employment of plant bacterial endophytes as bio stimulants is a valuable strategy for the improvement of crop health and ultimately for large-scale ecological farming with the perspective of strengthening the world's food security. The occurrence of endophytic bacteria in close vicinity of plants may reflect their crucial role in the growth enrichment of beneficially associated crops. This study aimed to establish the phylogeny of native bacterial species of sunflowers isolated from the endosphere and to assess their potential to promote plant growth by phenotypic approaches. For this purpose, 10 bacterial isolates were scrutinized for their growth promotion ability on manual re-inoculation into the native plant of sunflower (*Helianthus annuus* L.). Three different genera (*Bacillus, Priestia* and *Paenibacillus*) sharing the phylum of Firmicute with the supremacy of *Bacillus* species were identified by 16SrRNA phylogeny. *In Vitro* study has revealed their varying plant growth promotion abilities, with the foremost of auxin (46-104 µg/mL) and of siderophore (15-50 µg/mL) productions. Results in planta experiments, publicized the dominancy of bacillus inhabitants as promising bio stimulants for plant growth, reducing the chemical implementation in agricultural practices.

Keywords: Sunflower, Bacillus endophytes, Auxin, bio stimulants, plant growth promotion.

INTRODUCTION: One of the foremost tasks is the fulfilment of worldwide food production. To accomplish this task, the application of plant-associated growth-promoting bacteria has been a certified practice both under natural conditions as well as under stress-induced environments (Sharma *et al.*, 2008). Agriculture sustainability being the difficult problem can be very well solved by the employment of nutrient drivers of host plants. With the aim of this, the use of multiple microbial species as bio-inoculants has been an extensive global practice for many decades. Several studies have highlighted the significance of endophytic microbes as crop growth promoters which consecutively improve main crops (Yadav *et al.*, 2018). This emerging trend of applying endophytic bacterial growth promoters is due to the reason for their introduction into the plant tissues and organs which establishes their suitable strong connections with host plants as compared to other epiphytic or even rhizospheric bacteria. Moreover, these endophytes reside at entry sites or can spread into entire plant tissues without eliciting injurious alterations in them (Abdallah *et al.*, 2018). Deep inside plant tissues, bacterial endophytes make strong associations either directly or indirectly with the host plants. The most promising mechanisms of action for growth promotion include the fixation of nitrogen, solubilization of phosphorous, and production of siderophores, HCN and auxins (Yadav *et al.*, 2018). Previous research has been conducted on the study of bio-fertilizers, revealing the role of these microbes as providers of essentially required nutrients to various crops which become adequate for the enrichment of growth and yield of crops (Yadav *et al.*, 2018). Among the vitally acting endophytes in the host plants, the role of *Bacillus* species can never be ignored. *Bacillus* is the most abundantly occurring genera and its variant species have symbiotically prevailed among the host flora. The eco-friendly role of *Bacillus* species in specific association with agronomic crops was confirmed (Lacava *et al.*, 2022). In addition to their fundamental biotic roles, these are also known for abiotic tolerance in plants (Tsotetsi *et al.*, 2022). Thus, the dominancy of *Bacillus* genera can confer multifarious traits into the beneficially accompanying vegetation (Zahra *et al.*, 2023).

In worldwide agriculture, sunflower has been considered the fourth most important oil seed crop, yielding a large amount of oil per hectare area of land. Although Pakistan is an oil-producing country among others, but still its increasing demand does not meet the requirement for edible oil within the country. However, the provincial history of the homeland, regarding the annual production of sunflowers has declared Punjab as the top most producing province, executing the highest annual (2022-23) productions of the crop in tones, in the particular regions of D. G Khan (19433), Muzaffargarh (14534), Bakkhar (6493), Sialkot (6032) and Narowal (3110) according to Ministry of National Food Security and Research (GOP, 2022-2023). Hence, it has to import sunflower oil at the domestic level. However, large-scale production of oil-yielding sunflower varieties/cultivars can be a suitable strategy to rationalize the national oil requirements. Irrespective of its economic status, there is inadequate literature about PGP inoculations in sunflower crops (Majeed *et al.*, 2018).

OBJECTIVES: The present study is based on the following objectives:

(i) Isolation of bacterial natives of sunflower.
(ii) Molecular identification of super dominant bacterial genera of the crop.
(iii) *In Vitro* investigation of isolates for plant growth promoting traits.
(iv) Exploration of whether native bacterial endophytes upon re-inoculation promote the growth and yield of sunflower as safer bio-stimulants or not.

MATERIALS AND METHODS: Purification of native bacterial species: Three plant parts (leaf, stem and roots) of two different varieties named Hysun-33 & Hysun-39 of sunflower were considered for the study of isolation. These plant parts in triplicates were washed with running tap water and sterilized with 70% ethanol and finally the maceration of tissues using autoclaved mortar and pestle. Subsequently, these triplicate samples of each plant tissue were further subjected to the isolation of endophytic bacterial natives using nutrient agar as the growth medium. Following the incubation at a suitable temperature of 37°C for three consecutive days, the bacterial colonies were purified and considered for further study (Bashir *et al.*, 2020).

Identification of isolated bacteria: The isolated bacteria were initially identified by following the basic chemical protocol of gram staining (Giuliano *et al.*, 2019). Before the phylogenetic relationship of isolates, freshly cultured bacterial strains were sent to MACROGEN Korean and confirmed with 27F (5′ AGA GTT TGA TCA TGG CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) primers (Bashir *et al.*, 2021). The obtained partial gene sequences of 16S ribosomal RNA were converted into FASTA format and subjected to Basic Local Alignment Tool (BLAST) with further alignment by using the Muscle algorithm. These sequences were deposited in the National Center for Biotechnology Information (NCBI) data repository for identification of their respective GenBank accession numbers. The phylogenetic association between isolated strains and their type strains was constructed in the form of a maximum likelihood tree made with software Molecular Evolutionary Genetics Analysis (MEGA) Version 7.0 (Nel *et al.*, 2020).

***In Vitro* analysis of isolates for growth promotion potential of plant:** To judge the potential of bacterial isolates for their growth promotion in plants, auxin (Bashir *et al.*, 2021), siderophore (Castaldi *et al.*, 2021), HCN (Zainab *et al.*, 2021), nitrogen fixation (Jain *et al.*, 2021) and phosphate solubilization (Bhattacharyya *et al.*, 2020) tests were considered.

Quantification of auxin and siderophores: Furthermore, auxin and siderophores produced by 10 purified bacteria were quantified. For quantification of auxin, autoclaved Luria Bertani broth (Tryptone: 10g/L, Yeast extract: 5g/L and NaCl: 10g/L) well supplemented with L-tryptophan (0.1g/L) was used for inoculation of fresh bacterial cultures with 3 consecutive days of shaking at 37°C. Then, fully grown bacterial broths were immediately centrifuged at 7000 rpm for 10 min. and a change of colour was observed for auxin production with the addition of a reagent named Salkowski. At 530 nm wavelength, these supernatants were analysed under the spectrophotometer (Myo *et al.*, 2019). For the effective estimation of siderophores, bacterial samples were grown in a succinate medium for 2 days of growth at 27°C. Following the centrifugation, pellets were prepared at 9,000 rpm for 20 min. These pellets were discarded and the supernatant was utilized for spectrophotometry analysis (Nithyapriya *et al.*, 2021).

***In Planta* analysis of isolates for growth promotion potential of plant:** For this, the seeds (100g) of Hysun-33 and Hysun-39 were sterilized using mercuric chloride (0.1%) with frequent washings of ethanol (70%). Afterwards, seeds were soaked into bacterial suspensions (each with a CFU count of 10^9). Then these seeds were sown into the garden soil of sandy loam in texture having suitable physical properties (organic matter 0.40%, electrical conductivity 1.27 mS cm^{-1}, potassium 100 mg kg^{-1}, phosphorous 4.15 mg kg^{-1}, nitrogen 0.60 mg Kg^{-1}, pH 8.9), keeping one-inch depth and the distance of 25cm between the seeds. Appropriate watering and thinning were followed throughout the growing period. At 3 successive harvests: I (15 days of age), II (90 days of age) and III (160 days of age), multiple growth parameters (shoot & root lengths (cm/plant), shoot & root fresh and dry weights (gm/plant), relative water content (%), quantum yield (Fv /Fm) and total oil content (%) were measured. The relative content of water was calculated with the help of a formula given by Chen *et al.* (2022):

$$Relative\ content\ of\ water = \frac{Difference\ between\ fresh\ \&\ dry\ weight\ of\ leaves}{Difference\ between\ saturated\ \&\ dry\ weight\ of\ leaves} \times 100$$

Whereas, the quantum yield was measured by using Fluor Pen (FP100) as Fv /Fm ratio. For estimation of total oil content, the seed samples collected at harvest III of the plant were sent to Ayyub Research Institute (Faisalabad). The experiment was repeated thrice to obtain accurate results.

RESULTS: Purification of native bacterial species: On purification, a total of 189 bacterial colonies were successfully separated from multiple parts of the Hysun-33 & Hysun-39 varieties of sunflower. In the current study out of the total, 10 bacterial colonies labelled as SF-11 to SF-20 were considered.

Identification of isolated bacteria: When microscopy was done following the gram staining procedure, 10 bacterial colonies were identified as gram-positive type with their rod shapes. Further analysis by 16S ribosomal RNA sequencing displayed partial sequences which were utilized for sequential FASTA conversion and BLAST analysis. The accession numbers were presented in table 1. A tree represents phylogeny with the common phylum of Firmicute (figure 1). However, variations at genus and species level were found among these isolates. As there were three varying genera: *Bacillus* (MH475934, MH475935, MH475936, MH475937, MH475938, MH475940, MH475941, MH475943), Paenibacillus (MH475939) & Priestia (MH475942) and also eight different species among these bacterial natives: MH475934 (*Bacillus xiamenensis*), MH475935 (*B. maritimus*), MH475936, MH475937 and MH475938 (*B. licheniformis*), MH475939 (*Paenibacillus alvei*), MH475940 (*B. subtilis*), MH475941 (*B. albus*), MH475942 (*Priestia koreensis*), MH475943 (*B. glycinifermentans*) (figure 1). These results also depicted the supremacy of *Bacillus* species among the sunflower microflora.

***In Vitro* analysis of isolates for growth promotion potential of plant:** All bacterial isolates produced auxin and siderophores. Whereas, HCN production was found by a few strains (SF12, SF15, SF16, SF18, SF19 and SF20). In addition to these results, nitrogen fixation was found only in (SF12 & SF17) strains, while (SF11, SF15, SF18 & SF19) strains appeared to solubilize phosphorous (figure 2).

Quantification of auxin and siderophores: Bacterial strains: SF19 (104.6 μg/mL), SF18 (94.2 μg/mL) and SF16 (91 μg/mL) were quantified as the highest auxin producers. Whereas other strains (SF11, SF12, SF13 SF14, SF15, SF17, and SF20) produced auxin ranging from 46.1-86.7 μg/mL as compared with the control (10.6 μg/mL). Upon comparison with the control (5.1 μg/mL), siderophore quantification displayed significant values for siderophore productions (50 μg/mL by SF19), (45 μg/mL by SF18) and (44 μg/mL by SF17) strains with the minimum production (15μg/mL) by SF14 strain (figure 3).

***In Planta* analysis of isolates for growth promotion potential of plant:** In the experiments of growth-promoting potential of inoculated bacterial strains on two cultivars of sunflower, multiple vegetative parameters (shoot & root fresh and dry weights, shoot & root lengths, leaf area and relative water content) of inoculated plants were recorded as compared with an un-inoculated control plant (figure 4 and 5).

Sr No.	Isolates	Species	GenBank Accession No.
1	SF-11	*Bacillus xiamenensis* strain VITBJ4	MH475934
2	SF-12	*B. maritimus* strain KS16-9	MH475935
3	SF-13	*B. licheniformis* strain IEB-8	MH475936
4	SF-14	*B. licheniformis* strain FJAT-46659	MH475937
5	SF-15	*B. licheniformis* strain SS1	MH475938
6	SF-16	*Paenibacillus alvei* strain RG05	MH475939
7	SF-17	*B. subtilis* strain JP2	MH475940
8	SF-18	*B. albus* strain FS1	MH475941
9	SF-19	*Priestia koreensis* strain 3441BRRJ	MH475942
10	SF-20	*B. glycinifermentans* strain JTYP3	MH475943

Table 1: Molecular identification of isolated bacteria by 16S rRNA.

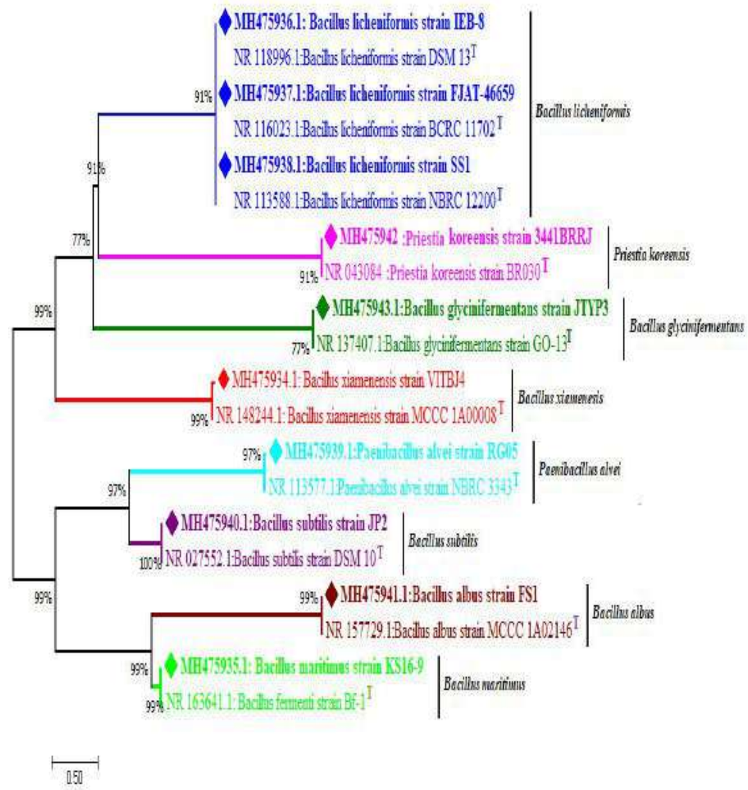

Figure 1: Phylogenetic analysis of bacterial isolates.

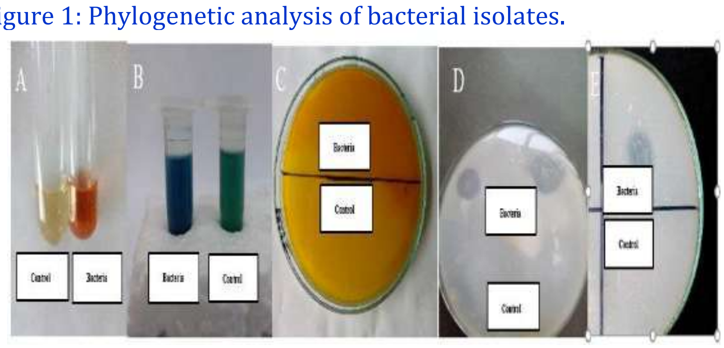

Figure: 2 *In Vitro* analysis of bacterial isolates for PGP traits.

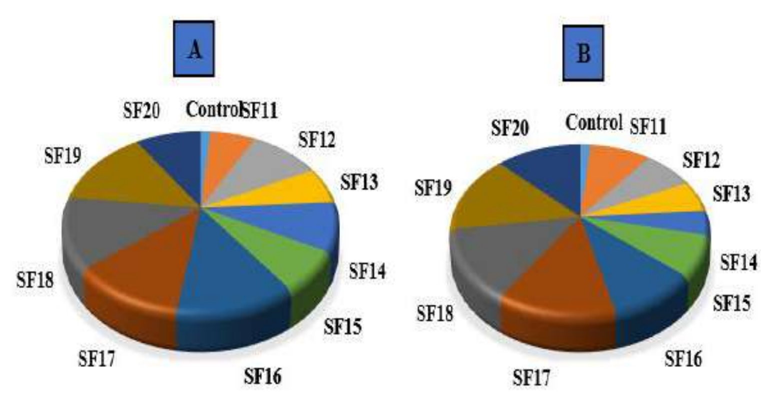

Figure 3: Quantification of auxin (A) and siderophore (B) productions by bacteria isolates.

Although all bacterial inoculations have caused increments in the growth of sunflower varieties as compared to control (figure 5). However, comparing the effects of all bacterial inoculations at all 3 harvests of 2 cultivars, there is a marked increase in relative water content (87-95%) and leaf area (23-431cm^2) of the 2 cultivars proceeding from 1st harvest to 3rd harvest upon comparison with control as shown in figure 4 and 5. Hence the growth of Hysun-39 inoculated plants is obvious as compared to the Hysun-33 variety. In addition to these results, when the yield parameters (quantum yield and total oil content) of two cultivars were measured under the effect of bacterial inoculations, progressive enhancement was recorded in quantum yield {(4-14 Fv/Fm in Hysun-33 & 0.5-16 Fv/Fm in Hysun-39)} b and total oil content {(9-16% in Hysun-33 and 10-43% in Hysun-39-%)}, specifically at final harvest as compared to control ones (figure 6).

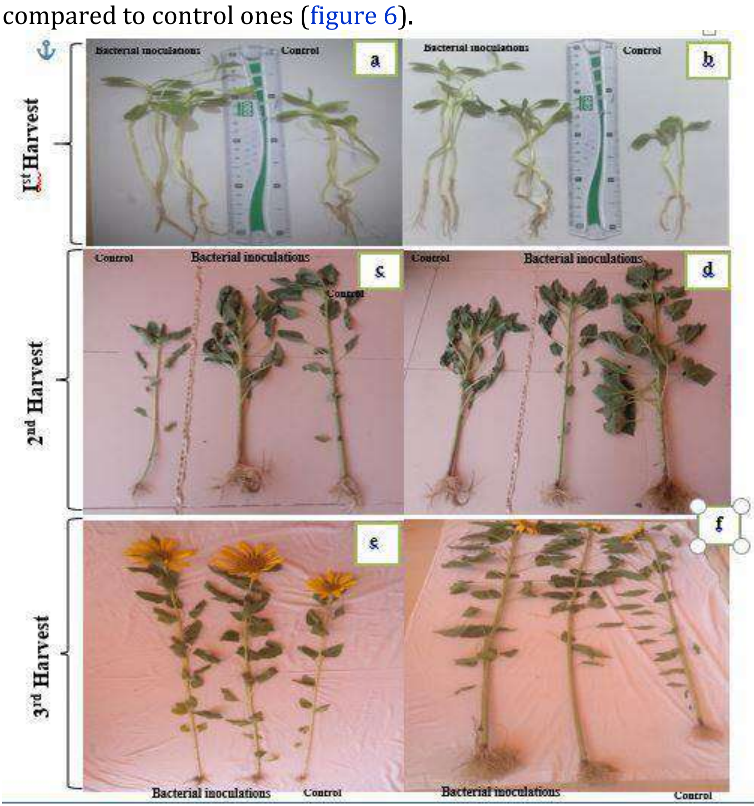

Figure 4 Effect of bacterial inoculations on 3 harvests of 2 varieties of sunflower.
Where a=Hyun-33, b= Hysun-39 of Ist harvest, c=Hyun-33, d= Hysun-39 of 2nd harvest, e=Hyun-33, f= Hysun-39 of 3rd harvest.

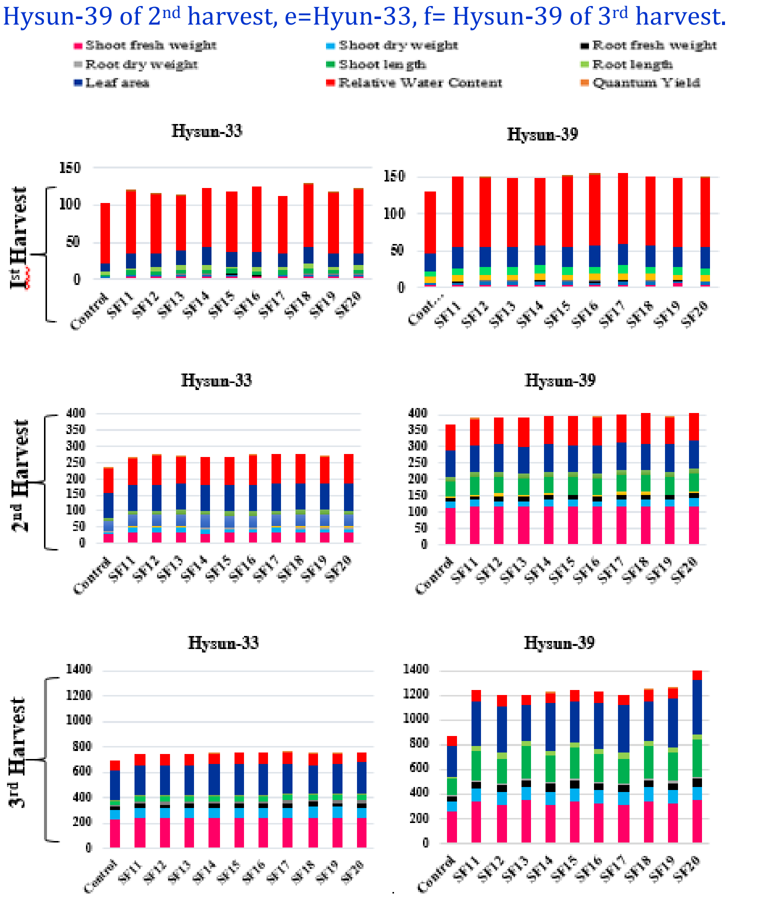

Figure 5: Effect of bacterial strains on growth & yield parameters of 2 varieties of sunflower at multiple harvests.

The correlation analysis of bacterial PGP traits (auxin and siderophores) with growth and yield parameters of 2 varieties (Hysun-33 and Hysun-39) of plants is shown in table 2. For bacterial auxin, the values of a correlation coefficient range from 0.212-0.730** for Hysun-33 parameters with the highest values for Plant height (0.730**) and plant biomass (0.697**). Similarly, a significant correlation (0.203-0.576**) has been found for Hysun-39 parameters with the greatest values for total oil content (0.576**) and plant biomass (0.550**). Table 2 confirmed a strong correlation between bacterially synthesized siderophore and plant parameters where correlation values of 0.101-0.677** for Hysun-33 parameters and 0.134-0.613** for Hysun-39 parameters.

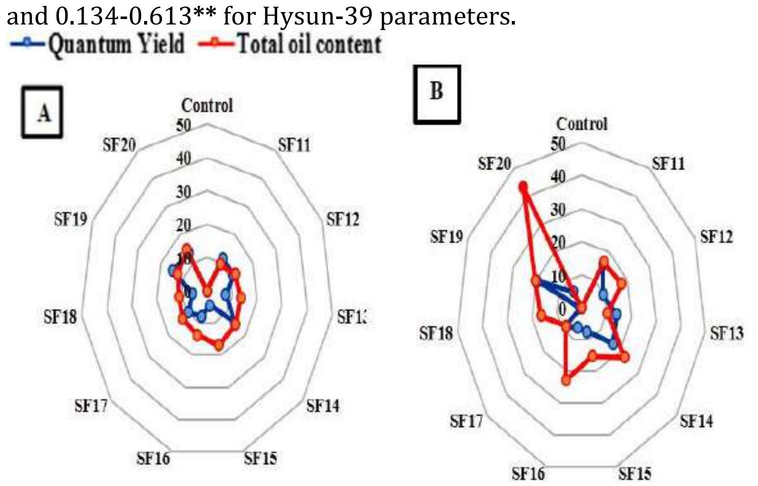

Figure 6: Radar graph showing effect of bacterial inoculations on quantum yield (Fv/Fm) and total oil content (%) of Hysun-33 (A) and Hysun-39 (B) cultivars of sunflower.

Among all parameters, highly influential plant parameters are plant biomass and plant height of both varieties as declared by correlation analysis (table 2)

Hysun-33		
Plant parameters	Bacterial PGP traits	
	Auxin	Siderophore
Plant Biomass	0.697**	0.677**
Plant Height	0.730**	0.668**
Relative Water Content	0.405*	0.223
Quantum Yield	0.212	0.101
Total Oil Content	0.406*	0.477*
Hysun-39		
	Bacterial PGP traits	
Plant parameters	Auxin	Siderophore
Plant Biomass	0.550**	0.613**
Plant Height	0.448**	0.585**
Relative Water Content	0.356*	0.250
Quantum Yield	0.203	0.134
Total Oil Content	0.576**	0.434*

Table 2: Correlation coefficient between bacterial products (auxin and siderophore) and plant parameters (vegetative and yield) of both varieties (Hysun-33 and Hysun-39).
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION: Bio-inoculants are such microbial formulations which are being used as suitable bio tools for the improvement of green land (Maitra *et al.*, 2021). In association with plants, microbes create dense communities, establishing beneficial interactions with host plants, and promoting growth as well as their development (Backer *et al.*, 2018). Among various microbes, the bacterial natives of plants which are found to reside in plant tissues as endophytes occupy the most significant position. This is because of their critical role in the assimilation and acquisition of essentially required nutrients into the plants, improvement of physico-chemical soil properties, and regulation of plant hormones along with the secondary metabolites (Leach *et al.*, 2017). However, there is little knowledge regarding the bio-inoculation practices on sunflower crops. Hence the present study was conducted to explore the application of sunflowers' bacterial endophytes as beneficial bio-inoculants.

Sequencing of the 16S rRNA gene has been considered an accurate approach for the phylogenetic identification of bacteria (Azaroual *et al.*, 2022). Thus, isolated bacterial natives of sunflower were successfully identified by 16SrRNA gene sequencing which revealed the taxonomic relationship of all isolated bacteria with phylum Firmicute with dominancy of Bacillus species (9/10 or 90% isolates) among these isolates (Saxena *et al.*, 2020). These results are well supported by the conclusions of Singh *et al.* (2022) who found supremacy of phylum Firmicute and Bacillus genera among isolates of *Momordica charantia*.

The screening of isolated bacterial strains for varying PGP traits under laboratory conditions is one of the convenient and valuable tactics for the estimation of their actual PGP potentialities (Massa *et al.*, 2022). The plant-associated bacteria are found to promote the growth of host plants by adopting multiple mechanisms. Among these, the foremost include phytohormone or siderophore production, fixation of necessarily required nitrogen and solubilization of phosphorous (Kumar *et al.*, 2016). The results of the current study showed auxin and siderophore production by all the tested strains. Whereas 60% of tested strains produced HCN, 40% of these solubilized phosphorous and only 20% of these strains fixed the nitrogen. Further quantification of bacterial isolates, revealed 46.1-104.6 µg/mL of auxin and 15-50µg/mL of siderophore concentrations indicating their closer association, and potential symbiosis, between host plant and bacterial natives (Fan and Smith, 2021).

Application of bacterial inoculants at the field level can be an appropriate assessment for their growth promotion action in crops. The results of the experiment based on the application of bacterial inoculants into 2 varieties of sunflower revealed their putative effects on plant biomass and yield as compared to un-inoculated control ones. The role of endophytic bacteria in plant growth promotion has also been documented by various studies (Purwaningsih *et al.*, 2019). Among varying parameters (shoot & root fresh and dry weights, shoot & root lengths, leaf area and relative water content), the most influential vegetative parameters were found to be the relative water content (95%) and leaf area (431cm^2) of inoculated plants over the comparison with control. Relative water content is considered a marked indication of water stress tolerance by endophytic bacteria under water deficit conditions (Aslam *et al.*, 2018). The rise of water status and leaf area by applied bacterial strains could be because these bacteria had sufficiently been supplying the required minerals and nutrients into inoculated plants by the development of an efficient root system which could promote their growth. Regarding the yield of plants, up to 14-16, Fv/Fm of improved quantum yield depicted the role of inoculated bacterial species in carrying the photosynthetic activity and maintaining the energy levels of both cultivars with the progressive plant growth stages. These conclusions are well supported by the findings of Salazar-Garcia *et al.* (2022). Furthermore, enhanced oil contents (16-43% in both cultivars) by tested bacillus species reflect the major impact of specifically, the Bacillus species in yield enhancement of plants (Gohil *et al.*, 2022). The present investigation declared a significant correlation between bacterially synthesized auxin (0.550**-0.697**) and siderophores (0.613**-677**) with plant biomass and plant height for both varieties. These results are by the report of Iftikhar and Iqbal (2019) who stated that provoked an increase (P≥0.05) in the growth of wheat & mung bean using auxin-producing bacterial strains. Current results also depicted a highly significant correlation between bacterial auxin and total oil content (0.576**) which highlighted the role of plant-associated bacteria in enhancing the endogenous level of auxin in host plant cells resulting in the accumulation of oil contents of seeds (Sharma *et al.*, 2008). According to Dağüstü *et al.* (2008), specific bacterial genes (hydroxylmethylglutaryl-CoA and phytoene desaturase) are involved in modifying sunflower oil yield. Contrary to these findings, phosphate-solubilizing bacteria may play a role in increasing the oil content of plants (Nosheen *et al.*, 2022). As a result, sunflower-associated bacterial species are declared as biostimulants specifically by harboring the auxin when applied under field conditions which can further be tested for other crop's enhancement as well.

CONCLUSION: Thus the study about bacterial natives of sunflower in particular the *Bacillus* species, reflects their role as fundamental bio inoculants which can efficiently stimulate crop productivity and yield while minimizing the application of hazardous and toxic chemical fertilizers. Therefore, considerate interaction between microbial natives and inoculated microbial communities in plants will contribute to the improvement of biological products.

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ETHICAL RESPONSIBILITY: This is original research, and it is not submitted in whole or in parts to another journal for publication purpose.

INFORMED CONSENT: The author(s) have reviewed the entire manuscript and approved the final version before submission.

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