Antimicrobial and Phytohormone production potential of *Azotobacter chroococcum* and its effect on the growth of non-leguminous plants

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Abstract
The overuse of chemical fertilizers in agriculture leads to a decrease in the fertility of soil and increases chemical pollution, which has adverse effects on living things. Biofertilizers have a solution towards chemical fertilizer to improve soil fertility and crop production. Therefore, the present study was undertaken to isolate different isolates of Azotobacter sp. to identify effective isolates for plant growth promoting activities and biological control. Six Azotobacter sp. were isolated and their plant growth promoting activities were checked. The isolate AC3 produced the highest amount (64 µg/ml) of IAA and produced HCN and 72 µg/ml of ammonia. The isolates AC1 and AC4 lacked the HCN production. Isolate AC3 showed a 26 mm zone of clearance on Pikovaskys agar, solublized the highest 71 µg/ml of inorganic phosphate and had the ability to produce siderophores.

The isolate AC3 showed good antimicrobial potential against pathogens. Isolate AC3 was identified as Azotobacter chroococcum on the basis of morphological and biochemical characteristics. The bioactive compound was produced by using Azotobacter chroococcum and purified by column chromatography. Fraction 1 showed good antimicrobial activity against all the pathogens. The highest zone of inhibition was 15 mm against F. oxysporum. FT-IR analysis of fraction 1 revealed carboxylic acid, alkane and carbonyl functional groups. Azotobacter chroococcum had a greater effect on the growth of non-leguminous plants in the bacterially inoculated pot than in the control.

Keywords: Biofertilizer, Antimicrobial, *Azotobacter chroococcum*, PGPR, Bioactive compound.

Introduction

Modern agriculture relies heavily on the use of chemicals such as fertilizers, fungicides and pesticides to increase yields. However, the use of chemicals can have a negative impact on the environment and can generate several environmental problems including a decrease in soil fertility, greenhouse effects, ozone layer depletion and acidification of water. Efforts to reduce the impact of environmental pollution on agricultural land include the use of environmentally friendly fertilizers, for example, microorganisms. In the last decade, biofertilizers have been used extensively as an eco-friendly approach to minimize the use of chemical fertilizers, to improve soil fertility status and to enhance crop production through their biological activity in the rhizosphere. Increased global trade, together with climate change and limitations in plant protection products, have favored the emergence and establishment of new plant diseases which in turn, cause significant crop losses[14,27].

A few microbes, including nutrients, plant growth regulators and biocontrol agents have many benefits for plants[15]. Plant growth promoting rhizobacteria (PGPRs) are indigenous to the soil and plant rhizosphere and play a major role in the biocontrol of plant pathogens, as they can suppress a broad range of bacterial, fungal, viral and nematode diseases. A major group of rhizobacteria i.e. Azotobacter, Azospirillum, blue green algae, Azolla, phosphate solubilizing microorganisms and mycorrhizae was found[24]. The multiple mechanisms of biocontrol include the ability to produce a wide variety of antibiotics, chitinolytic enzymes, siderophores and HCN[2,13].

Cysts forming aerobic nitrogen fixing organisms of the genus Azotobacter are important PGPR that benefit plants in multiple ways. The beneficial effects of Azotobacter on the growth and yield of various agriculturally important crop plants include the potential to fix nitrogen and produce vitamins and growth substances including indole acetic acid, gibberellins and cytokines[26] which enhance root growth and aid in nutrient absorption and inhibit phyotopathogenic bacteria and fungi through antibacterial and antifungal substances respectively[18].

In fact, Azotobacter species has beneficial effects on plant yields due to its ability to fix nitrogen[25] to solubiliz phosphates[7,9] and to secrete microbes that stimulate phytohormones, such as gibberellins, auxins and cytokinins. The major issue in production of efficient biofertilizers using Azotobacter sp. is the search for efficient strains possessing an array of beneficial characteristics viz. a high rate of dinitrogen fixation. The ability to produce growth promoting substances and broad spectrum antifungal activity toward phytopathogens were observed[12].

Therefore, the present work planned to isolate effective Azotobacter sp. as a PGPR agent as well as a biocontrol

agent through *in vitro* screening for antagonistic activity and field trials via pot experiment.

Material and Methods

Collection of soil samples: Three soil samples were collected from wheat, cotton and onion cultivated fields in Nanded (Maharashtra) at depths of 1-15 cm. The soil samples were air dried and sieved (<2mm) and stored in sterile bags at 4°C in a refrigerator until the microbes were isolated.

Test organism: Test organisms were obtained from the Department of Life Sciences, S.R.T.M.University, Nanded and maintained on agar slants for further use. The bacterial cultures used were: *Pseudomonas aeruginosa* (ATCC27853), *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 443), *Staphylococcus aureus* (NCIB6571) and the fungal cultures used were *Fusarium oxysporum* (ATCC62705), *Fusarium melanochlorum* (ATCC16069), *Alternaria solani* (ATCC38918), *Candida albicans* (ATCC90028) and *Aspergillus flavus* (ATCC9643).

Enrichment and isolation of Azotobacter: One gram of soil sample was inoculated in 250 ml conical flasks containing 100 ml of Jensen medium. The flasks were incubated on a rotary shaker for 7 days to enrich the nitrogen fixer at 120 rpm at 30°C. After incubation, Gram staining of the pellicle was performed for microscopic observation of the nitrogen fixer. For the isolation of Azotobacter sp., nitrogen free mineral glucose agar was used. The loopful of enriched broth culture was streaked on agar plates which were incubated at 30°C for 72-96 hrs. The well isolated colonies were selected and subjected to culture and morphological characterization. The isolates were characterized by Gram staining, motility and cyst formation.

Screening of isolates for PGPR traits: The following methods were used for assessing the characteristics of the Azotobacter strains revealed by PGP.

IAA production: The selected isolates were grown individually in triplicate in glucose nitrogen free mineral broth supplemented with tryptophan (0.5 gm/ml) and incubated at 30°C for 7 days. Each culture was centrifuged at 10000 rpm for 15 min and cell free supernatants were used foe IAA analysis. 1 ml of supernatant was taken and mixed with 1-2 drop of Salkowskis reagent and development of pink colour indicated positive results. The concentration of IAA produced was determined spectrophotometrically at 530 nm using standard IAA as reference.

Hydrogen cyanide production (HCN): The bacterial cultures were streaked on pre-poured plates of Kings medium-B containing 4.4 gm/lit glycine. A Whatmann filter paper no. 1 was soaked in 2% sodium carbonate in 0.5% picric acid solution and placed in the lids of Petri plates. The Petri plates were sealed with parafilm and incubated at 30°C for 7 days; the uninoculated control was kept for comparison. The plates were observed for a change in the colour of the filter paper from yellow to orange brown to dark brown indicating HCN production[16].

Ammonia production: The isolates were subsequently grown in peptone water at 30°C for 7 days. After incubation, ammonia production was tested by using Nessler's reagent. The development of a brown to yellow colour was considered a positive test for ammonia production. Ammonium chloride (NH_4Cl) in the range of 1-10 µg/ml was used to prepare a standard curve. The concentration of ammonia was determined by using a standard curve.

Phosphate solubilization: The isolates were spot inoculated on Pikovaskys agar plates and incubated at 30°C for 7 days. The appearance of clear zones around the colony indicated phosphate solubilization. The zones of clearance around the colonies were measured by using a scale.

Siderophore production: The siderophore production ability of the isolates was detected by inoculating the isolates on Chrome Azurol S (CAS) agar[23] and incubating them at 30°C for 7 days. The colonies that showed an orange zone around their growth, were considered to indicate siderophore production.

Antimicrobial potential: All the isolates were screened for *in vitro* antibacterial and antifungal potential against *Pseudomonas aeruginosa* (ATCC27853), *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 443), *Staphylococcus aureus* (NCIB6571) and *Fusarium oxysporum* (ATCC62705), *Fusarium melanochlorum* (ATCC16069), *Alternaria solani* (ATCC38918), *Candida albicans* (ATCC90028)and *Aspergillus flavus* (ATCC9643) on nutrient agar and PDA plates respectively using an agar well diffusion assay. The isolates were subsequently grown in glucose containing nitrogen free broth media at 30°C for 7 days. The antibacterial and antifungal potentional of the resulting cell free supernatant was tested. A total of 0.1 ml of suspension from 24 hrs old cultures of bacteria or fungi was asceptically spread on nutrient agar or potato dextrose agar respectively. Then, 0.1 ml of cell free supernatant (CFS) was added to the wells and the plates were kept in a refrigerator for diffusion for 30 min. The plates were incubated at 30°C for fungi and 37°C for bacteria and the zone of inhibition was observed around the wells.

Identification of potential isolate: Based on the ability of isolate to produce PGPR traits and antimicrobial potential, the isolate AC3 was selected as a promising isolate. It was identified on the basis of their morphological and biochemical characteristics following the Bergey's Manual of Systematic Bacteriology.

Production of bioactive compound: 500 ml of production media was inoculated with 24 hrs of active culture of *A.*

chroococcum which was subsequently incubated at 30°C for 7 days in a shaking incubator. The media were centrifuged at 10,000 rpm for 10-15 min after which the CFS was concentrated by evaporation at 40°C.

Extraction and purification of bioactive compound: The bioactive compound was extracted by using a double volume of ethyl acetate and chloroform. The extract obtained was evaporated, dried and purified by using thin layer chromatography (TLC) and column chromatography.

Column chromatography: The bioactive components were purified by using silica gel column (mesh 150) chromatography. The elution was carried out using graded concentration of n-butanol and chloroform. The active fractions were tested against bacterial and fungal cultures.

TLC: The extracts were dissolved in methanol and spotted on TLC plates which were developed by using n-butanol: acetic acid: water (4:3:2) as the solvent. The separated compounds were detected in an iodine chamber and the R_f values were determined.

Characterization of bioactive compound

FT-IR: The IR spectra of the purified bioactive compounds were recorded with a Perkin-Elemer model 297 IR spectrophotometer. The compound was scanned at a speed of 1µl/min.

Biofertilizer production: The active culture of *A.chroococcum* was inoculated in 50 ml of medium and incubated at 30°C for 3-4 days in a shaking incubator as a starter culture. 5 ml of starter culture was inoculated in 1000 ml Erlenmeyer flask containing 500 ml of medium and incubated at 30°C for 5-8 days. The produced biomass was blended with sterilized carrier (peat or lignite), packed in sterile polythene bags and kept at 25°C for curing. The peat culture was stored at 4°C until use.

Seed treatment: 1% sugar solution was prepared and heated for 15 min. Forty grams of gum arabic were added and the mixture was cooled to room temperature. The inoculum was mixed and a slurry was prepared. The seeds were added to the inoculum slurry and mixed well by hand. The seeds were subsequently dried in the shade.

Pot assay: The effect of biofertilizer i.e. *A. chroococcum* was detected on wheat, cotton and onion. A pot experiment was designed with 2 sets i.e. one with a control and another with seeds treated with biofertilizer. All pots were kept in a natural environment and watered on alternate days. The growth of the plants was observed for a period of 10 days to determine shoot height.

Isolation of Azotobacter: In the present study, six isolates of azotobacter were isolated from three soil samples. The isolates were selected on the basis of their colony characteristics. All the isolates were Gram negative, motile, had cyst formers and produced large mucoid, opaque and brown colonies on Jensen medium. The Azotobacter spp. strains from the rhizosphere of different crops were isolated.

IAA production: The IAA production of the isolates is shown in table 1. IAA was produced by all six isolates. The isolate AC3 isolated from the rhizosphere of wheat produced significantly greater amounts of IAA than the other isolates. The quantitative estimation of IAA was performed to determine the amount of IAA produced by the isolates. The highest amount of IAA was produced by isolate AC3 (64 µg/ml) and the lowest was produced by Azotobacter AC1 (15 µg/ml).

Azotobacter spp. produced 38.82 µg/ml IAA in culture medium supplemented with tryptophan. Mutluru and Konada[20] reported that IAA production by bacteria can vary among different species and strains and is influenced by culture conditions, growth stage and substrate availability.

Ammonia extraction and HCN production: Ammonia was released by all six Azotobacter isolates. There was a significant difference in the amount of ammonia released by the isolates. The ammonia released by Azotobacter sp was in the range of 15-72 µg/ml. The isolate AC3 was able to excrete the most ammonia (72 µg/ml) whereas isolates AC6 and AC1 excrete 15 and 17 µg/ml of ammonia respectively.

HCN production was greatest only for isolates AC2, AC3, AC5 and AC6. HCN production was absent in isolates AC1 and AC4 (Table 2). Day et al[6] reported that IAA production promotes plant growth and that HCN production has been proposed as a defense regulator against phytopathogens. The hydrogrn cyanide is a powerful antifungal compound produced by PGPR and is involved in the biological control of the pathogens[8].

Table 1
IAA production of the isolates

Isolates	Pink colour develop	IAA (µg/ml)
AC1	+	16.13±1.002
AC2	++	37.61±0.537
AC3	+++	64.08±0.144
AC4	++	34.81±1.732
AC5	++	23.93±1.677
AC6	++	30.46±1.286

Phosphate solubilization: The isolates were spot inoculated on tributyrine containing agar plates. All the Azotobacter species were able to solublize phosphate according to the agar spot method, pH drop method and soluble phosphate amount. The isolate AC3 showed the highest 26mm zone of clearance around the spot. The lowest zone was 11 mm long as shown by the isolate AC5 (Table 3). The isolate AC3 produced the highest amount 71 µg/ml of soluble phosphate. The lowest soluble phosphate concentration was 28 µg/ml for isolate AC5. Katiyar and Goel[11] reported that many bacteria have phosphate solubilization ability and are used as plant growth promoters.

Siderophore production: Siderophore production was observed in 5 out of the 6 isolates. The isolate AC3 produced dark orange zone around its growth.

Antimicrobial potential: The antibacterial and antifungal efficacy of the Azotobacter isolates was tested. The isolate AC3 had a greater effect than the other isolates (Table 4). Isolate AC3 showed antimicrobial potency against all test organisms except *S. aureus* and *F. melanochlorum*. The isolate AC5 showed antimicrobial activity against only *F. melanochlorum* and *A. solani*.

Results and Discussion

Many researchers have reported the antifungal activity of Azotobacter sp. isolated from different soil samples. Cavaglieri et al[4] studied the effects of Azotobacter sp and Arthrobacter sp. on the root colonization of *Fusarium verticillioides* and reported the growth inhibition and *in vitro* suppression of fumonisin B1 production by *Azotobacter armaniacus* RC2. *Azotobacter vinelandii* isolated from soil samples[3] also exhibited antifungal activity against *Fusarium oxysporum*. Similarly, Mali and Bodhankar[18] showed the inhibition of *Aspergillus terrus*, *Alternaria alternata*, *Aspergillus flavus* and *Fusarium oxysporum* by *Azotobacter chroococcum* isolated from the soil of the Sangli district. Agarwal and Singh[1] also reported the antifungal activity of Azotobacter sp. against *Fusarium oxysporum* and Aspergillus sp.

Identification of potential isolate: The isolate AC3 showed the highest production of IAA, ammonia extraction, phosphate solubilization, significant siderophore production and promising antagonistic activity. The isolate AC3 also showed significance in terms of HCN production and bacterial and fungal inhibition, hence it was selected as a potential strain and identified as *Azotobacter chroococcum* on the basis of its morphological and biochemical characteristics (Table 5). Microscopic and macroscopic examinations revealed that the isolate AC3 was Gram-negative bacilli that was capable of forming cysts and white, transparent, viscous and moist colonies that turned dark brown after 5-7 days of incubation on Jenson agar media (Fig. 1). The results of biochemical tests revealing the identification of the AC3 isolate are shown in table 5.

Production, extraction, purification and characterization of bioactive compounds produced by *A. chroococcum*: The bioactive compound was produced from *A. chroococcum* by using Jenson medium. The bioactive compounds produced were extracted via the solvent extraction method. The solvents were separated and collected in a beaker. The beaker was kept in a hot air oven at 40-45 ^{o}C for 24 hrs. The extracted bioactive compound was purified by using a silica gel column. The 5 fractions were collected and each fraction was checked for bioactivity against the test pathogen.

Table 2
Ammonia and HCN production.

Isolate	Ammonia production (µg/ml)	HCN production
AC1	17.13±0.275	-
AC2	25.01±0.225	+
AC3	72.18±0.236	++
AC4	38.61±0.275	-
AC5	41.35±0.409	+
AC6	14.91±0.381	+

Table 3
Phosphate solubilization.

Isolate	Zone of clearance (mm)	Soluble phosphate (µg/ml)
AC1	14.66±0.577	37.41±0.381
AC2	16.66±0.577	48.25±0.250
AC3	26.00±0.000	71.08±0.144
AC4	18.66±0.577	63.6±0.529
AC5	11.66±0.577	28.35±0.312
AC6	15.66±0.577	44.36±0.510

Table 4
Antimicrobial activity.

Isolates / Test organism	Antimicrobial activity					
	AC1	**AC2**	**AC3**	**AC4**	**AC5**	**AC6**
E. coli	+	-	+	+	-	+
S. aureus	-	+	-	-	-	+
P. aeruginosa	-	-	+	+	-	-
K. pneumoniae	+	-	+	-	-	-
B. subtilis	-	-	+	-	-	+
F. oxysporum	+	+	+	-	-	-
F. melanochlorum	-	+	-	-	+	+
A. solani	-	-	+	+	+	-
A. flavus	-	+	+	-	-	+
C. albicans	+	-	+	-	-	-

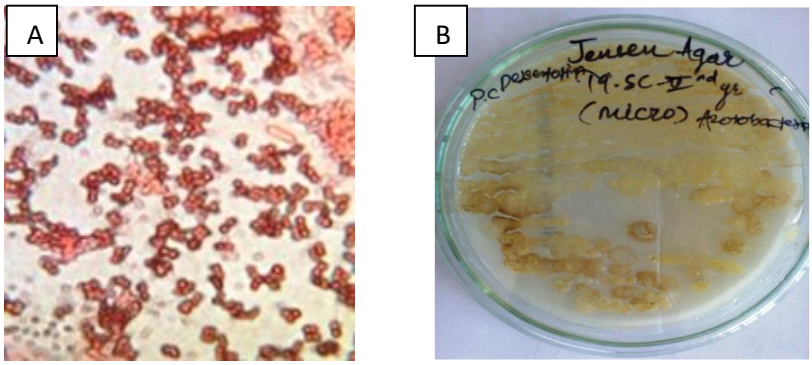

Fig. 1: Microscopic (light microscope 100x) and macroscopic examination of the Gram negative bacilli. A. Gram-negative bacilli, B. colonies of *A. chroococcum* on Jenson agar medium.

Table 5
Biochemical tests of isolate AC3.

S.N.	Biochemical test	Isolate AC3
1	Catalase	+
2	Oxidase	+
3	Nitrate reduction	+
4	Sucrose	+
5	Melibiose	+
6	Xylose	+
7	Glucose	+
8	Manitol	+
9	Rhamnose	-
10	Innosital	-
11	Cyst formation	+
12	Movement	+

Fraction number 1 showed potentional activity against bacterial as well as fungal phytopathogens compared to the other fractions (Table 6 and fig. 2) for the bacterial culture of streptomycin and for the fungal species, amphotericin-B was used a as standard. TLC of purified fraction number 1 showed that a single spot had an Rf of 0.81 value (Fig.3).

The purified fraction number 1 was characterized by using FT-IR spectroscopy for the determination of functional groups present in the compound. The FT-IR spectrum is shown in fig. 4. The FT-IR spectra confirmed the presence of 2852-2922 C-H stretch (alkane), 2544 OH (acid group), 1680-1730 C=O (carbonyl group) and 894-1028 COOH (carboxylic acid group) functional groups in the bioactive compound.

Similarly, Bhosale et al[3] produced bioactive compounds from *Azotobacter vinlandii* and analyzed them by TLC, column chromatography and FT-IR. A single spot was observed with an Rf value of 0.53. The spectra of the compounds purified by using an FT-IR spectrometer showed major peaks at 2700.34, 2331.94, 1734.01, 1541.12, 1288.45

and 1039.63, indicating the presence of aldehydes, C-N esters, aromatic rings, P-H stretches and C-N stretches of alkyl amines as prime functional groups in the bioactive compound.

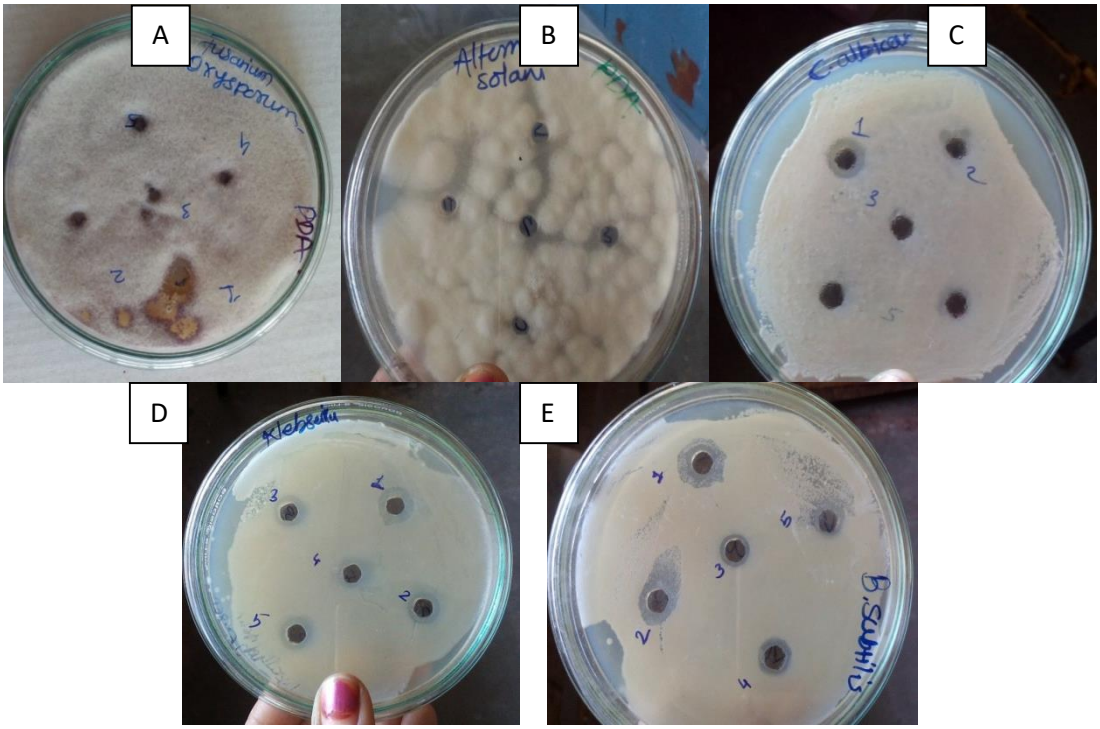

Fig. 2: Antimicrobial potential of the fractions. A) *Fusarium oxysporum* B) *Alternaria solani* C) *Candida albicans* D) *Klebsiella pneumoniae* and E) *Bacillus subtilis*

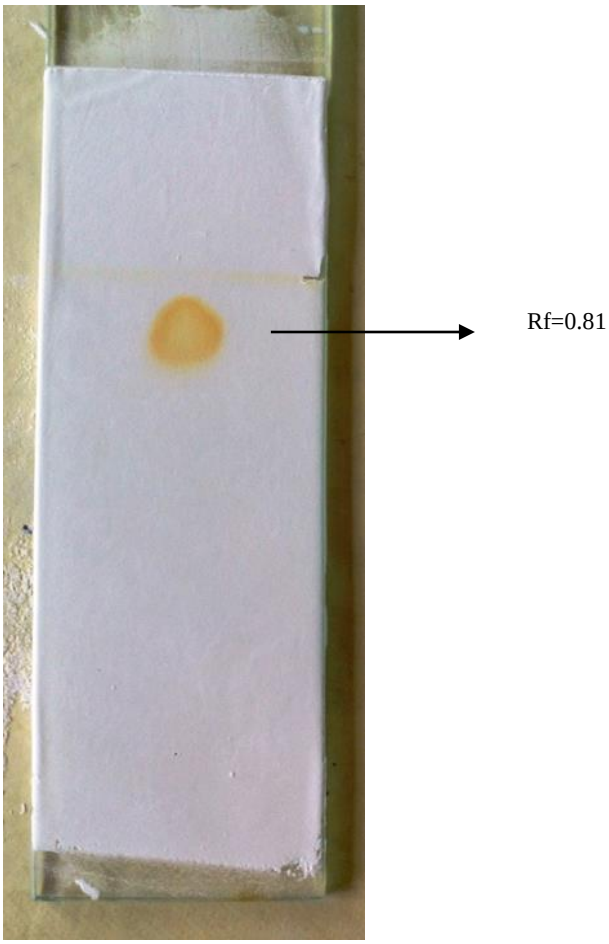

Fig. 3: TLC image of purified fraction number 1.

Seed treatment and pot assay: The produced biofertilizer was used for the treatment of onion, wheat and cotton seeds. After treatment, the seeds were ready for sowing. The resulting biofertilizer culture was activated by using a sugar solution before the plants were treated. After sowing, the plants were watered regularly at intervals of one day. After 20 days of incubation, all the experimental plants i.e. onion, wheat and cotton plants, grew well compared to the control plants. The biofertilizer produced from *A. chroococcum* enhanced the growth of non leguminous plants.

Sachin et al[22] reported that inoculation with *Azotobacter chrococcum* had a positive effect on the growth parameters of bamboo and maize in pot experiments. In addition, Kanchana et al[10] reported that the dry weight of chilli significantly increased in Azotobacter treated plants.

Table 6
Zone of inhibition of fractions against fungal and bacterial pathogens.

Fractions / Test organism	Zone of inhibition (mm)				
	1	**2**	**3**	**4**	**5**
Fusarium oxysporum	14.66±0.57	09.66±0.57	-	-	-
Aspergillus flavus	12.33±1.15	10.00±0.00	10.00±0.57	-	-
Alternaria solani	11.00±0.00	-	11.66±0.57	-	-
Candida albicans	11.66±0.57	09.00±1.00	-	-	-
Fusarium ….	10.00±0.00	11.00±0.00	09.33±0.57	-	-
Pseudomonas aeruginosa	11.66±0.57	09.66±0.57	-	-	-
Klebsiella pneumoniae	12.00±0.00	09.00±1.00	10.00±0.00	-	11±0.00
Bacillus subtilis	14.00±0.00	13.00±0.00	10.66±0.57	10±0.00	09±0.00
E.coli	12.00±1.00	08.33±0.57	-	-	-

Amphotericin-B standard for fungal pathogen and Streptomycin standard for bacterial pathogen.

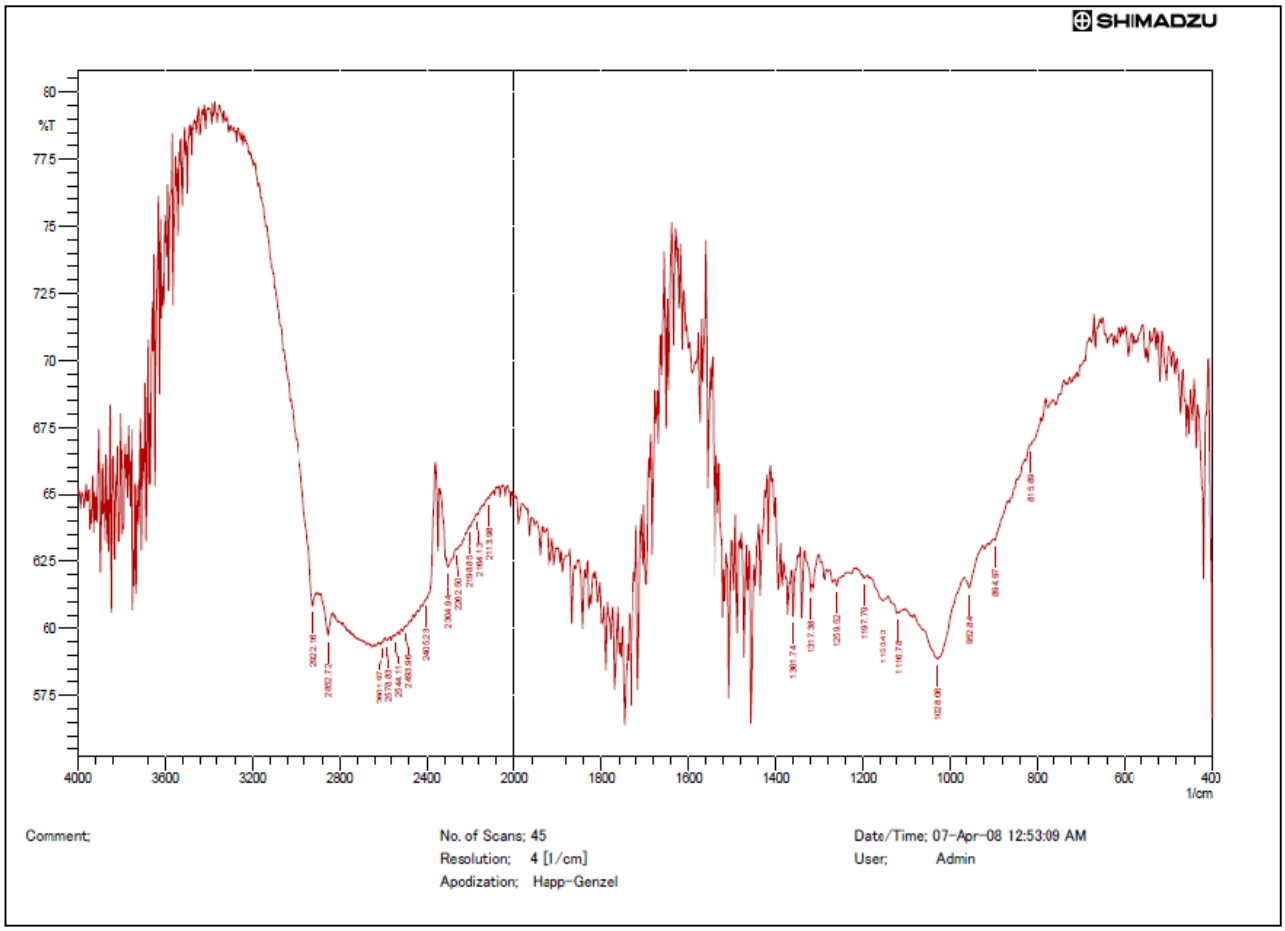

Fig. 4: FT-IR spectroscopy of purified bioactive compounds.

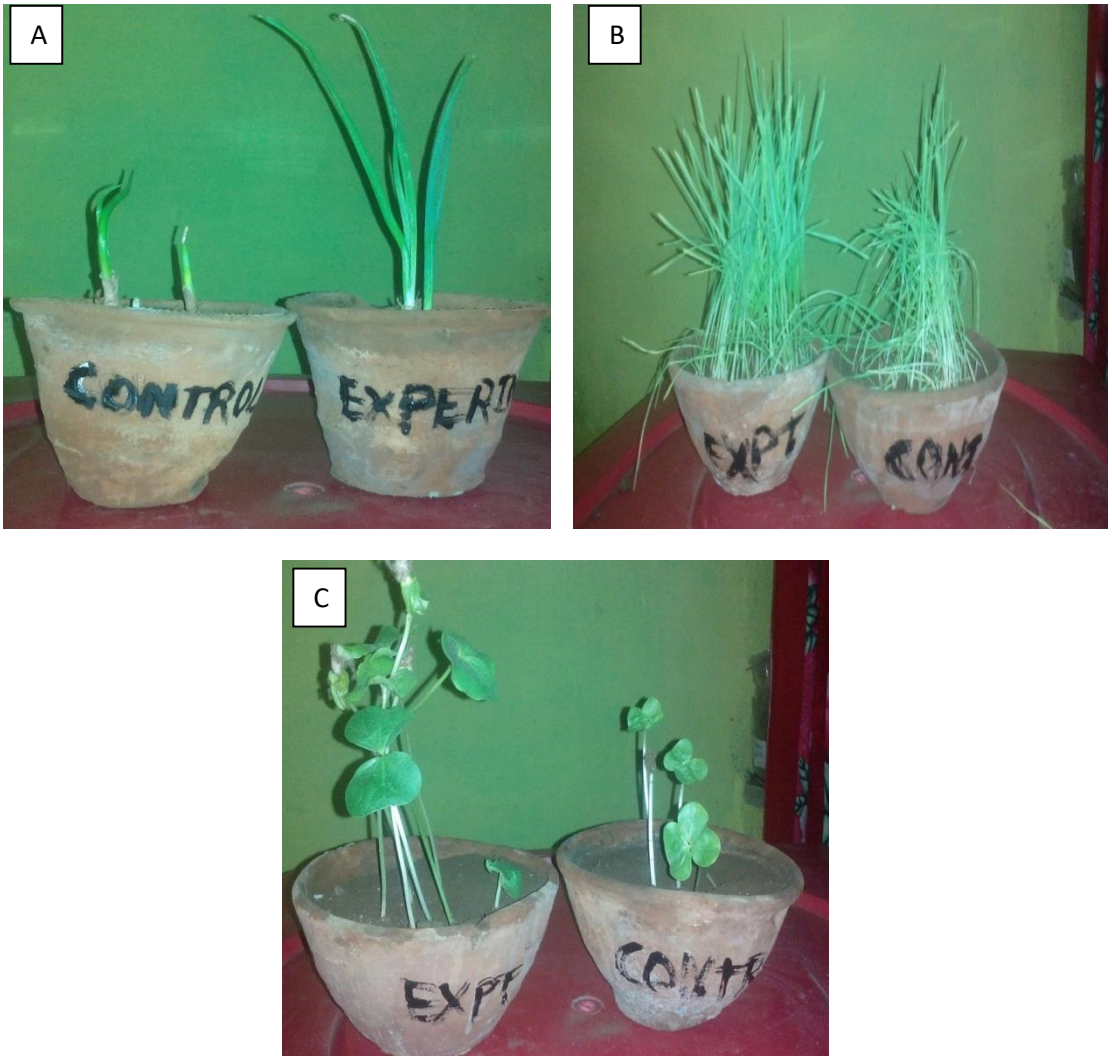

Fig. 5: Growth of plants in the experimental and control groups after 10 days of incubation. A) Onion, B) Wheat, C) Cotton.

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