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PLANT GROWTH PROMOTING ACTIVITY OF AZOSPIRILLUM ISOLATED FROM PADDY FIELD OF KANKOL, KANNUR DISTRICT

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ABSTRACT

Azospirillum sp are the gram negative rhizobacteria, which are found in association with plant roots, especially with Maize, Rice and other plants.The aim of the study was to isolate *Azospirillum* sp. from Rice plant root (*Oryza sativa* var IR 20) collected from paddy field of KANKOL. The phenotypic characterization of the isolates based on the Bergey's manual of Determinative Bacteriology, carrying out microscopy (morphology) Gram's staining. The isolates were cultured on N-Free malate medium developed by Dobereiner. The growth and metabolisms of bacteria were indicated by the color change in the medium from green to blue due to the production of metabolic end products. In this study the isolates were assessed for their Plant Growth Promoting Activity (PGPR) activity by demonstration of IAA production and GA production, siderophore production, Nitrate reductase activity study. The PGPR activity indicate the isolates of *Azospirillum* sp also be used as a biofertilizer and the Nitrate reduction activity improves the quality of grains along with growth promoting activities of microbes improves growth rate of plant.

KEYWORD: *Azospirillum* sp, *Oryza sativa* var IR 20, PGPR activity, IAA and GA.

INTRODUCTION

Nitrogen is an essential macro nutrient, and is the most commonly-deficient nutrient, contributing to reduced agricultural yields throughout the world (Saikia and Jain, 2007).

About 83 million tons of N fertilizers are produced each year by the Haber-Bosch process (Jenkinson, 2001). With increasing the costs of chemical fertilizers, concern about environmental pollution, and increasing demand for organically-grown agricultural and horticultural products has given much more interest in promoting biological nitrogen fixation (BNF) as a biofertilizer to meet crop nitrogen requirements (Vessey, 2003). Agricultural crops have been constant interactions particularly with different microorganisms in the rhizosphere (Singh *et al.*, 2004). The nitrogen fixation process were performed by rhizosphere microbes. A portion of these rhizospheric bacteria are capable of exerting growth stimulatory effects on plants and are referred to as plant growth promoting rhizobacteria PGPR. The mechanisms by which PGPR stimulate plant growth can broadly be categorized as direct or indirect mechanisms (Glick, 1995). Mechanisms include enhanced plant growth through production of plant hormones such as indole acetic acid (IAA), gibberelic acid (GA) and cytokinins. Based on the capability of the PGPR to make more nutrients available to plants, stimulate the plant growth by different determinants, especially phytohormones, degradation of organic pollutants and to produce antimicrobial compounds (Somers *et al.*, 2004). *Azospirillum* is a Gram-negative, associative symbiotic, plant-growth- promoting bacterium (PGPB), it has repeatedly been isolated from the rhizosphere of many grasses, (Steenhoudt and Vanderleyden, 2000). They play an major role in growth promotion capacity producing various phytohormones that improve growth, absorption of water and minerals that eventually yield more productive plants (Dobbelaere *et al.*, 2001). The current study also focused on the use of *Azospirillum* as a PGPR bacterium, which can be directly added to soil to improve soil fertility.

MATERIALS AND METHODS

SAMPLE COLLECTION

Root samples of Rice plant (Oryza sativa var.IR 20) were collcted from the paddy fields of KANKOL, a panchayath in kannur district.

The root samples were collected from the marsh about 15 cm depth along with rhizosphere soil. The collected samples were carried to the laboratory in a safe manner without harming the roots, and are surface sterilized and kept in refrigeration for further use.

Isolation and characterization of *Azospirillum* from *Oryza sativa var.IR20*

Screening of N-fixing organisms from the rhizosphere of different crops was carried out by the enrichment culture technique using NFb semi-solid medium developed for Azospirillum

by Döbereiner and Day (1976)and further characterized by gram staining, glucose assimilation, biotin requirement (Baldani and Dobereiner 1980; Tarrand et al. 1978), PHB staining, hanging drop technique etc.

PROCEDURE

- Prepare semisolid malice acid medium in test tubes in 5 ml; quantity and sterilize them at 121°c for 5 minutes
- Collect fresh root samples from Rice plant(***Oryza sativa var.IR20)***
- Wash the roots in tap water, remove the adhering soil particles.
- Using a sterilized knife/blade, cut the roots in to small bits of 1-2 cm size.
- Surface sterilize the roots by immersing in either 80% Ethanol or 0.1% Mercuric chloride for 1 minutes.
- Wash the root bits with sterile distilled water for 3-4 times to remove excess ethanol or Mercuric chloride
- Using a sterile forceps, transfer aseptically 2-3 root bits to the test tubes containing N-Free semisolid Malic acid medium
- Incubate the tubes under room temperature 28±2°c for 2-3days
- Maintain one tube as a control without root bits.

NITRATE REDUCTASE ACTIVITY

Nutrient broth supplemented with 1% sodium nitrate were inoculated with the *Azospirillum* sp isolates and incubated for 4 days at room temperature. One ml of α-naphthalamine reagent were added to the culture tubes, the change in color was observed. The appearance of pink color indicated the presence of nitrate reductase activity. To the tubes showed no color change, a small amount of zinc powder was added, shaken and allowed to stand for 10 min. Pink color development shows the absence of nitrate reductase activity.

EVALUATION OF PGPR ACTIVITY ASSAY OF IAA PRODUCTION

One ml of cultures at exponential phase was inoculated in 100 ml medium containing 0.5Mm filter sterilized L-Tryptophan (0.01%w/v). All the flasks were wrapped with black paper to avoid photo inactivation of the biologically active compounds. The flasks were incubated at room temperature for 7 days. The cells were harvested by centrifugation at 100000 rpm for 5 min and supernatant were collected and concentrated to 25 ml.

EXTRACTION OF IAA and ESTIMATION

The culture flasks was adjusted the pH 2.8 with 1N HCL and equal volumeof ice cold (4°c) diethyl ether was added. The contents were shaken well and allowed to stand in dark for 4 h with intermittent shaking. Using a separating funnel, the aqueous phase was separated from organic phase and the extraction was repeated 3 times. Discarding the aqueous phase, the organic phase were pooled and evaporated to dryness in the dark. The residue was dissolved in 2 ml of absolute methanol. The estimation can be done by the methods of Gordon and Paleg, 1957.

GIBBERELIC ACID PRODUCTION

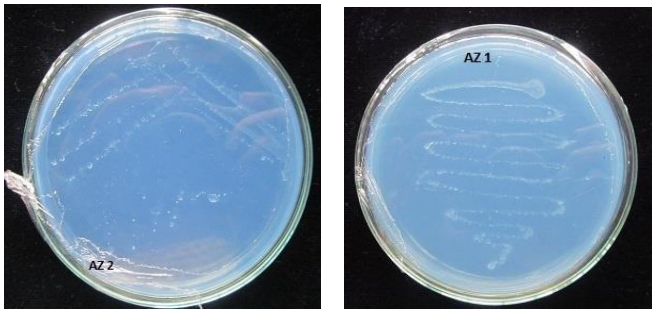
Extraction of gibberellic acid from the culture at exponential stage by following the standard Procedures by Borrow et al, 1995. Estimation is done by following procedures. Two ml of zinc acetate solution (21.9 g zinc acetate was dissolved in 80 ml distilled water containing 1 ml glacial acetic acid and made up to 100 ml) was added to the dissolved residue. After 2 min, 2 ml of potassium ferrocyanide solution (10.6 g potassium ferrocyanide dissolved in 100 ml distilled water) was added and the mixture was centrifuged at 10,000rpm for 10 min. Five mi of supernatant was added to 5 ml of 30%hydrochloric acid and the mixture was incubated at 20*c for 75 min. The blank was prepared with 5% HCL The absorbance was measured at 254nm in VU-VIS spectrophotometer (ELICO Ltd. Taiwan).

SIDEROPHORE PRODUCTION BY *AZOSPIRILLUM* ISOLATES

Qualitative assay followed by Schwyn and Neilands, 1987 and the quantitation is done by the methods by Reveee et al., 1983. The quantity of siderophore synthesized were expressed in µg ml^{-} of culture filtrate.

RESULT AND DISCUSSION

N-free malate medium containing inoculates grown as white colored pellicles, formed about the middle of the medium and the isolation were carried out in solid N-Free medium, in which small regular circular mucoid colonies, and the media color changes from green to blue due to the result of growth and metabolism (Fig 1). The colonies formed were Microscopically observed as Gram negative long spiral shaped rods exhibiting motility on wet mount preparations by following the reports of Holt *et al*. in 1994. By applying stressful conditions, the bacteria were capable of producing PHB granules(Poly β hydroxyl granules, by Del Gallo & Haegi, 1990).



NITRATE REDUCTASE ACTIVITY

In these experiment the nitrate reductase activity were tested and high intense pink color were produced in samples AZ 1, AZ 2, and AZ 4, while adding α-naphthalamine reagent. The AZ 3 shows pink color only after the addition of Zinc powder that indicated the absence of nitrate reducatse activity. Nitrate reductases are molybdoenzymes that reduce nitrate ($NO^{-}3$) to nitrite ($NO^{-}2$). This reaction is critical for the production of protein in most crop plants, as nitrate is the predominant source of nitrogen in fertilized soils. The test samples AZ 1, AZ 2, and AZ 4 shows nitrate reductase activity so as the isolates can be used as a biochemical tool for predicting grain yield and grain protein production in crops.

ASSAY OF IAA PRODUCTION and ESTIMATION

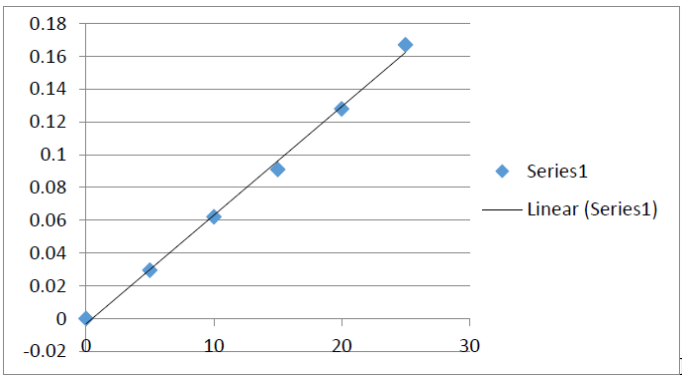
The indole acetic acid production by utilizing Tryptophan in the medium, and it shows the phytohormone production ability of each strain as per the concentration or amount estimated. In these test all the strains shows IAA activity but their concentration varies depends on the strains.

The test samples AZ 1, AZ 2, AZ 3 and AZ 4 shows pink color formation similar to the works explained in 1995 by El-Khawas El-Khawas, H.M. after an incubation period of 1 hrs from addition of Salper's reagent under dark. The experimental result indicated that the sample AZ 4 shows most activity and AZ 3 have slight different but almost similar activity like AZ 4

,others shows lesser activity than AZ 3 and AZ 4. The IAA production amount variation may be due to strain difference and also due to some variations during repeated sub culturing of the strains in vitro.

The amount of IAA production was estimated and the values were analysed statistically by ploting the graph. The concentration of IAA produced was calculated by plotting the absorbance on the standard graph, it shows the corresponding concentrations.

From the graph obtained, by plotting the standard graph, the amount of produced indole acetic acid were detected and that indicate phytohormone production ability of each isolated strains that directly affect the associated plant growth.



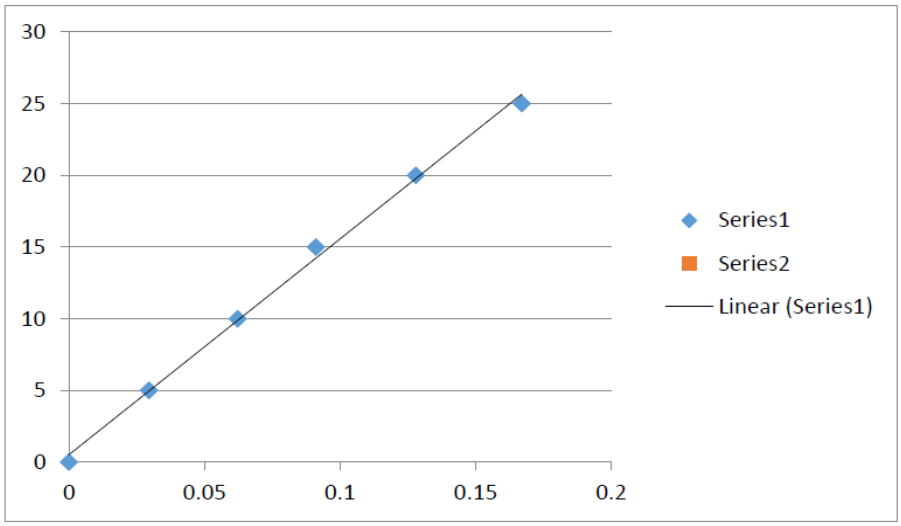
IAA production graph

GIBBERELIC ACID PRODUCTION

The PGPR activity of *Azospirillum sp*. Is due to the production of phytohormones especially Auxins and Gibberelins. The amount of gibberellic acid produced were estimated by using Salper's reagent method and the resulting values were plotted on the standard graph and the concentrations of hormone were calculated. The resultant graph in which all the strains shows almost similar activity as compare to IAA production. The test strain shows almost similar hormone production level rather than IAA production experiment, so as the test results are plotted in a straight line graph.

The strain AZ-1 shows high hormone production ability and the sample AZ 3 shows least activity. The standard strains have a range of activity which lies in between 2-2.9 at 254nm, and all the test samples lies in between the range, so as we can predict that all the strains tested are *Azospirillum*, but they shows some variations either in strain level or may be due to repeated culture in-vitro resulted in mutations. The plant growth promoting ability of bacteria

improves the fertility of rhizosphere soil and that directly improves the use of strains industrially for the biofertilizer production.



Gibberelic acid production

SIDEROPHORE PRODUCTION BY *AZOSPIRILLUM* ISOLATES QUALITATIVE ASSAY

Siderophores are the iron chelating compounds resulting in advanced intake of soil minerals by associated plants of the particular rhizospheric regions from where the samples are collected. Here the production of iron chelating compounds was qualitatively estimated by using Chrom azurol S as an indicator dye solution, as per increasing the rate of mineralization the indicator dye forms complex with the mineralized iron that shows the fluorescent yellow color formation as explained and demonstrated by Schwyn and Newlands in 1987. All the strains tested were shows siderophore production ability, in which AZ 1 shows high rate of production than AZ 2 and AZ 3, comparatively AZ 2 shows slow rate of chelating ability. In these the test sample AZ- 1 and the AZ 3 shows almost same area of fluorescence zone. All the samples were qualitatively estimated that the samples are able to produce chelating compounds and are help full to promote plant growth as per the result of chelation, the soil fertility of rhizosphere soil were enhanced in an extent that ultimately results in promote growth rate of the rice plant in the field. The properties of Iron mineralization also provide an advancement to provide phytopathogenic resistance against some fungal diseases.

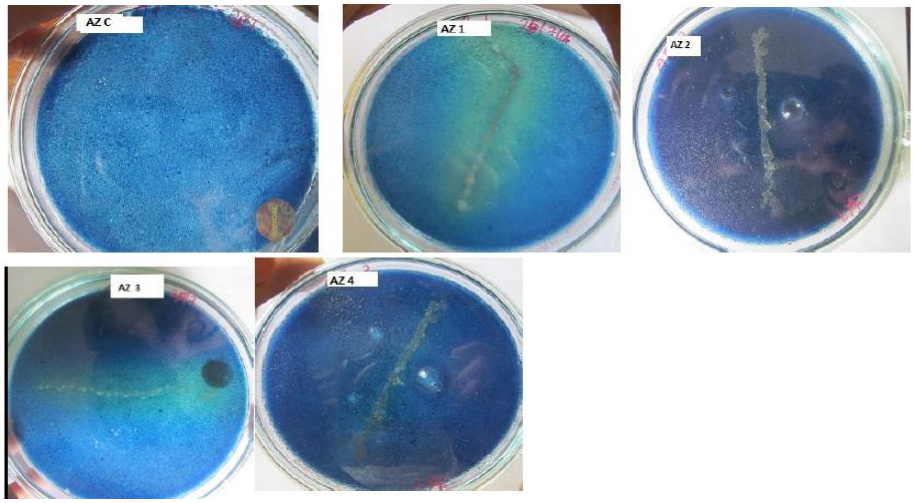
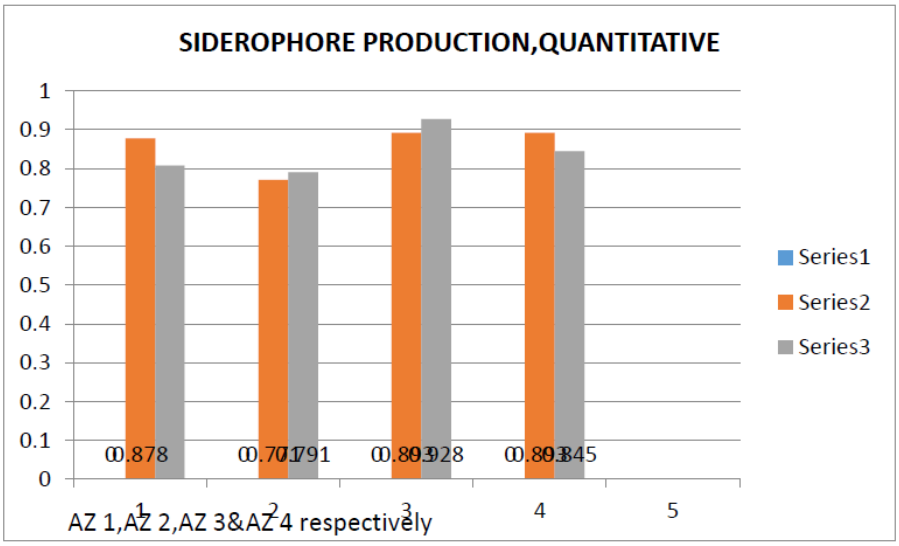


Fig 1.

QUANTITATIVE ASSAY

The production of iron chelating compounds was detected by the qualitative assay, and the quantitative assay was carried out by using DHBA as a standard. The amount produced was estimated and are plotted on graph by following statistical methods. In the quantitative assay also AZ 1 shows similarity with AZ 4 values and the AZ 3 also gives almost similar result. But the quantity of siderophore produced by AZ 2 is more variable than all other samples. The quantitative assay provides the quantity of iron mineralized. The property of siderophore production improves the use of *Azospirillum* strains to improve soil fertility and use of the bacteria for iron mineralization.



CONCLUSION

Azospirillum sp were isolated from the paddy field shows high rate of PGPR activity and reached the conclusion that all the isolated strains AZ 1,AZ 2,AZ 3,AZ 4; Shows high rate of metabolic activity so as we can use them as biofertilizers on different fields, that may improve the crop yield. The isolates were assessed for various other attributes like nitrate reductase activity, which shows the evidence for nitrogen fixation by the nif H genes, the siderophore production were detected and estimated, which shows high rate of chelation within small period of time. The current study provide idea to farmers of Kankol, Kannur district about their soil nature, the total productivity of grains and the use of *Azospirillum* as a growth promoting agent who shows high rate of PGPR activity and also siderophore production,

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