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The Pesticidal Activities of Rhizospheric Bacteria Isolated from *Holarrhena pubescens*, their Plant Growth Promotion and IAA Production Optimization -

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Abstract

Plant growth-promoting rhizobacteria produce indole acetic acid (IAA) in a wide variety of crop plants. The present works deals with the isolation of rhizo-bacteria from the rhizosphere *Holarrhena pubescens,* screening the isolates for insecticidal activity, IAA production with different types and concentrations of C sources and N sources and the effects of superior strains on some crop plants in sterile soil for growth promotion. Selection of the best IAA producing bacterial isolates was made using a response surface methodology. Sugar type, sugar concentration, and strains were manipulated for IAA production. Out of 20 isolates, four of them were screened as efficient PGPRs based on different plant growth promoting attributes. Isolates MHA-01, MHA-07, MHA-09 and MHA-11 showed better production of IAA at pH 8 (91.7 mg/ml^{-1}) and at temperature 37°C (81.7 mg/ml^{-1}). Dextrose (1.25%) was found to be the best carbon source for isolate MHA- 01 with 100 mg/ml^{-1} IAA production. The seed germination rate of treated plants with MHA-01 strain had increased by 33.78% compare with the control whereas the vigour index was increased by 90.14% in respect to control plant up to 10 days. The application of MHA- 01 increased shoot fresh and dry weight (9.5 ± 0.47 g, 6.3 ± 0.31 g) and root fresh and dry weight (6 ± 0.3 g, 2.9 ± 0.15 g) of treated plants compared to untreated plant after 30 days. The highest chlorophyll (chl-a = 0.23554 µg/ml and chl-b = 0.34842 µg/ml) and carotenoid content (0.00872 µg/ml) observed by the inoculation of MH-11 strain in *Zea maize* followed by *Vigna radiata*, *Pisum sativum,* and *Cicer arietinum.* The current study demonstrated that rhizobacteria that produce IAA could be harnessed to enhance plant growth. Response surfaces are also a valuable tool for controlling their production, making them very useful for optimizing.

Keywords: *Holarrhena pubescens;* Rhizobacteria; Insecticidal activity; Plant growth; Indole-3-acetic acid; Carbon sources; Response surface methodology

INTRODUCTION

The agricultural sector is a major contributor to national income and earnings in developing countries, since it is crucial for food security and employment. Sustainable farming meets the increased need for food production in today's agricultural sector by using sustainable farming practices [1]. The use of conventional agriculture methods is significant in meeting the food needs of an increasing population, but it has also led to an increasing reliance on chemical fertilizers [2]. Chemical fertilizers became increasingly essential to farmers in the past few decades as a relatively reliable method of crop protection, helping them improve their economic stability. The exploitation of chemical fertilizers results in eutrophication of groundwater and airborne pollutants, as chemical fertilizers are constructed using known quantities of nitrogen, phosphorus, and potassium [3]. There have been a number of factors that have negatively affected farmers' crop yields worldwide, including infertility of soil, heavy metal contamination, infestation of plants with pathogenic microorganisms [4], development of pathogen resistance, and environmental impact from non-targeted agricultural practices [5]. A way to address this problem is to reduce land degradation and use plant-beneficial Rhizobacteria as bioinoculants that require a good understanding of a sustainable agricultural vision where crops need to be resistant to disease, salt and drought, and must be able to with stand heavy metal stress [6]. Bio-based organic fertilizers are gaining popularity because they offer an alternative to agrochemicals and are more innovative in their approach to crop production [7]. Root-residing microorganisms improve the development and protection of a plant [8]. Plants benefit greatly from Rhizobacteria, as they can fix atmospheric nitrogen, dissolve inorganic phosphate, and produce IAA, ammonia and siderophores that are beneficial to plants [9]. In view of this need for biocides, which can promote plant growth and control pathogenic microorganism's attacks, they are one of the possible solutions, and can also greatly improve crop yield and overcome challenges caused by agrochemicals. To increase nutrient uptake capacity and water use efficiency, soil microorganisms i.e. bacteria, fungi, algae may be able to increase the above crop properties [10]. There are a number of potential soil microorganisms, but Plant Growth-Promoting Rhizobacteria (PGPR) appear to be the most promising. PGPR therefore could be used to improve plant health and accelerate plant growth without polluting the environment [11]. Among the beneficial microbiomes in the rhizosphere, there are PGP rhizobacteria, nitrogen-fixing symbionts, endophytes, mycorrhizal fungi, disease-controlling microorganisms, protozoa, and mycoparasitic fungi [12]. Inoculants of plant-beneficial Rhizobacteria are preparations that contain cells from various strains of bacteria that can be applied to soil or plants to boost their metabolic processes and prevent them from being harmed by microbial pathogens and pests. In the last few decades, a variety of PGPR species has been studied and some have been commercialized, including *Pseudomonas, Bacillus, Enterobacter, Klebsiella, Azobacter, Variovorax, Azosprillum,* and *Serratia* [13]. As plants synthesize plant hormones for growth promotion, including auxins for weed suppression [14], gibberellins, ethylene, abscisic acid, jasmonic acid, cytokinins, salicylic acid and strigolactones for diseases suppression [15,16], indirect mechanisms come into play to suppress fungal, bacterial, nematode, and viral path. Plants have special preference for microbe's positive impact on plant growth, which impacts rhizospheric microbe communities [17]. Different plant growth related parameters such as rate of seed germination, vigour index, shoot fresh weight, root fresh weight, shoot biomass, root biomass, plant canopy size and fruits number found to be influenced by Indole Acetic Acid producing rhizobacteria. Since, different bacteria, fungi and algae are capable of producing physiologically active amounts of IAA which has been found to be very important for plant growth and development. Plants and microbes were synthesized IAA via tryptophan dependent and tryptophan independent pathways. Microbes released many secondary metabolized such as amino acids, sugars and organic acids which helps the plants in various physiological activities. The rhizospheric bacteria produced IAA huge amount due to the supply of substrates required for this secondary metabolite production [18]. The plant growth promoting activity reported by many researchers in *Zea maize, Vigna radiata*, *Cicer arietinum*, and *Pisum sativum* [19-22]. Improvement in crop yield and to attain success in sustainable agricultural systems use of Plant Growth Promoting Rhizobacteria (PGPR) is a promising alternative to chemical fertilizers, pesticides, fungicides etc… [23]. There have been problems with insects and diseases that threaten crop production. There are two insect pests that affect mangos in India: mango mealy bugs and fruit flies. A polyphagous insect, the mango mealy bug feasts on a wide variety of plants. Consequently, there is no operative and effective chemical method for controlling mealy bugs infesting citrus and other horticultural crops [24]. The use of biopesticides is relatively safe and environmentally friendly, with fewer or no side effects [25,26].

The aim of this study aim was to screen for rhizobacteria that perform insecticidal activity, produce IAA and optimize factors leading to a high concentration of IAA using response surface methodology. Furthermore, the present study investigated some differences in growth parameters between crops of *Zea maize, Vigna radiata, Cicer arietinum,* and *Pisum sativum* by using the growth parameters MHA-01, MHA-07, MHA-09 and MHA-11.

MATERIALS AND METHODS

Isolation of rhizospheric bacteria from soil

Soil samples were collected from the Sreegopal Banerjee College's Medicinal plants garden of Bagati, Mogra, Hooghly. The root with intact plant was dug out carefully with 15 cm soil slab. The roots clumps soil which tightly bound were carefully stored in sterile polyethylene and used for isolation of PGPRs. The bacterial isolation from the soil was done by standard tenfold serial dilution method [27]. The excess moisture was removed through soil air dried technique. 1 gm of soil was suspended in 10 ml autoclaved distilled water and 1 ml of soil solution from each tube was passed on to the next tube and subsequently a dilution range of 10^{-1} to 10^{-10} was prepared. 10 µl of soil solution was spread on sterile nutrient agar plates and incubated at 37°C for 24 hrs. Morphologically distinguishable colonies were picked and streaked on another fresh nutrient agar plates from the several bacterial colonies that were grown on NA plates. Pure cultures colonies were obtained by re-streaking method. Then colonies were inoculated in IAA detecting media for selected IAA-producing isolates. The maximum amount of IAA producing isolate was further selected for the optimization of IAA production. The selected isolates were named according to their collection sites and analyzed for various plant growth promoting activities. Pure cultures were stored in nutrient agar slants at 4^0C in sterile conditions for further use.

Collection of mealy bugs

The specimens of adult female mealy bugs were collected with a camel hair brush from mango (*Mangifera indica* L.) orchards located on the campus of the University of Gour Banga and transported to the Department of Botany's laboratory. The two generations were maintained in the laboratory at 26 ± 2°C and 75 ± 5% relative humidity on fresh pumpkin fruits for about two weeks. Toxicological bioassays were conducted on healthy and active 2^{nd} instar nymphs and adult females obtained from a field collection of 2^{nd} generation mealy bugs.

Detection of IAA production

The IAA production was detected by the method of Brick, et al. [28] with some modification. The freshly grown cultures was inoculated in 30 ml nutrient broth and kept at 37°C for 36 hrs at 120 rpm in an incubator shaker. Fermentation cultures were centrifuged at 8,000 rpm for 10 min. at room temperature. Then 2 ml Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) added in 1 ml Cell Free Supernatant (CFS) in a test tube. Further, two drops of orthophosphoric acid added to it and kept in dark for color formation. The optical density was recorded at 530 nm after 2 hrs. IAA concentrations were determined using the standard plot of IAA.

Assays of standard IAA

Crude IAA was purchased from HiMedia and different concentrations (20, 50, 100, 150, 200, 250 and 300 µg/ml-^{1}) were prepared in distilled water. Salkowski reagent was added for color formation in different dilution of IAA (1:2) followed by the addition of two drops of orthophosphoric acid and kept in dark. The optical density measurements were made at 530 nm and standard curve was plotted.

Production optimization of IAA

The culture medium was inoculated with 24 hrs grown cultures (O.D 0.5) of isolates MHA-01, MHA-07, MHA-09 and MHA-11 for IAA production. The experiment was based on four different parameters viz. Temperature, pH, and carbon source and nitrogen sources were taken for the study. The basic IAA production medium consisted of the peptone 10 g/l^{-1} yeast extract 6 g/l^{-1} and NaCl 5 g/l^{-1}. All cultures were incubated at 37°C at 120 rpm for 36 hrs for observation. pH is one of the most important physicochemical parameters for IAA production. The pH range of 5-11 was examined for its effect on IAA production by different isolates. The IAA production depend on the temperature which is also an important parameter for the growth of bacteria affected by low or high temperature and IAA production is dependent upon the correct growth of microorganism. The IAA production was tested at 25, 30, 35, 37, 40 and 45°C at 120 rpm. Six different sugars viz. Dextrose, maltose, sucrose, starch, mannose and mannitol at different concentrations of 0.5%, 0.75%, 1.0%, 1.25%, 1.50% and 1.75% were tested. Different nitrogen sources viz. beef extract, soybean meal, malt extract, and potassium nitrate at varying concentrations of 0.50%, 0.75%, 1%, 1.25%, 1.50% and 1.75% were used for the study. The amount of IAA production was quantified through the standard plot.

Isolates		Carbon source		
Strain	Code	Sugar	Code	Concentration
MHA-01	01	Sucrose	1	0.5
MHA-07	07	Starch	2	0.75
MHA-09	09	Maltose	3	1
MHA-11	11	Dextrose	4	1.25
		Mannitol	5	1.5
		Mannose	6	1.75

In vitro and *in vivo* effect of selected isolates in crop plants

Seeds of three crop plants viz. *Zea maize, Vigna radiata*, *Cicer arietinum*, and *Pisum sativum* were purchased from jayashree beej ghar, Malda market. Fresh bacterial cells were collected by centrifugation at 8000 rpm for 5 min at 4°C from large volume of broth culture of shake flask and pellet was washed twice with sterile distilled water, and suspended in 10 ml sterile distilled water approximately 10^6 CFU/seed (O.D .08), vortex and used for seed treatment. Approximately 15-20 seeds of all crops were surface sterilized with 5% sodium hypochloride (NaoCl, Merk, India) for 1 min and washed three times in sterile distilled water. The bacterial suspension and dry seeds were mixed gently by the stirred for 5 min. Bacterized seeds were spread on a petri dish and air dried overnight at room temperature. The number of bacterial cells per seed was determined via serial dilutions and was set to approximately 10^6 CFU/seed (O.D = 0.8). To assess the effect of the strain LBF-1 on germination and seedling vigour, 15 seeds inoculated with bacterial strain were incubated in two 9 cm Petridishes on two layers of moistened filter paper (Whatman No. 1). As a control treatment, seeds treated with distilled water instead of bacterial suspensions were also established. Maintain sufficient moisture for germination, the Petri dishes were moisture in every alternate days, with sterile distilled water and incubated at 28°C ± 2°C in a light incubator. The germination percentage was recorded every

24 hrs for 10 days. Root and shoot length were measured after 10 days (the experiment was repeated thrice). The germination rate and vigour index were calculated using following formula [29].

Germination rate (%) = (number of seeds germinated/ total number of seeds) × 100

Vigour index = % germination × total plant length

After 10 days seedling was transfer in soil pot containing double autoclave soil and allow to grown up to 30 days. Then plants were carefully uprooted and the shoots and roots were separated and dried in an oven at 50°C for 48 hrs to calculate the dried root and shoot biomass.

Photosynthetic pigments analysis

To analysis of photosynthetic pigments, 100 mg tissue from the terminal opened tender leaf of the bacterial treated and control plants were separately cut into small pieces and mixed with 7 ml of DMSO (dimethyl sulphoxide) in test tubes at 65°C for 3 h. After crushing the samples, DMSO was poured onto make up the volume up to 10 ml and the absorbance of the clear extract was measured using UV-vis Spectrophotometer (UV-Vis1800, Shimadzu, Japan) at 645 and 663 nm marking blank as pure DMSO [30]. Extraction and estimation of Photosynthetic pigments from leaves of maize plants were ground with 80% acetone. Estimation of the chlorophylls and carotenoid were followed the method of Lichtenthaler, 1987 [31]. Photosynthetic pigments viz. total chlorophyll, chlorophyll a, chlorophyll b and carotenoids were expressed as mg/g of fresh leaf tissue. The absorbance for chlorophyll-a, chlorophyll-b and carotenoid was recorded at 663, 645, and 470 nm, respectively.

Extraction of crude extract from cell free supernatant

The active strain MHA-01, MHA-07, MHA-09 and MHA-11 were inoculated in 100 ml sterile Nutrient Broth containing 250 ml Erlenmeyer flask and incubated at 37°C in B.O.D incubator (Labtek, India) for 24 hrs using previously described method. After 10 days fermentation, broth was centrifuge at 8000 rpm for 10 min at 4°C and collected the Cell Free Supernatant (CFS) in a 50 ml centrifuge tube. Different solvents with increasing polarity viz. n-Hexane, Diethyl ether, Dichloromethane, and Ethyl acetate were used (1:1, v/v) for extraction of antifungal compounds from cell-free supernatant. The solvent supernatant mixture was agitated gently for 10 min in separating funnel and stand by 3-5 days for completely separated solvent phase from cell free supernatant. The solvent phase containing antifungal compounds was collected from each used solvent and concentrated by rotary vacuum evaporators (RotaVap, R-150, Superfit, Mumbai, India) and assayed for their antifungal activity by well agar diffusion method [29]. After repeated solvent fractionation purification, concentrated Dichloromethane crude extract was passed through a silica gel column using MERCK silica gel (100-200 mesh) as the stationary phase and n-Hexane ethyl acetate (1:1) as the mobile phase and then dried using rotary evaporator.

Toxicity bioassay of bacterial crude extract

The toxicity of pesticides was assessed by bioassay for 2nd instar nymphs and adult females of laboratory-reared mealy bugs. The bacteria extracts were extracted using Dichloromethane solvent. The effectiveness of five different bacterial extract concentrations, i.e. 0.0, 15.0, 30.0, 45.0, 60.0 and 75.0%, against 2nd instar mealy bugs was investigated in four replicates for each. A total of four replications of each crude extract were conducted against adult female mealy bugs at concentrations of 0.0, 15.0, 30.0, 45.0, 60.0 and 75.0 %, a Completely Randomized Design (CRD) was used in this study. The crude extracts were serially diluted using distilled water. Nymphs were subjected to bioassays using the leaf dip method, and female adult insects were subjected to bioassays using freshly cut citrus twigs. After dipping leaf discs and twigs in treatment solutions for 30 seconds, they were air dried on towel paper at room temperature (22°C) for 15 minutes, before being transferred onto moist filter paper discs in sterilized glass petridishes (diameter: 9 cm). Leaf-discs and twigs were dipped in treatment solutions for 30 seconds and were air dried at room temperature (22°C) on towel paper for 15 min before their transfer on moist filter paper discs in sterilized glass petridishes (dia. = 9 cm). A camel-hair brush was used to release fifteen mealy bug nymphs or five adult females on the treated mango leaf discs (and/or twigs).

Statistical analysis

The experiments performed in the present study were done in triplicates and all the experiments repeated twice using totally randomized design. Exploratory data analysis was done. Normality of the data was checked by Shapiro-Wilk. One-Way ANOVA was used for analysis of variance in the data. RSM model was created for detecting main effects and interaction effects of predictor variables (Strains, Sugar type, sugar concentration) on IAA production. Linear regression models were created between sugar type and sugar concentration to understand the IAA production process. All the analysis was done, and plots were made in R 3.6.3 software (www.r-project.org) and the analyses were run in the software environment R Studio 1.2.5042 (http://www.rstudio.com).

RESULTS

Screening of IAA producing bacteria

The rhizobacterial strains were screened for IAA production and showed that all the strains were able to produced significant amount of IAA in tryptophan-supplemented medium, whereas strains MHA-01, MHA-07, MHA-09 and MHA-11 were potent to IAA production in the medium devoid of tryptophan (Table 1).

Effect of temperature and initial pH for IAA production

The isolated strain MHA-01 was able to produce good amount of IAA throughout the pH range tested and the highest amount of IAA (85.7 $\mu g/ml^{-1}$) produced at pH 8 (Figure 1). The strain MHA-07 increased the IAA production from 5-8 pH rang, decreased from 9-11 pH ranges and it showed maximum production (77.71 $\mu g/ml^{-1}$) at pH 8. The stain MAH-09 produced 67.16 $\mu g/ml^{-1}$ IAA at pH 8 and 32 $\mu g/ml^{-1}$ IAA at pH 11 and strain MAH-11 produced 72.12 $\mu g/ml^{-1}$ IAA at pH 8 and 42 $\mu g/ml^{-1}$ IAA at pH 11.

The effect of temperature was studied in the range 20-45°C whereby maximum yield was observed at 37°C by all isolated strains (Figure 2). The strain MHA-01 produced maximum 80.24 $\mu g/ml^{-1}$ followed by 77.16 $\mu g/ml^{-1}$, 70.36 $\mu g/ml^{-1}$, 72.12 $\mu g/ml^{-1}$ IAA by MHA-01, MHA-07, MHA-09 and MHA-11, respectively.

Effect of carbon source for IAA production

The most important carbon source was Dextrose producing 100 µg/ml IAA at 1.25% and 93 $\mu g/ml^{-1}$ at 1% concentration by the strain MHA-01. The strain MHA-07, MHA-09 and MHA-11 produced maximum 89 $\mu g/ml^{-1}$, 70 $\mu g/ml^{-1}$ and 80 $\mu g/ml^{-1}$ IAA, respectively. The production of IAA enhanced by the addition of sucrose as a carbon source in NB media. The highest IAA production showed in

1.25% sucrose concentration. The MHA-01 strain produce 98 µg/ml IAA followed by 87 µg/ml, 70 µg/ml and 75 µg/ml IAA by the strains MHA-07, MHA-09, and MHA-11, respectively. Starch was one of the important carbon sources for IAA production. It produced highest 80 µg/ml IAA by the strain MHA- 01 by the addition of 1% starch in NB media. The others strains viz. MHA-07, MHA-09 and MHA-11produced highest 75 µg/ml, 55 µg/ml and 60 µg/ml of IAA in addition of 1% concentration of starch. The production of IAA significantly increased by the amended of 0.75% maltose sugar. MHA-01 produced maximum (79 µg/ml) amount of IAA followed by MHA-07 (75 µg/ml), MHA-09 (65 µg/ml), and MHA 11(70 µg/ml), respectively. In the presence of mannose sugar, the highest IAA producing activity found in 1% concentration by the isolated strain MHA-01 which was 67 µg/ml. The other strains MHA-07, MHA- 09 and MHA-11 produce highest 56 µg/ml, 43 µg/ml, and 39 µg/ml IAA, respectively. Similarly in the presence of mannitol sugar, all strains synthesized highest IAA in 1% concentration which were 65 µg/ml, 50 µg/ml, 45 µg/ml and 55 µg/ml by the strains MHA-01, MHA-07, MHA-09 and MHA-11, respectively (Figures 3a,b). According to one way ANOVA analysis, there a no significance difference between strains in different sugars and between sugars in different strains (Figures 4a,b).

Table1: Indole acetic acid production by cultures isolated from the rhizospheric soil of Kurchi (Holarrhena pubescens).

Strains	IAA Production(µg/ml)	
	Tryptophan dependent	Tryptophan independent
MHA 01	607.8	350.02
MHA 02	35.2	0
MHA 03	26.4	0
MHA 04	96.8	5.8
MHA 05	29.7	0
MHA 06	152.9	12.02
MHA 07	590.02	340.9
MHA 08	78.9	0
MHA 09	458.8	255.8
MHA 10	26.7	0
MHA 11	563.8	324.8
MHA 12	134.7	9.02
MHA 13	78.7	0
MHA 14	36.4	0
MHA 15	48.9	0
MHA 16	104.8	13.8
MHA 17	110.7	16.9
MHA 18	78.9	0
MHA 19	70.8	0
MHA 20	68.9	0

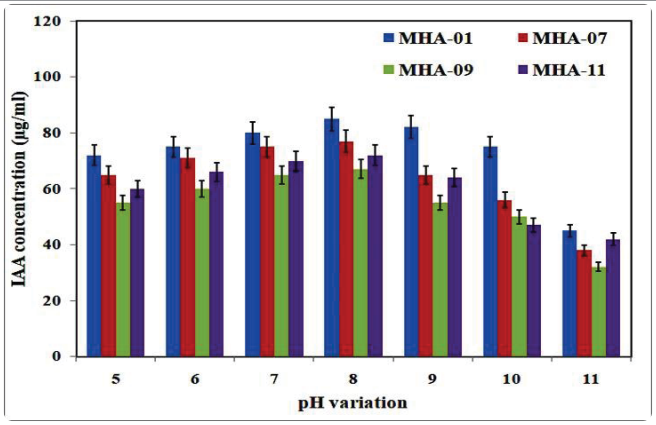

Figure 1: Effect of pH on IAA production by PGPR.

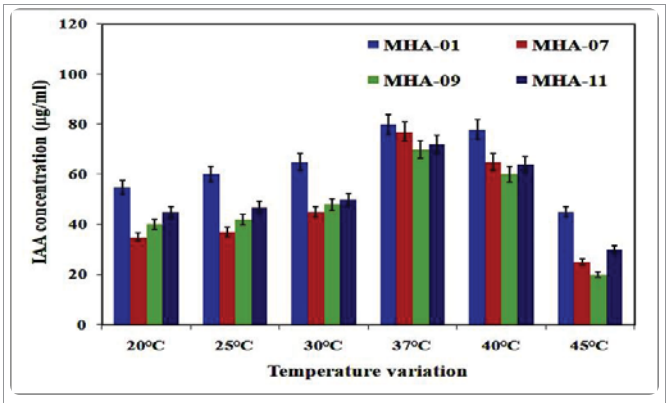

Figure 2: Effect of temperature on IAA production by PGPR.

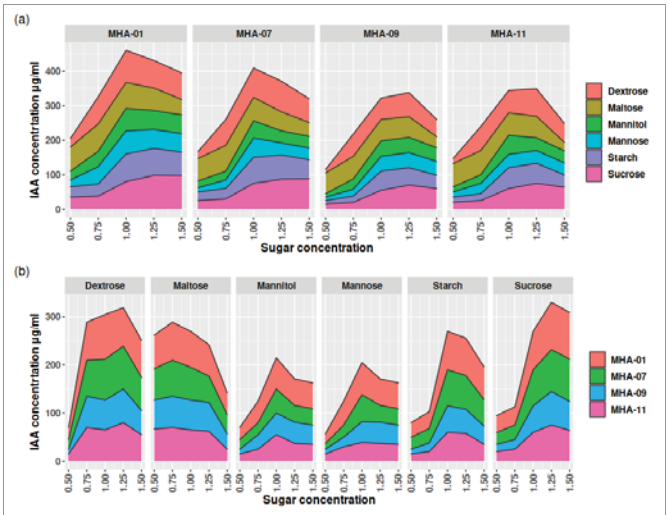

Figure 3: Comparison of IAA production by permutation combination of sugar type, sugar concentration and strain (a, b).

Effect of nitrogen source for IAA production

The used of malt extract in NB media showed that the significant amount of IAA production which was shown in figure 5A. The strain MHA-01 produced highest 95 µg/ml IAA followed by 79 µg/ml, 60 µg/ml, 91 µg/ml, by the strains MHA-07, MHA-09, MHA-11, respectively. The used of Beef extract showed the maximum IAA production in 1.25% concentration by the all strains as shown in figure 5B. The strain MHA-01 showed highest 80 µg/ml IAA followed by 75 µg/ml, 55 µg/ml and 77 µg/ml by the strains MHA-07, MHA-09, MHA-11, respectively. The application of Soyabean meal enhanced the maximum IAA production 80 µg/ml and 75 µg/ml by the strains MHA-01 and MHA-07 in 1% concentration. The strains MHA-09 and MHA-11 produced 60 µg/ml and 79 µg/ml IAA in 1.25% concentration as shown in figure 5C. The used of Glycine significantly increased the production of IAA in 1% concentration which was shown in figure 5D. The highest IAA production showed by the strain MHA-01 in terms of 60.25 mg/ml followed by the 34.12 mg/ml and 51 mg/ml by the strain MHA-07 and MHA-11, respectively. The strain MHA-09 utilized the glycine 1.25% for the better production of IAA. Similarly in case of Ammonium nitrate, all the strain used and increased IAA production up to 1% concentration which was shown in figure 5E. The strain MHA-01 produced 65 µg/ml IAA followed by 35 µg/ml, 30 µg/ml and 56 µg/ml by the strains MHA-07, MHA-09 and MHA-11, respectively. The strain MHA-01, MHA-07 and MHA-11 utilized the

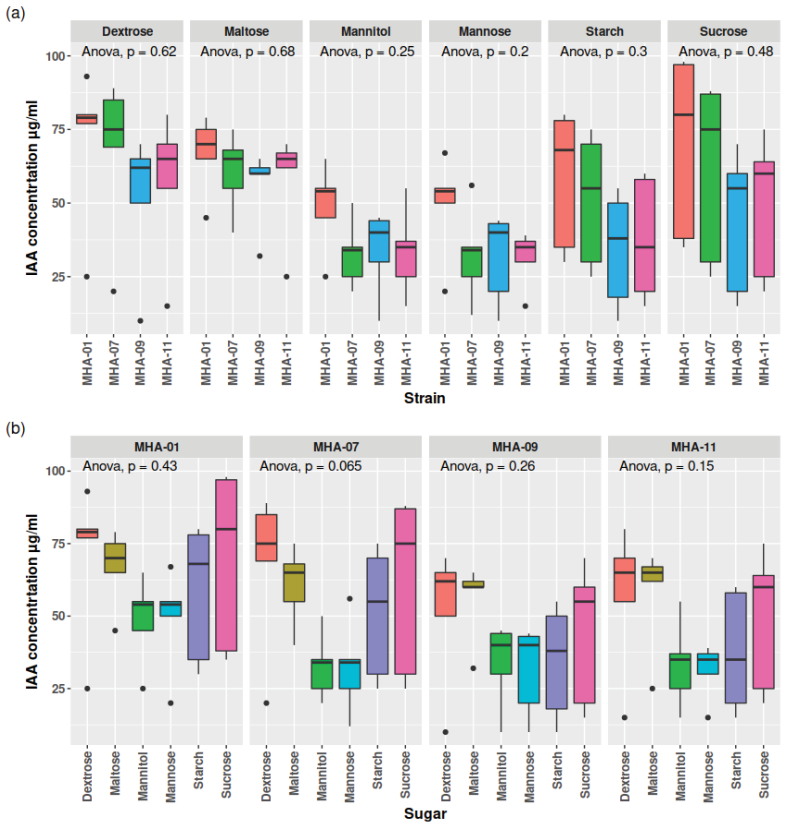

Figure 4: Analysis of variance in IAA production between strains in different sugars (a) and between sugars in different strains (b).

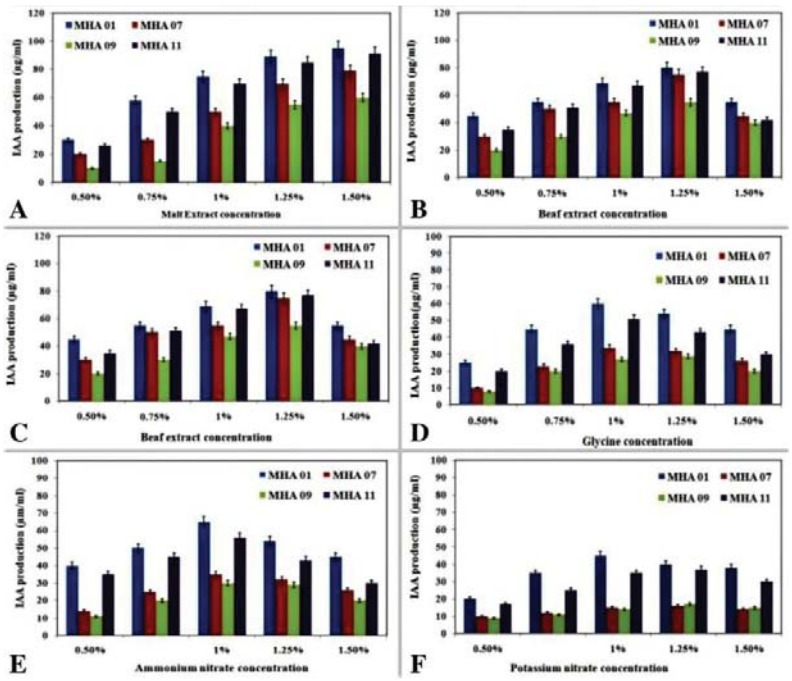

Figure 5: Graph showing effect of various nitrogen sources for IAA production;
A) Malt extract concentration.
B) Beef extract concentration.
C) Soybean meal concentration.
D) Glycine concentration.
E) Ammonium nitrate concentration.
F) Potassium nitrate concentration.

potassium nitrate up to 1% concentration in ratio of IAA production in terms of 45 µg/ml, 15 µg/ml and 35 µg/ml as shown in figure 5F. The strain MHA-09 produced 17 µg/ml IAA in 1.25% potassium nitrate concentration and decreases the IAA production.

Seed germination rate assessment of bacterized seeds and vigour index

Assessment of rate of germination of bacterized seeds, vigour index of treated seedling after 10 days and plants growth parameters of *Zea maize, Vigna radiata*, *Cicer arietinum*, and *Pisum sativum* plants were compared with control which was shown in figure 6. The seed germination rate of *Zea maize* plant treated with strain MHA-01 had increased by 33.78% compared with the control whereas the vigor index was increased by 90.14% whereas the seed germination rate 25.57%, 28.37%, 21.62% and vigour index 81.08%, 81.75%, 79.72% treated with the strains MHA-07, MHA-09, and MHA-11 respectively. The seed germination rate of *Vigna radiata* plant treated with strain MHA-01 had increased by 38.01% compared with the control whereas the vigor index was increased by 82.67% whereas the seed germination rate 29.57%, 35.21%, 28.16% and vigour index 73.29%, 73.94%, 75.35% treated with the strains MHA-07, MHA-09, and MHA-11 respectively. The seed germination rate of *Pisum sativum* plant treated with strain MHA-01 had increased by 42.02% compared with the control whereas the vigor index was increased by 112.90% whereas the seed germination rate 37.68%, 36.23%, 22.53% and vigour index 111.29%, 113.70%, 116.93% treated with the strains MHA-07, MHA-09, and MHA-11 respectively. The seed germination rate of *Cicer arietinum* plant treated with strain MHA-01 had increased by 37.06% compared with the control whereas the vigor index was increased by 79.86% whereas the seed germination rate 23.61%, 25.77%, 27.77% and vigour index 71.38%, 69.44%, 68.03% treated with the strains MHA-07, MHA-09, and MHA-11 respectively. The results suggest that rhizobacterial treatment could improve the germination and vigour of *Zea maize, Vigna radiata*, *Cicer arietinum*, and *Pisum sativum* seeds.

Assessment of plant growth promotion of some crop plants

Assessment of plant growth parameters viz. shoot fresh weight, root fresh weight, shoot biomass and root biomass of *Zea maize, Vigna radiata*, *Pisum sativum,* and *Cicer arietinum* plants were compared with control which was shown in figure 6. The application of MHA-01 increased shoot fresh and dry weight (9.5 ± 0.47 g, 6.3 ± 0.31 g) and root fresh and dry weight (6 ± 0.3 g, 2.9 ± 0.15 g) of maize plants compared to untreated maize plant at 30 days followed by strains MHA-07, MHA-09 and MHA-11. The shoot fresh weight 7.5± 0.37 g, 8 ± 0.4 g, 9 ± 0.45 g and root fresh weight 3 ± 0.15 g, 5.6 ± 0.28 g, 4.9 ± 0.24 g increased with the application of strains MHA-07, MHA-09

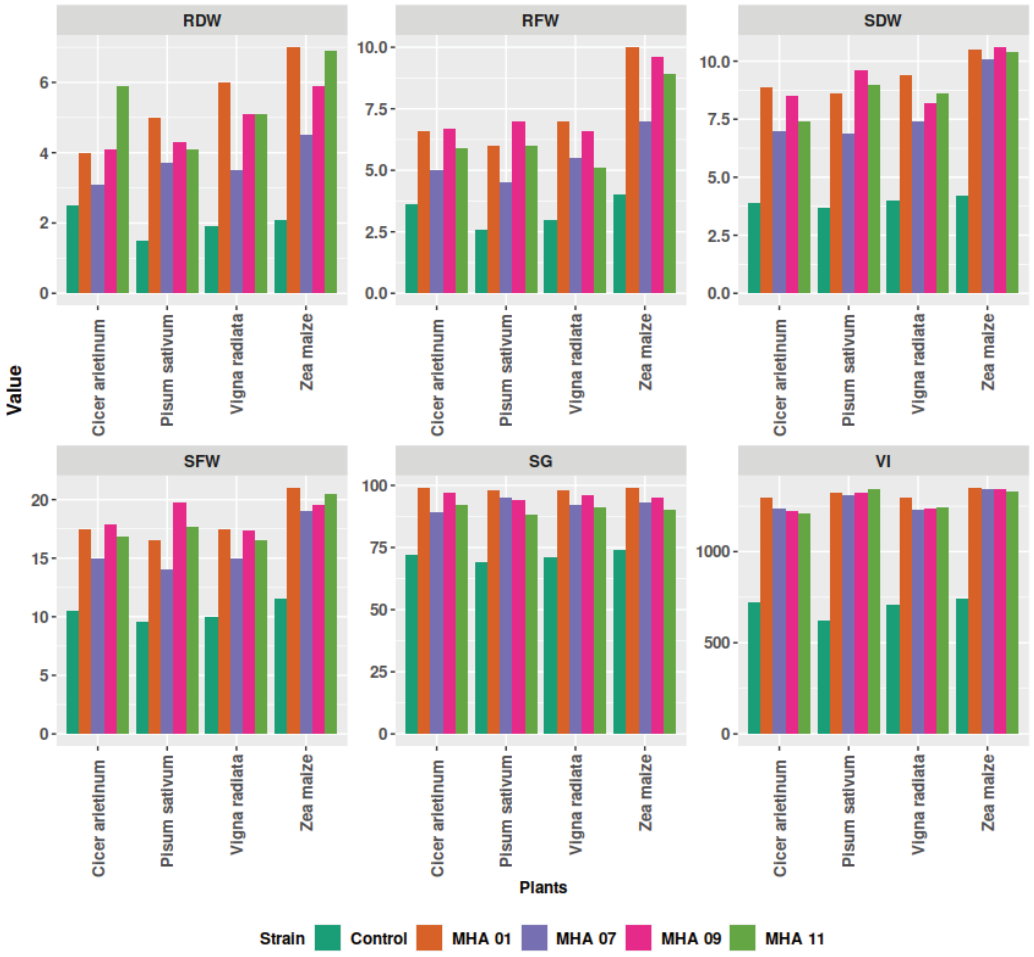

Figure 6: Assessment of seed germination, vigour index, plant growth parameters and comparison between strains. RDW: Root Dry Weight; RFW: Root Fresh Weight; SFW: Shoot Fresh Weight; SDW: Shoot Dry Weight; SG: Seed Germination; VI: Vigour Index.

and MHA-11, respectively. The application of bacterial strain MHA-01 significantly increased highest shoot dry weight 6.3 ± 0.31g root dry weight 4.9 ±0.24 g among the bacterial isolates. Similarly the highest plant growth promotion observed in case of *Vigna radiata* by means of shoot fresh weight 7.5 ± 0.38 g, shoot biomass 5.4 ± 0.27 g and root fresh weight 4 ± 0.2 g, root biomass 4.1± 0.20g with the application of MHA-01 strain. Isolates MHA-09 and MHA-011 showed better response on treating the seeds of *Pisum sativum* with production of 9.9 ± 0.49 g and 8.1 ± 0.4 g and of shoot fresh weight and 5.9 ± 0.29 g and 5.3 ± 0.26 g dried root biomass compared to control plant. In case of *Cicer arietinum* the highest shoot fresh weight was shown 7± 0.35 g and 7.4 ± 0.37 g and shoot biomass and root biomass was 5 ± 0.25 g and 4.6 ± 0.23 g by isolate MHA-01 and MHA-09 among the bacterial strains.

Photosynthetic pigments analysis

The results of photosynthetic pigments estimation by application of MH-01, MH-07, MH-09 and MH-11 rhizobacterial strains in *Zea maize, Vigna radiata*, *Pisum sativum,* and *Cicer arietinum* pots showed in figure 7. Among the pots crops, *Zea maize* pot showed that highest chlorophyll (chl-a = 0.23554 µg/ml and chl-b = 0.34842 µg/ml) and carotenoid content (0.00872 µg/ml) observed by the inoculation of MH-11 strain followed by the MH-01, MH-07, MH-09. Similarly in case of *Vigna radiata*, *Pisum sativum,* and *Cicer arietinum,* MH-11 significantly performed the highest photosynthetic pigments production than the other three strains.

Toxicity assay

The toxicity assay showed that the crude extracts of MHA-01, MHA-07, MHA-09, and MHA-11 were biopesticidal and killed the mealybug at different concentrations. The percentage of dead increases as concentration increases, as shown in figure 8.

DISCUSSION

The isolated bacteria screening for plant growth promoting rhizobacterial (PGPRs) properties and among them four were selected and used for optimization of IAA production. The most potent four IAA producing isolates produce IAA in the presence of tryptophan and absence of tryptophan in NB media (Table 1). The isolated strains produce of IAA only in the presence of L-tryptophan indicates that the tested strains utilize L-tryptophan as a precursor for IAA production during their growth in the medium. Maximum IAA production was obtained with isolate MHA-01. The plants growth can be affected by soil pH in the plant rhizispheric soil because of metal ions could reach toxic levels in the soil at Lower pH limits [32]. The physiological and metabolic process of plant depends on soil pH and metal cations, therefore, impact of pH range of 5-11 was only checked for IAA production (Figure 2). Bharucha, et al. [33] showed that the maximum IAA production at pH 7.5 by *Pseudomonas putida* UB-1 and whereas Shanti, et al. [34] suggested that production varied between pH 6.4 and 7.8 by *Rhizobium* sp. isolated from root nodules of *Vigna mungo* L. Sachdev, et al. [35] showed that the maximum production observed at pH 6-8 and optimum at pH 7.2 by *klebsiella pneumonia* isolated from wheat rhizosphere. Similar result was shown in other studies [36] where 37°C was the best temperature for IAA production for *Rhizobium* and *Bacillus* spp. The production of IAA optimized by the used of several carbon sources that are also used in production of others secondary metabolites. Six different types of sugars (Sucrose, Starch, Maltose, Mannitol, Mannose, and Dextrose) were used in this study to check their effect on IAA production (Figures 3a,b). The used

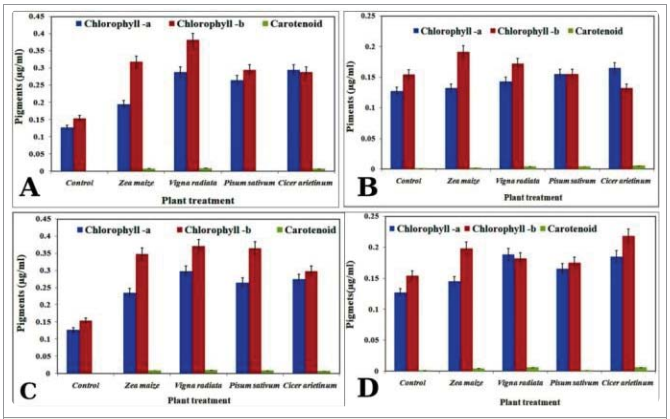

Figure 7: Photosynthetic pigments analysis of crop plants treated by PGPR stains.
A) MHA-01
B) MHA-07
C) MHA-09
D) MHA-11

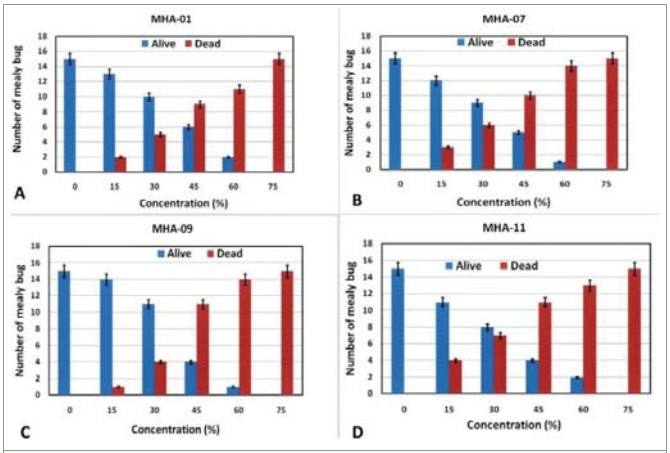

Figure 8: Toxicity assay of bacterial crude extract against mango mealy bugs in different concentration.
Here A) Indicated the effect of MHA-01strain; B) Effect of MHA-07 strain; C) Effect of MHA-09 strain and D) Effect of MHA-11 strain.

of various carbon sources showed that monosaccharide's were better sources than disaccharides and polysaccharides. In this content, dextrose was found as the best sugar source for IAA production. The individual carbon sources affect the IAA production which was studies on bacteria by Shanti, et al. [34] and Sridevi, et al. [37]. The production IAA optimized by the used of several carbon sources that are also used in production of others secondary metabolites. Six different types of sugars (Sucrose, Starch, Maltose, Mannitol, Mannose, and Dextrose) were used in this study to check their effect on IAA production (Figures 3A-F). The used of various carbon source showed that monosaccharide's were better sources than disaccharides and polysaccharides. In this content, dextrose was found as the best sugar source for IAA production. The individual carbon sources affect the IAA production which was studies on bacteria by Shanti, et al. [34] and Sridevi, et al. [37]. The effect of different concentrations of sugar sources in basal media was different due to variable utilization of sugars by bacteria during their growth. *Acetobacter diazotrophicus* and *Pseudomonas fluorescence* produced IAA 48 mg/ml^{-1} by the addition of ammonium chloride as nitrogen sources [38]. According to Balaji [39] *Pseudomonas* species produced IAA 210 mg/ml^{-1} after addition of yeast extract and 18.08 mg/l^{-1} after addition of soyabean meal as

the best nitrogen sources. According to recent study by Nutarata, et al. [40] showed that the application of *Enterobacter* sp. DMKU-RP206 improve IAA production 13.4-fold which was higher amount of IAA than previously reported for the genus *Enterobacter*.

The statistically analysis of variance in IAA production between strains in different sugars (a) and between sugars in different strains (b) (Figure 4). From the RSM model, main effects or the First Order effect of sugar type (p = 0.0038947) and sugar concentration (p = 2.966e-12) found significant but strain not found significant (p = 0.3516260). Interaction effects between Strain: Sugar-type (p = 0.6210806) and Strain: Sugar-concentration (p = 0.3513582) not found significant but interaction between sugar-type: Sugar-concentration (p = 0.0381735) found significant. The model found moderately good and significant (R-squared = 0.5768, Adjusted R-squared = 0.5422, DF = 110, p < 2.2e-16, alpha = 0.05). According to the surface plot Starch (Sugar-type 2), with concentration of 1.25 is most suitable for high amount of IAA production (Figure 9).

From the violin plot there seems clear difference in IAA production due to different sugar types, due to different strain types (Figure 10A). There is overall significant difference in IAA production between all the strains (p = 0.0093), but no significant difference found between MHA-01:MHA-07, MHA-07:MHA-09, MHA-07:MHA-11, MHA-09:MHA-11, although significant difference found between strains MHA-01:MHA-09, MHA-01:MHA-11 (Figure 10B). Overall significant difference (p = 8.8e-05) in IAA production in different sugar types was found (Figure 10C). Overall significant difference (p = 1.3e-08) in IAA production in different sugar concentration was found (Figure 10D).

Correlation between IAA production and sugar concentration was found positive and significant in Dextrose (R = 0.57, p = 0.0093), Manitol (R = 0.57, p = 0.008), Mannose (R = 0.59, p = 0.006), Starch (R = 0.62, p = 0.0034), Sucrose (R = 0.84, p = 3.2e-06) but in Maltose the correlation was found negative and significant (R = -0.73, p = 0.00028) (Figure 11).

The seeds of *Zea maize, Vigna radiata*, *Pisum sativum,* and *Cicer arietinum* treated with strains MHA-01, MHA-07, MHA-09 and MHA-11 significantly increased the percentage of seed germination by 33.78%, 38.01%, 42.02%, and 37.06% compared with the control,

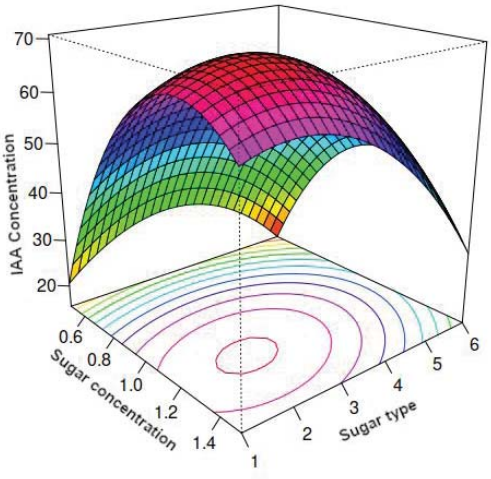

Figure 9: Response surface plot showing interaction effect of sugar type and sugar concentration on IAA production. Sugar types are in coded value (1 = Sucrose, 2 = Starch, 3 = Maltose, 4 = Dextrose, 5 = Mannitol, 6 = Mannose).

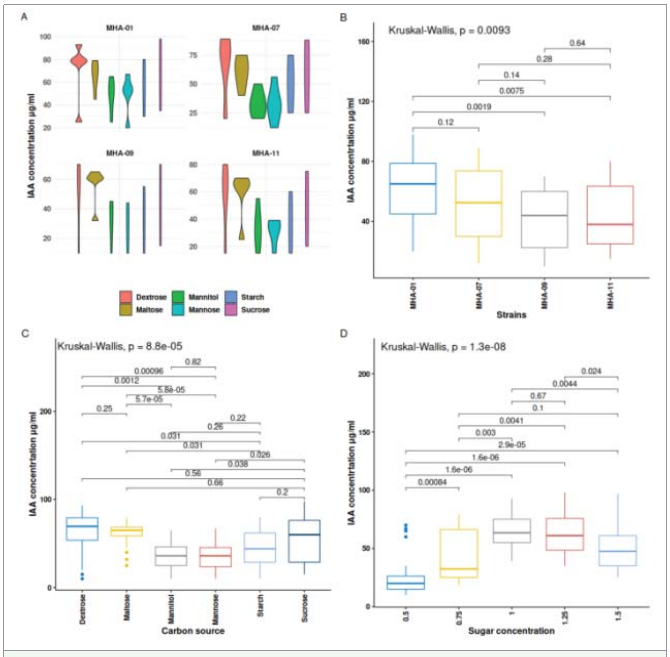

Figure 10: Violin plot A) shows overall data distribution of IAA production in four strains. Box plot showing differences in IAA production between four strains B), between six sugars C) and between sugar concentrations D).

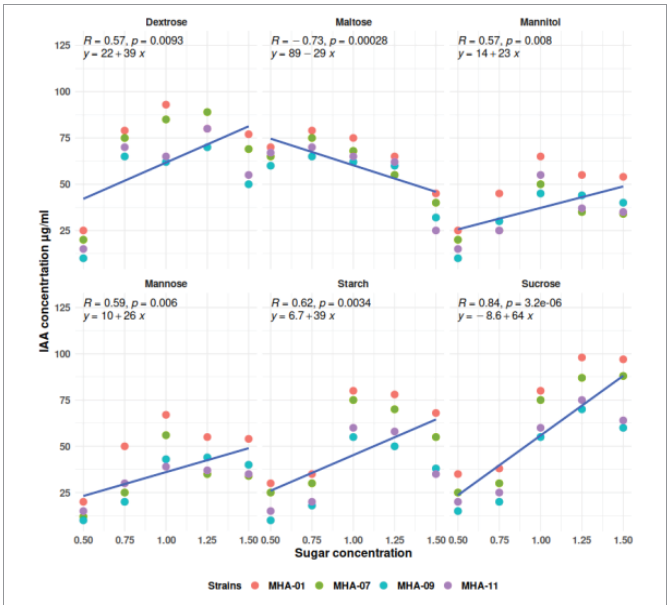

Figure 11: Linear regression models showing correlation between IAA production and sugar concentration in different strains.

while the vigour index was increased by 90.14%, 82.67%, 112.90%, and 79.86%, respectively. The application of MHA-01 increased shoot fresh weight 82.60% in case of *Zea maize,* 75% in case of *Vigna radiata,* 71.87% in case of *Pisum sativum* and 66.66% in case of *Cicer arietinum* above control (Figure 5). Similarly root fresh weight increased 150% in case of *Zea maize* 133.33% in case of *Vigna radiate,*130.76% in case of *Pisum sativum* and 83.33% in case of *Cicer arietinum* above control. The application of MHA-11 in case *Zea maize, Vigna radiata*, *Pisum sativum,* and *Cicer arietinum* showed the moderate percentage of growth increase shoot fresh weight 73.91%, 65%, 84.37% and 60% and root fresh weight 122.5%, 70%, 130.7% and 63.88%, shoot biomass 147.61%, 115%, 143.24% and 89.74% and root

biomass 228.57%, 168.42%, 173.33 and 136% higher than the control. Similarly the application of strains MHA-07 and MHA-09 in case of *Zea maize, Vigna radiata*, *Pisum sativum* and *Cicer arietinum* showed that shoot fresh weight, root fresh weight, shoot biomass and root biomass significantly increased than the control plants (Figure 5). The application of *Burkholderia and Herbasprillum* in Maize (*Zea mays* L.), sugarcane grass, shorgum increased the growth and production by synthesis of indole-3-acetic acid (IAA) [41-43]. The growth and yield of many important crops, including maize, banana, and Bt cotton increased by the application of PGPR [44]. *Pseudomonas aeruginosa* PS24, an antagonistic bacteria showed the multiple plant growth promoting attributes such as phosphate solubilization activity, indole acetic acid (IAA), siderophore,and HCN production [45]. The enteric bacteria *Salmonella* showed the production of IAA, colonizing plant tissues and help in interaction with plants and animals providing new incentives to gain insight into the function of this plant hormone in a larger biological context [46] .The screening of beneficial microorganisms from different plants rhizosphere based on IAA producing properties which is considered as effective tool rhizobacterial isolates on plant growth [47]. The involvement of rhizobacterial isolates for enhancing the plant growth by synthesizing IAA which was confirmed previous studies. Root elongation of *Sesbania aculeate* and *Brassica campestris* was found to occur by inoculation with *Azotobacter* spp. and *Pseudomonas* spp., and by *Bacillus* spp, [48], in *Vigna radiata* by *Pseudomonas putida* [49], and in *Pennisetum americanum* by *Azospirillum brasilense* [50]. Effect of IAA producing isolate was also observed in *Solanum lycopersicum*, where it significantly increased the shoot and root biomass and chlorophyll (a and b) contents as compared to control plants.

Application of MHA-11 significantly enhanced total chlorophyll and carotenoid content of leaves in *Zea maize* by 52.63% and 82.75% over untreated control plants followed by 60.29%, 56.45% and 51.78% of *Vigna radiata*, *Pisum sativum,* and *Cicer arietinum* respectively. Similarly application of MHA-01, MHA-07, and MHA-09 in *Vigna radiata*, *Pisum sativum,* and *Cicer arietinum* pot increased the total chlorophyll and carotenoid content in treated plant over the control. Chlorophyll biosynthesis has been considered as an indicator of net physiologically available iron to the plant. Higher absorption of iron is correlated with higher contents of chlorophyll a, chlorophyll b and total chlorophyll [23,51]. The increase in chlorophyll content could be due to the utilization of microbial siderophore by the plants. The best way to control insecticide-resistant pest species is to screen out alternate options that cause the least off-target effects. In the modern era of bio-intensive agriculture, bacterial-derived chemicals have emerged as more environmentally friendly and safer alternatives to synthetic pesticides [52]. An insecticidal effect of four different bacterial extracts on mango mealybug nymphs and adults was determined in this study using two bioassays. There was no effect of mealybug on the control treatments. In contrast, adult female mealybugs were more susceptible to bacterial extract than second-instar nymphs, most likely due to their more intricate body integument (Waxy layer of mealy powder covered with intricate body integument) [24].

CONCLUSION

There is an increasing need for bacterial pesticides to replace toxic and hazardous synthetic chemicals, thus providing effective biorational options against mealybugs and other homopterous pests, and so they should be incorporated into future pest management programs. In this study, we employed a RSM approach to optimize the production of IAA by MHA-01, MHA-07, MHA-07 and MHA-11 with the goal of exploring formulations best suited for commercial agriculture. IAA production was enhanced by optimizing the culture conditions using a statistical design method and a suitable medium for improved IAA production was developed. The maximum yield of IAA was obtained at pH 8 (85.7 $\mu g/ml^{-1}$) and at temperature 37°C (80.24 $\mu g/ml^{-1}$) by isolate MHA 01. Dextrose (1%) was found to be the best carbon source for isolate MHA 01 with 93 $\mu g/ml^{-1}$ IAA production and malt extract was the good nitrogen source for IAA production showed maximum production by MHA-01 95 µg/ml at 1.5% concentration. strains MHA- 01, MHA-07, MHA-09 and MHA 11 significantly increased the percentage of seed germination by 33.78%,38.01%, 42.02%, and 37.06% compared with the control, while the vigour index was increased by 90.14%, 82.67%, 112.90%, and 79.86%. The application of strains MHA- 01, MHA -07, MHA-07 and MHA-11 in case of *Zea maize, Vigna radiata*, *Pisum sativum,* and *Cicer arietinum* showed that shoot fresh weight, root fresh weight, shoot biomass and root biomass significantly increased than the control plants. Therefore, further research is needed to confirm that rhizobacteria have the potential to contribute to plant development, plant defense, and plant productivity in order to ensure sustainable agriculture.

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Compliance with ethical standards

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the author.

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