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Production and Estimation of Plant Growth Regulators in Fungi Isolated from Western Ghats

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ABSTRACT

Introduction: The Rhizosphere is the narrow region of the soil tightly adhered to the plant root system and is under the influence of metabolites released by the associated soil microorganisms. Metabolites act as chemical messengers and attract motile microorganisms, representing a nutrient source to support their growth. These microorganisms are often called plant growth-promoting microorganisms, including bacteria, fungi, actinomycetes, and cyanobacteria.

Aim/Objectives: The plant growth regulators were produced from the fungi obtained from Western Ghats. The potent PGPF was selected for HPLC and pot study analysis among the isolates obtained. Also, 18s rRNA sequencing was performed to identify the genetic similarities.

Methodology: Samples were collected from the rhizosphere of the Western Ghats region. They are used for isolation and partial identification of fungi. Later, organisms were subjected to IAA and GA determination up to 15 days. The estimated IAA and GA were subjected to HPLC analysis and compared with the standards. Further, the cultured filtrate of isolated organisms was tested for IAA and GA activity by performing pot analysis with *Vigna radiata.* The data obtained from pot studies was statistically analyzed. The potent isolated organism was further identified using molecular sequencing and bioinformatics tools.

Results: The isolated organisms were partially identified as species from *Aspergillus, Rhizomucor*, *Fusarium, Cladosporium,* and *Trichoderma.* Among the isolated organisms, *Rhizomucor* had the maximum concentration of IAA (15 days incubation), and a species of Rhizomucor and Fusarium had maximum GA (15 days incubation). The presence of IAA and GA was confirmed by comparing the standard peaks obtained from HPLC analysis. Pot study analysis showed an initial increase in shoot and root length from the 5th day onwards. Based on the statistical analysis, auxin root length showed significance at 95% interval levels. Compared with databases, the sequenced data had a 99.82% identity with *Mucor irregularis*.

Conclusion: Auxins and gibberellins produced by the isolated organisms enhanced the root and shoot length and thus can be chosen as bioinoculants.

Key Words: Bioinoculants, Chemical fertilizers, HPLC, Metabolites, Auxins, Gibberellins

INTRODUCTION

The Rhizosphere is the narrow region of the soil tightly adhered to the plant root system and is under the influence of metabolites released by the associated soil microorganisms. Metabolites act as chemical messengers and attract motile microorganisms, representing a nutrient source to support their growth.[1] These microorganisms are often called plant growth-promoting microorganisms, including bacteria, fungi, actinomycetes, and cyanobacteria. Microorganisms within the Rhizosphere are crucial in shaping the plant host. Some of them are helpful for the overall growth and development of plants. Microorganisms establish symbiosis with plants based on mutual benefits, also known as mutualism.[2] The next group of organisms includes pathogenic fungi, oomycetes, bacteria, and nematodes. Various organisms inhabit the Rhizosphere, and the fungi colonizing this habitat are called plant-growth plant- promoting fungi (PGPF).[3]

Plant Growth Promoting Fungi comprise species of the genera such as *Aspergillus, Fusarium, Penicillium, Trichoderma, Piriformospora, Phoma,* and *Rhizoctonia.* [4] Rhizosphere microorganisms play an essential role in releasing nutritional cations from the soil minerals and are helpful for their nutri-

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tion and plant nutrition. Biological Nitrogen Fixation is the best-known example of plant growth promotion.[5] Arbuscular Mycorrhiza (AM), a mutual symbiosis of plant roots and fungi, increases the absorbent surface of seeds. It provides better access to water, minerals, and environmental and biotic stress resistance. Many PGPFs release plant growth regulators known as phytohormones.[6]

Plant growth regulators or hormones are further classified into plant growth promoters and inhibitors based on their mode of action. Plant growth promoters promote cell division, cell enlargement, flowering, fruiting, and seed formation, which include Auxins and Gibberellins.[7] In contrast, inhibitors inhibit growth and encourage dormancy. Inhibitors also promote abscission in plants containing abscisic acid. Ethylene and other gaseous phytohormones act as either promoters or inhibitors, but most are inhibitors.[8]

The extended use of chemical fertilizers has led to pollution worldwide, which includes soil and water pollution. Replacing them with bio-origin products like phytohormones reduces pollution and regulates plant growth and development.

The present study aims to isolate the phytohormones, particularly auxins and gibberellins, from Plant Growth growth-promoting fungi (PGPF) and to estimate the maximum phytohormone producer among the selected PGPF. Further studies are aimed at the application of the phytohormone to increase the growth of the plants.

Plant Growth Promoting Fungi (PGPF):

Plants are associated with various microorganisms, particularly Rhizosphere fungi and bacteria. These microorganisms positively and negatively impact plant growth and development. Fungi associated with the plant root improve plant growth and are called Plant Growth Promoting Fungi (PGPF).[9]

PGPF belongs to a diverse category of saprotrophic, non-pathogenic fungi. They are classified broadly into endophytic, which exists inside the roots and directly exchanges metabolites with plants; epiphytic, which exists freely on the surface of the roots; and free-living PGPF, which exists outside of plant cells, i.e., in the Rhizosphere.[10] They frequently engage in intricate interactions with plants and evolve unique tactics to facilitate host plant productivity, growth, flowering, and seed germination enhancements. In addition to mediating favorable effects on plant growth and development, PGPF also has an advantageous impact on suppressing phytopathogenic bacteria.[11]

Under all circumstances or in connection with all plant hosts, not every organism classified as PGPF will promote plant development. Most extensively reported and widely distributed PGPF include *Aspergillus, Fusarium, Penicillium, Phoma,* and *Trichoderma*. The ability of PGPF to encourage plant growth and development strongly supports the fact that they possess certain advantageous features for plants. Both immediate and long-term impacts on germination and subsequent plant performance can be controlled by PGPF.[12] The most frequently prescribed effects by PGPF include an improvement in germination, seedling vigor, shoot growth, root growth, photosynthetic efficiency, blooming, and yield. A certain PGPF may exert all, some, or all of these effects to influence plant growth.[13]

Plant-hormones (Phytohormones):

The term "Phytohormone" (or plant growth substances) was first used by Thiman.[14] Plant hormones/phytohormones, also known as plant growth substances, are produced naturally by plant tissues (i.e., endogenously) and enhance their growth and development. "Plant Growth Regulators" are synthetic chemical substances, such as polyamine, that can modify plant growth and development by similar physiological activities to those of plant growth substances. They do this by managing plant growth processes like the development of leaves and flowers, controlling, modifying, or regulating plant growth processes, such as the formation of leaves and flowers, the lengthening of stems, and the growth and ripening of fruits.[15]

This concept of plant growth regulators came from an experiment on phototropism. The investigation showed that in the germination of oat seedlings exposed to a lateral light source, a transported signal originating from the plant apex. It promoted differential cell elongation in the lower parts of the seedling and caused it to bend toward the light source.[16]

Synthetic hormones can control similar processes, such as the development and growth of buds, flowers, fruits, and roots. These hormones occur at low concentrations in plant tissue, which presents significant challenges in isolating, identifying, and extracting appropriate amounts for laboratory tests. In agriculture, viticulture, and horticulture, plant growth regulators are frequently used to promote growth under unfavorable or stressful conditions (such as short growing seasons, low soil fertility, and diseases) as well as to increase yields and facilitate harvesting (by preventing immature fruit drop, accelerating maturity, and ripening, for example).

Auxins:

The Auxin plays an essential role in plant growth and developmental processes. Phototropism,

Geotropism, and the role played by auxins were reviewed by Fankhauser and Christie. [17]

"Avena Coleoptile Curvature test" is the bioassay for auxin detection and was described by Dutch botanist Went [18], who also discovered Auxin.

Indole-3-acetic acid (IAA), a standard product of L-tryptophan metabolism by several microorganisms, is an active substance that was found to be similar to Auxin. K.V. Thimann first isolated IAA in a culture of the fungus *Rhizopus sinus*.[14] The most naturally occurring auxins, including IAA, Indole-3-butyric acid (IBA), and 4-chloroindole-3-acetic acid (4-Cl-IAA), contain an aromatic indole ring in their structure.[19] Physiologically active quantities of auxins produced by diverse soil microorganisms may profoundly impact plant growth and establishment.

Decarboxylation and deamination of L-tryptophan in seeds, young leaves, and other tissues results in IAA synthesis. Due to the abundant availability of substrates, microbial isolates from the Rhizosphere of various crops have a higher potential to produce and release IAA as secondary metabolites. Isolated microbial species and strains produce varying amounts of IAA depending on the multiple substrates available. Most root-promoting microorganisms synthesize IAA, and their effect on plants is similar to that of exogenous IAA.[20]

Young seedlings benefit from the rapid establishment of roots because it improves their capacity to cling to the soil and absorb water and nutrients from their surroundings, increasing their chances of survival. Adding sucrose (0.5%) and calcium nitrate (0.1%) supplements provide carbon and nitrogen sources to the L-tryptophan medium, thereby increasing IAA production.

Cell enlargement and division, tissue differentiation, and response to light and gravity are the essential physiological processes that IAA controls in plants. Microorganisms that produce IAA can be either pathogenic or beneficial to plants and can impede plant growth by upsetting the auxin balance when they interact with the plants. In pathogenic microorganisms, IAA participates in developing plant tumors and makes its way from tryptophan via the intermediate indoleacetamide.[21] IAA is primarily produced by beneficial microorganisms using a different tryptophan-dependent pathway, which involves indole-pyruvic acid.[22]

Indole-pyruvic acid results from the reductive deamination of tryptophan, and the final products of the reaction are indole, pyruvic acid, ammonium (NH4+), and energy in which tryptophanase enzyme catalyzes the amine (-NH2) group and requires pyridoxal phosphate as a coenzyme in the reaction.[23]

The sub-cultured fungal isolates were batch cultured by inoculating the fungal colonies into a 250ml Erlenmeyer flask containing 100 ml of Potato Dextrose broth supplemented with tryptophan and NaCl at a concentration of 2g/L and 5g/L, respectively. Three consecutive days—Days 3, 6, 9, 12, and 15— were used to measure IAA production in the inoculated flasks. They were raised at 27°C in a shaker incubator concurrently.

Gibberellins:

Gibberellins is a type of plant and fungal phytohormone. Gibberellins are a large family of tetracycline diterpenoids with an ent-gibberellin ring structure containing 20 or 19 carbon atoms.[24]

Plant hormones, by the entire life cycle of the plant, regulate many different forms of plant growth and development, including the promotion of cell division and elongation, seed germination, stem and hypocotyl elongation, root growth, and flowering. Scientists have discovered one hundred thirty-six gibberellin molecules, but only some are bioactive, such as GA1, GA3, GA4, and GA7. [25]

The terpenoid pathway in plastids produces the active form of gibberellins, which are then modified in the endoplasmic reticulum and cytosol to make active gibberellins. Some inhibitors, such as AMO- 1618, Phosphon-D, and B-995, are also present, and these significant chemical substances are called anti-gibberellins.[26]

Understanding GAs was first developed in plant pathology, with studies on "foolish seedling" disease in rice.27 Fungus *Gibberella fujikuroi* (*Fusarium moniliformae*) causes Foolish seedling disease. The active compound in the disease-causing fungus, *Gibberella fujikuroi*, was isolated by Yabuta and Sumuki.28

In crystalline form, which is relatively heat stable, it is named gibberellic acids or gibberellins A and B (GA3). The precursor of Gibberellins is Acetyl CoA.

Holbrook estimated gibberellic acid concentrations with slight modifications by the spectrophotometric method.[29] Phytohormones have a wide range of applications for better growth and development of plants. The present study mainly reviews the plant growth promoters (Auxins and Gibberellins); the following are the functions and applications of the phytohormones towards plant growth.

Functions of Auxins:

- Aids in the elongation of cells, cell division in tissue, and organ culture.
- Beneficial for secondary growth, necessary for coleoptile and stem growth.
- Essential for apical dominance.
- It enhances adventitious root initiation in cuttings, raises the female flower number and decreases the number of male flowers in plants, prevents premature fruit drop, delays leaf abscission, prolongs dormancy, and eliminates weeds.

Functions of Gibberellins:

- In citrus fruits and leaves, senescence may be postponed by it.
- It can defer plant senescence by preventing androecium growth.

- It causes sterility in plants by inhibiting androecium growth.
- It is necessary for parthenocarpy in apple, tomato, and cucumber.
- It is essential for genetic dwarfism and the germination of seeds.

The delivery of agriculturally essential microorganisms has been smartly implicated by encapsulating the organism in a gelatin capsule; this encapsulating technology is known as bio-capsules. In the case of PGPR and *Trichoderma harzianum*, this technology has been developed, field-tested, and successfully commercialized by the ICAR-Indian Institute of Spices Research, Kozhikode.[30] The microorganisms encapsulated in the capsule are either immobilized or inactivated, which can be activated by dissolving the capsule in water.

Agriculture uses chemical fertilizers, which may be organic or inorganic. Inorganic fertilizers contain heavy metals like mercury, cadmium, lead, and copper, which are considered toxic to the environment, resulting in poor soil fertility and low crop yield.

The main objective of the present study is to estimate the maximum PGR (Auxins and Gibberellins) producers from fungal isolates collected from the Western Ghats soil sample. The present study was mainly designed to isolate plant growth-promoting rhizobacteria and identify their role in increasing the plant's growth. Plant growth hormone auxin was extracted from the efficient isolate and applied as an inoculant to the plant.

MATERIALS AND METHODS

Materials required:

All the chemicals used in the present study were procured from the Standard Indian Chemical companies. All the HPLC solvents were high-grade and obtained from Sigma Aldrich Company.

Methodology:

Sample collection:

Soil samples were collected from the Rhizosphere in the Western Ghats (Achankovil, Kerala, India) and brought to the lab in a sterile zip-lock pouch within 48 hours.

Isolation and Identification of Fungi

The sample collected (fig. I) was used to isolate fungi by pour plate method in Potato Dextrose Agar (PDA).31 The serially diluted soil sample was utilized to isolate fungi. After plating all the dilutions, the plates were incubated at room temperature (20-27°C) for 3-5 days. Then, the fungal colonies were identified microscopically by the lactophenol Cotton Blue staining method. The fungal colonies were then sub-cultured on fresh PDA plates.

Preparation of Salkowski Reagent:

Add 49 ml 35% Perchloric acid to 1 ml of 0.5M ferric chloride solution to get the Salkowski reagent.[32]

Indole-3-acetic acid (IAA) Determination:

The sub-cultured fungal isolates were subjected to fermentation by inoculating the fungal colonies into 250 ml Erlenmeyer flask containing 100 ml Potato Dextrose Broth supplemented with tryptophan and NaCl at 2g/L and 5g/L, respectively. The medium was incubated in a shaker incubator at 27±2°C, and IAA production was determined colorimetrically on Days 3, 6, 9, 12, and 15. During IAA determination, 5ml of fungal filtrate was pipetted onto 15ml centrifuge tubes and was centrifuged at 10,000rpm for 10 mins. After centrifugation, a supernatant of 1 ml was reacted with 4 ml of Salkowski's reagent and was incubated for 30 minutes in dark conditions. The pink color indicates the IAA production, and absorbance was read at 530 nm. The maximum IAA producer among the fungal isolates was identified by plotting the graph of OD at 530 nm on the Y-axis v/s Days on the X-axis.[33]

The above procedure is followed for the commercial-grade IAA standard, and a comparison was made between the two. The sample was further analyzed quantitatively by HPLC.

Identification of IAA by HPLC:

The fungal culture supernatant was analyzed for IAA quantification using a High-performance liquid Chromatography (HPLC) system containing a C18 column and ultraviolet (UV) detector that absorbs at 254 nm. The sample injection volume is 20µl, and the mobile phase used was acetonitrile and acid water (0.01% H_3PO_4) with a ratio of 60:40, respectively, having a flow rate of 1.0 ml/min, and the retention time of the sample peaks compared to those of the IAA standard.

Gibberellic Acid (GA) Determination:

The Gibberellic acid concentration was estimated spectrophotometrically, as suggested by Holbrook. et al.[29] with slight modifications, and shows the following steps. The sub-cultured fungal isolates were subjected to fermentation by inoculating the fungal colonies into a 250ml Erlenmeyer flask containing 100 Potato Dextrose Broth supplemented with NaCl at the concentration of 5g/L. The medium was incubated in a shaker incubator at 27±2°C, and Gibberellic acid production was determined spectrophotometrically on Days 3, 6, 9, 12, and 15. During Gibberellic acid determination, 15 ml of fungal filtrate was pipetted and centrifuged at 5,000rpm for 10 minutes.

To the supernatant 2 ml of Zinc acetate reagent (21.9g Zinc acetate + 1 ml of glacial acetic acid was added, and the volume was made up to 100 ml with distilled water) was added and left aside for 2 minutes, after which 2 ml of Potassium ferrocyanide solution (10.6% in distilled water) was added. 15ml of the supernatant containing Zinc acetate reagent and Potassium ferrocyanide solution was centrifuged at low speed (2,000rpm) for 15 minutes.

To 5ml of the supernatant, 5ml of 30% HCl solution was added and incubated at room temperature for 75 minutes. 5 ml of 5% HCl solution was used as a blank. Absorbance was read at 254 nm using a UV/Vis spectrophotometer.[34] The maximum Gibberellic acid producer among the fungal isolates was determined by plotting a graph with OD at 254 nm on the Y-axis v/s Days on the X-axis. The procedure mentioned above is followed for the commercial-grade GA standard, and a comparison was made between the two. The sample was further analyzed by HPLC analysis.

Identification of Gibberellins by HPLC:

The sample preparation of Gibberellins was done according to the procedure mentioned by Shwetha Sharma et al.[34] The fungal supernatant was mixed with 250 ml of saturated NaHCO3 solution in a separating funnel. The aqueous phase collected was extracted with 300 ml of ethyl acetate (2X), and the organic phase was discarded. 5N HCl was added to the aqueous phase to make the PH 2.5. Later, equal volumes of ethyl acetate were added and shaken vigorously for 5 min. Further, separate the ethyl acetate fraction and re-extract with an aqueous layer with 300 ml of ethyl acetate solvent. All the ethyl acetate fractions were pooled. Later, 1ml of acidic Ethyl acetate extract was evaporated under vacuum by rotary evaporator at 40°C and dissolved in 250 µl acetonitrile (HPLC grade), and 5µl of this solution was injected into Shimadzu HPLC instrument under the following conditions:

Column dimension W 260 × H 210 × D 500mm, excluding protruding parts; mobile phase: acetonitrile and acid water (0.01% H3PO4) in the ratio 60:40; flow rate 1 ml/min; variable wavelength detector at 254 nm.

Pot Study:

The seeds *Vigna radiata* were surface sterilized to remove the contaminants that are adhered to the seed coat. First, the seeds were treated with 95% ethanol and 2.5% Sodium hypochlorite (NaOCl) for 10 minutes, respectively. The seeds were cleaned thrice with distilled water to remove the chemicals. Soil for pot study was collected and autoclaved at 121°C for 15 minutes with a pressure of 15 lbs. After sterilization, the soil samples were mixed with the respective fungal culture filtrates and filled into the pots with *Vigna radiata.* The pots are regularly monitored and watered periodically. The plants are then examined for the shoot and root length as mentioned by Bhattacharya et al.[35]

Biostatistical analysis:

Data obtained during the study was statistically analyzed using M.S Excel software.

Identification of the potent PGPF by Molecular Characterization:

The potent fungal isolate isolated, which showed the highest IAA production, was selected for further identification by molecular characterization by standard protocol (Spin column) of Barcode Life Sciences Ltd.

Extraction of DNA sample:

The test sample and 1 ml of lysis buffer were incubated at 600°C for 2 hours. Add 1 ml of Chloroform: Isoamyl alcohol (24:1) and vortex it. Centrifuge at 13,000rpm for 8 min. Take the supernatant into a new vial, add 850µl of binding buffer, and mix it well. Transfer it to the column tube subjected to centrifugation at 12,000 rpm for 1 minute. Repeat the step if necessary. Add 700 µl of wash buffer and centrifuge at 12,000 rpm for 1 min. Repeat the same action and increase the time to 2 minutes. Everything is done in the same collection tube. Dry spin the same collection tube at 14,000 rpm for 4 minutes. Transfer the spin column into a 2ml tube. Add 50 µl of elution buffer and store for the further process. Thermo Fisher Scientific DNA analyzer 3730XL (Table 1) was used to determine the sequence of the extracted DNA.36

Bioinformatics studies:

The data obtained after performing sequencing was analyzed using bioinformatics tools like BLAST. [37] Further, they were investigated to show the relationship among the similar sequences constructing a phylogenetic tree.

RESULT AND DISCUSSION

Isolation and Identification of Fungi:

The fungi identified belonged to *Aspergillus spp, Rhizomucor spp, Fusarium spp, Cladosporium spp, and Trichoderma spp* (fig.II).

Auxin Determination:

The amount of auxins produced by fungal isolates was determined by colorimetric method at 530 nm on Days 3, 6, 9, 12, and 15. A standard curve was obtained using 100µg/ml stock solution of IAA prepared by dissolving 1mg IAA in 10 ml of acetone and mixing well. Different IAA concentrations (Table 2) were taken in a test tube and treated with 2 ml of Salkowski's reagent (fig. III, fig. IV).

A species of *Cladosporium, Fusarium, Rhizomucor*, and *Trichoderma* showed maximum production of IAA, among which IAA from *Rhizomucor* was further analyzed by HPLC

quantification. The following table (Table 3) indicates the concentration of IAA (in µg/ml) produced by respective fungal cultures every three consecutive days up to 15 days. A species of *Rhizomucor* was found to have a maximum concentration of IAA when compared to other fungal isolates.

Quantitative estimation of IAA by HPLC:

The fungal filtrate supplemented with tryptophan and NaCl with the concentration of 2g/L and 5g/L was centrifuged, and 20µl of the supernatant was injected in HPLC. The retention time of the sample peak was found to be 3.100, while the IAA Standard was 3.554[38]. The area and height of the sample peak were 41706814 and 828809, respectively (fig. V).

Gibberellic Acid:

The amount of Gibberellic Acid produced by fungal isolates was determined spectrophotometrically at 254 nm on Days 3, 6, 9, 12, and 15. A standard curve was obtained using a 100µg/ml stock solution of Gibberellic Acid prepared by dissolving 0.04 mg Gibberellic Acid in 100 ml absolute alcohol and mixing well. Different concentrations of Gibberellic acid were taken in a test tube and treated with 2 ml Zinc acetate solution followed by 2 ml Potassium ferrocyanide solution after incubation for 2 minutes. The contents in the tubes were centrifuged at 2,000 rpm for about 15 minutes, and an equal volume of 30% HCl was added to the collected supernatant. Absorbance was read spectrophotometric at 254 nm after 75 minutes of incubation (fig.VI, fig. VII, Fig **VIII** Table 4).

A species of *Fusarium, Rhizomucor*, and *Trichoderma* showed maximum IAA concentration, among which HPLC further analyzed IA from *Fusarium*.

The following table (Table 5) indicates the concentration of Gibberellic Acid (in µg/ml) produced by respective fungal cultures on Days 3, 6, 9, 12, and 15. A species of *Fusarium* and *Rhizomucor* were found to have the maximum concentration of GA when compared to other fungal isolates.

Quantitative estimation of Gibberellic Acid by HPLC

The fungal filtrate supplemented with NaCl at 5g/L was solvent extracted with ethyl acetate, and 20µl of the aqueous phase was considered for HPLC. The retention time of the sample peak was found to be 3.261, while the IAA Standard was 3.651.[39] The area and height of the sample peak were 22198341 and 285547, respectively (fig VII).

Pot Study

The pot study includes mixing the sterile soil with respective fungal filtrates. Observations on day 15 include measuring the root and shoot length. In the pot study of IAA, the root and shoot length of the plant were found to be maximum in the soil supplemented with a species of *Rhizomucor* (fig. IX, fig. X, fig. XI, Table 6). The ultimate root length indicates the plant has increased absorption ability for water, minerals, and nutrients deep inside the ground.

In a pot study of Gibberellic Acid, the root and shoot length of the plant were found to be maximum in the soil supplemented with a species of *Fusarium* (fig. XII, fig. XIII, fig. XIV Table 7). The total root length indicates the plant has increased absorption ability for water, minerals, and nutrients deep inside the ground.

Statistical Analysis:

All the experiments were performed as triplicates, the results were expressed as their mean, and statistical analysis was done.

The null hypothesis states that there is no difference in the mean length of the shoots in plants treated with different fungal isolates (Auxins) can be rejected as the p-value is 0.023714, which is less than 0.05. Hence, the results are significant at a 95% significance level.

Also, to substantiate the above observations, the p and f critical values obtained from the ANOVA analysis were cross-checked and found to be in sync with the p-value results.

The null hypothesis states that there is no difference in the mean length of the shoots in plants treated with different fungal isolates (Gibberellic acid) can be rejected as the p-value is 0.000008, less than 0.05. Hence, the results are significant at a 95% significance level. Also, to substantiate the above observations, the p and f critical values obtained from the ANOVA analysis were cross-checked and found to be in sync with the p-value results.

The null hypothesis that there is no difference in the mean length of the roots in plants treated with different fungal isolates (Gibberellic acid) can be rejected as the p-value is 0.014138, less than 0.05. Hence, the results are significant at a 95% significance level. Also, to substantiate the above observations, the f and f critical values obtained from the ANOVA analysis were cross-checked and found to be in sync with the p-value results (Table 8).

Identification of the potent PGPF by Molecular Characterization:

18S ribosomal RNA sequencing was performed to identify the isolated organism partially. BLAST search analysis was conducted for the sequences procured from the 18s rRNA sequencing using the NCBI- Genbank database. The results showed sequence identity of 99.82 % to *Mucor irregularis strain* LHL1-3 and 99.64% to *Rhizomucor Variabillis* (fig. XV, fig. XVI).[40] BLAST analysis revealed alignment of the sequence with many species of the genus Mucor; among them, we have considered the maximum similarity percentage.

CONCLUSION

Using chemical fertilizers in agricultural fields has increased the risk of pollution and health risks among humans and animals. The alternative to chemical fertilizer is the use of bio-inoculants. The bio- inoculants include bacteria, fungi, and actinomycetes. The metabolites produced by the bio-inoculants promote growth and hence are called "Plant Growth Regulators."

The phytohormones are the plant growth regulators, which include Auxins, Gibberellins, Abscisic Acid, Cytokinins, and Ethylene. The bio-inoculants are known to release these phytohormones as secondary metabolites. Auxins and Gibberellins promote better development and growth of plants, including improvement in germination, seedling vigor, shoot growth, root growth, photosynthetic efficiency, flowering, ripening, and yield.

The amount of Auxins and Gibberellins produced by Plant Growth growth-promoting fungi (PGPF) was estimated. The maximum phytohormone-producing fungal isolate is considered the best bio- inoculant and an alternative to chemical fertilizers.

The use of bio-inoculants not only reduces the pollution level by altering the chemical fertilizer but is also cost-effective, has a better yield, is organic in nature, etc.

Future aspects of this study include the development of biocapsules, which are gelatin capsules encapsulating inactivated or immobilized microbes. These immobilized or inactivated microbes can be activated by dissolving the capsule in water. Since gelatin capsules are products obtained from vegetables, collagen of animal skin, or bones, they do not harm the environment.

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Conflict of Interest:

All authors declare that they have no conflicts of interest.

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Authors' Contribution:

All authors' equally participated in the preparation of manuscript.

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Table I: Primers used

Primers used:

Fungi- ITS1/ITS4 (5.8S) – N 600 bp (Cycle 2)

Parameters	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
Temperature	95^0 C	95^0 C	50^0 C	72^0 C	72^0 C
Time	5min	30 sec	30 sec	45 sec	5 min

35

Table II: Standard curve for IAA

Volume of Standard IAA (ml)	Concen tration of IAA (µg/ml)	Volum e of Distill ed Water (ml)	Volume of Salkowski 's reagent (ml)	Incubate at Room Temperatu re for about 20 minutes.	Volume of Dis-tilled Water (ml)	OD at 530 nm
1.0(PDB)	00	0.0				0.0
0.1	10	0.9				0.09
0.2	20	0.8				0.17
0.3	30	0.7				0.28
0.4	40	0.6				0.32
0,5	50	0.5				0.41
0,6	60	0.4				0.49
0.7	70	0.3				0.54
0.8	80	0.2				0.60
0.9	90	0.1				0.65
1.0	100	0.0				0.69
			2.0		7.0	

Table III: Concentration of IAA in µg/ml ("*" indicates that the samples were diluted in a 1:1 ratio)

Fungal Isolates	Day 3	Day 6	Day 9	Day 12	Day 15
Aspergillus spp.	21.3	25.3	28.0	32.0	33.3
Cladosporium spp.	22.6	34.6	45.3	57.3	80.0
Rhizomucor spp.	28.0	62.6*	82.6*	89.3*	132.0
Trichoderma spp.	17.3	94.6*	97.3*	108.0*	70.6
Fusarium spp.	17.3	25.3	80.0	89.3	61.3

Table IV: Standard Curve for Gibberellic Acid

Volume of standard Gibberel-lic acid (ml)	Concentrati on of standard gibberellic acid (µg/ml)	Vol-ume of distilled water (ml)	Volume of zinc acetate (ml)	Incubatio n at room temperat ure for about 2 minutes	Volume of potassium ferrocyani de (ml)	Centrifuge at 2000rpm for 15 min-utes	Volume of 30% HCL	Incubati on at room tempera ture for 75 minwutes	OD at 545nm
5.0ml (Blank –30%HCl	0.0	0.0							0.0
0.2	20	0.8							0.311
0.4	40	0.6	2.0		2.0		5.0		0.486
0.6	60	0.4							0.600
0.8	80	0.2							0.734
1.0	100	0.0							0.860

Table V: Concentration of Gibberellic acid in µg/ml (d indicates that the samples were diluted 1:5 ratio)

Fungal Isolates	Day 3	Day 6	Day 9	Day 12	Day 15
Aspergillus spp.	13.61	65.74d	71.80d	93.51d	98.93d
Cladosporium spp.	11.38	83.08d	90.53d	97.34d	101.27d
Rhizomucor spp.	12.34	79.89d	82.34d	102.23d	106,06d
Trichoderma spp.	13.29	99.68d	101.0d	104.36d	105.74d
Fusarium spp.	14.57	68.82d	77.02d	81.38d	105.63d

Table VI: Pot Study – Shoot and Root Length of plants in the presence of IAA

Fungal isolates	Length (in cm)			
Control	Shoot length Root length	25.0 09.2	21.0 02.9	18.5 04.0
Aspergillus	Shoot length Root length	24.9 07.2	24.5 04.6	22.5 04.3
Cladosporium	Shoot length Root length	18.0 04.4	17.5 02.8	14.0 01.3
Rhizomucor	Shoot length Root length	25.0 10.1	23.5 09.9	23.3 03.5
Trichoderma	Shoot length Root length	24.0 09.5	22.5 08.4	19.7 08.1
Fusarium	Shoot length Root length	24.7 09.5	18.8 06.7	17.0 04.8
Mixture	Shoot length Root length	21.0 04.9	20.0 04.3	19.5 01.9

Table VII: Pot Study – Shoot and Root Length of plants in the presence of Gibberellic Acid

Fungal isolates	Length (in cm)			
Control	Shoot length Root length	21.0 06.5	20.0 04.4	14.3 02.2
Aspergillus spp.	Shoot length Root length	15.3 04.6	14.7 04.1	14.1 03.5
Cladosporium spp.	Shoot length Root length	09.8 03.1	08.3 03.0	04.3 01.7
Rhizomucor spp.	Shoot length Root length	17.4 03.6	15.5 03.5	13.7 03.5
Trichoderma spp.	Shoot length Root length	06.5 03.3	06.0 03.2	05.5 03.1
Fusarium spp.	Shoot length Root length	22.0 06.7	18.9 05.9	18.0 05.3
Mixture	Shoot length Root length	14.7 04.7	14.0 04.6	11.9 04.3

Table VIII: Statistical Analysis (ANOVA) of IAA and Gibberellic Acid

Component	F - value	p - value	F critical
Auxin - Shoot length	3.553413	0.023714	2.847726
Auxin - Root length	2.455674	0.077902	2.847726
Gibberellic acid - Shoot length	17.67192	0.000008	2.847726
Gibberellic acid - Root length	4.081964	0.014138	2.847726

All the experiments were performed as triplicates, results were expressed as their mean, and statistical analysis was done using ANOVA. When treated with both Auxins and Gibberellins, the results are significant at a 95% significance level.

Figure I: Soil Sample collection area: Achankovil Road, Aruvappulam, Kerala, India

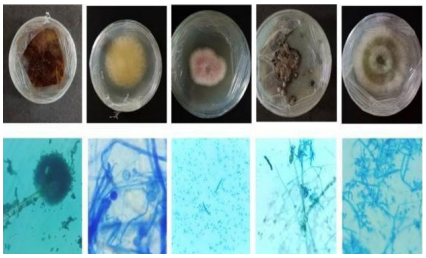

Figure II: Fungal colony and microscopic observation (From left: A species of Aspergillus, Rhizomucor, Fusarium, Cladosporium and Trichoderma).

Figure III: Determination of IAA

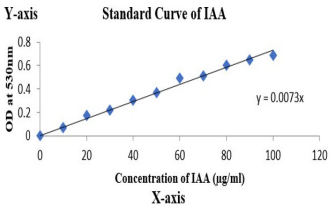

Figure IV: Standard curve for IAA

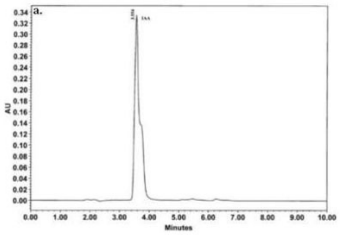

Figure Va: HPLC chromatogram of Standard IAA38

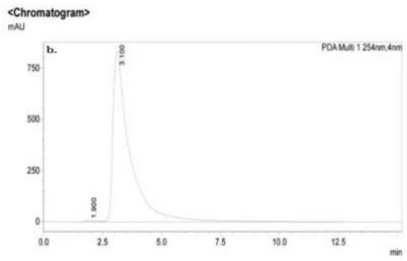

Figure Vb: HPLC chromatogram of Sample

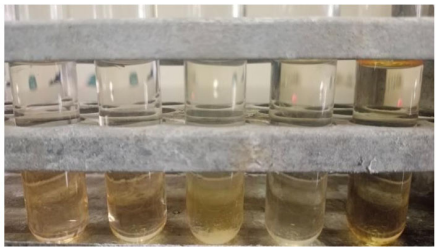

Figure VI: Gibberellic Acid Determination

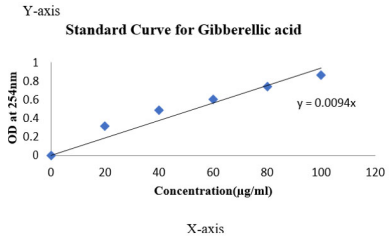

Figure VII: Standard curve for Gibberellic acid

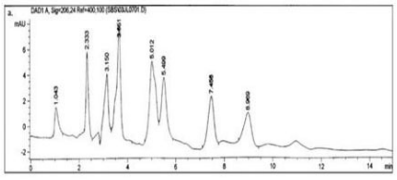

Figure VIII a: HPLC chromatogram of GA39

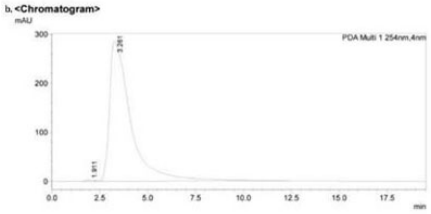

Figure VIII b: HPLC chromatogram of Sample

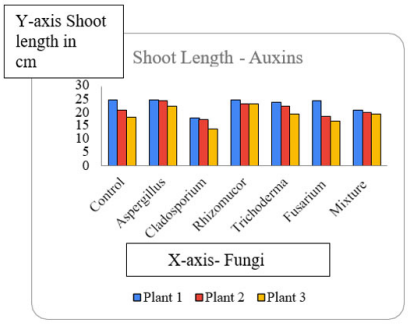

Figure IX: Shoot length of plants in presence of IAA

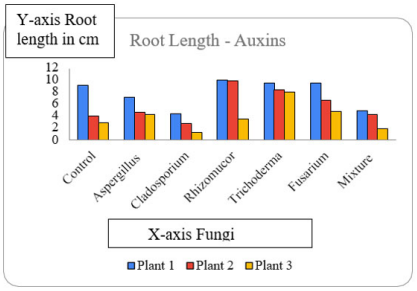

Figure X: Root Length of plants in the presence of IAA

Figure XI: Pot Study - Indole-3-Acetic Acid

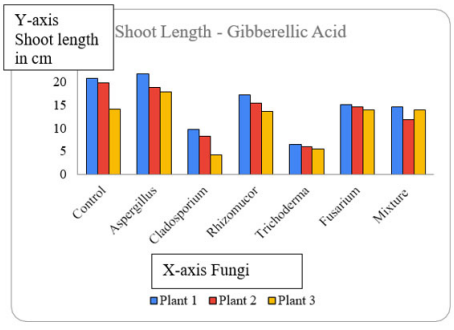

Figure XII: Shoot Length of plants in the presence of Gibberellic Acid

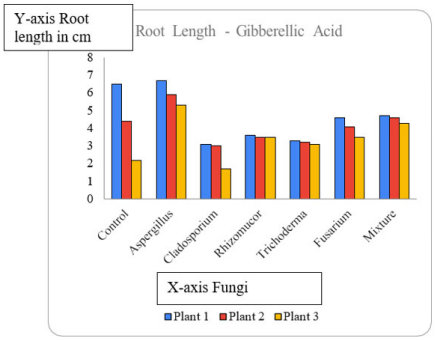

Figure XIII: Root Length of plants in the presence of Gibberellic Acid

Figure XIV: Pot Study - Gibberellic Acid

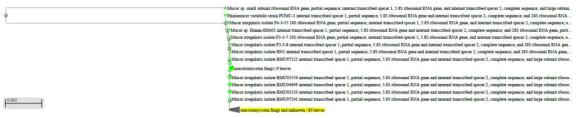

Figure XV: Phylogenetic Tree (ITS1) [Yellow highlighted: Query, green highlighted: hits]

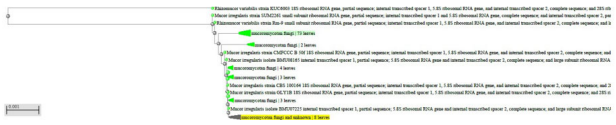

Figure XVI: Phylogenetic Tree (ITS4) [Yellow highlighted: Query, green highlighted: hits]