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PRODUCTION, PURIFICATION AND APPLICATION OF PLANT GROWTH HORMONES (IAA AND GIBBERELLIC ACID) BY *BACILLUS SUBTILIS*

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Abstract

To isolate bacterial strains from chickpea rhizosperic soil and nodules, to characterize and identify bacterial strains by using 16s rRNA gene sequencing. Plant growth promoting rhizobacteria are being preferred nowadays as inoculants for influencing crop via multiple direct or indirect mechanisms but screening to find out the effective PGPR strain is one of the crucial steps. This research is aimed at keeping in view their potential for phosphate solubilisation, indole acetic and ammonia production.

Keywords: IAA, GA3, PGPR**,** FTIR**,** indole acetic acid, thin layer chromatography**.**

I. INTRODUCTION

Plant growth promoting *bacillus licheniformis* (PGPB) are beneficial bacteria which have the ability to colonize the roots and either promote plant growth through direct action or via biological control of plant diseases (Kloepper and Schroth 1978). They are associated with many plant species and are commonly present in varied environments. Strains with PGPB activity, belonging to genera *Bacillus licheniformis*, have been reported (Hurek and Reinhold-Hurek 2003). The species of *Bacillus* are the most extensively studied. These bacteria competitively colonize the roots of plant and can act as biofertilizers and/or antagonists (biopesticides) or simulta-neously both.

Gibberellic acid (GA3) is an important member of the gibberellins family and acts as a natural plant growth hormone, controlling many development processes, which is gaining great attention all over the world due to its effective use in agriculture, nurseries, tissue culture, tea gardens, etc. (Davies, 2004; Shukla *et al*., 2005). Reduction in its production costs could lead to wider applications for a variety of crops (Kumar and Lonsane, 1989; Tudzynski, 1999; Linnemannstons *et al.*, 2002). Industrially GA3 is produced by submerged fermentation (SmF) using the ascomycetous fungus *Gibberella fujikuroi*, renamed *Fusarium fujikuroi* (O'Donnell *et al*., 1998). The cost of GA3 production using SmF is very high, mainly due to extremely low yield and expensive downstream processing. As plant roots grow through soil they release water-soluble compounds such as amino acids, sugars and organic acids that supply food for the microorganisms. In return, the microorganisms provide nutrients for the plants. All this activity makes the rhizosphere the most dynamic environment in soil.

Rhizosphere microorganisms produce vitamins, antibiotics, plant hormones solubilization of mineral nutrients, synthesis of vitamins, amino acids, auxins, cytokinins and gibberellins which stimulate plant growth and antagonism with potential plant pathogens and communication molecules that all encourage plant growth. The action and interaction of some growth regulators like auxins regulate most of the physiological activities and growth in plants. Chemically it is Indole acetic acid the ability to synthesize phytohormone is widely distributed among plant associated bacteria. 80% of the bacteria isolated from plant rhizosphere are to produce IAA. (Zakharova Zakharova *et al*., 1999)

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According to Halda-Alija (Halda-Alija 2003), up to 74% of rhizobacteria identified and tested to produce IAA.The associative nitrogen-fixing bacteria tested produced IAA, especially with tryptophan as a precursor (Pedraza *et al*., 2004).

The rapid growing population has eventually raised the demand of food production. The researchers emphasized the need to optimized for alternative yield raising techniques without affecting the soil health and environmental quality (Kantar, Hafeez *et al*-2007). Bacteria, with a vital role for plant growth known as plant growth promoting rhizobacteria (PGPR) make possible the improved plant growth by colonizing the plant roots. Seeds coinoculated with rhizobia, PGPR and PSB (phosphorus solubilization bacteria) have been found out to enhance crop growth and productivity (Dashti , Zhang *et al*., 1998 and Rudresh DL, Shivaprakash MK *et al*.,2005).

Siderophores produced by some PGPR scavenge heavy metal micronutrients in the rhizosphere (e.g. iron) starving pathogenic organisms of proper nutrition to mount an attack of the crop. Antibiotic producing PGPR releases compounds that prevent the growth of the pathogens. Bioprotectants are currently being studied by the laboratories of Fernando and Daayf in the Department of Plant Science, University of Manitoba. Biofertilizers-nitrogen fixing bacteria are also available for increasing crop nutrient uptake of nitrogen from nitrogen fixing bacteria associated with roots (*Azospirillum*). Nitrogen fixing biofertilizers provide only a modest increase in crop nitrogen uptake (at best an increase of 20 Kg N acre-1).

Gibberellic acid is synthesized by *Gibberella fujikuroi, Sphaceloma manihoticola, Neurospora crassa, Aspergillus niger, Sphaceloma* sp., *Rhizobium phaseoli, Azospirillum brasilense, Pseudomonas* sp. and *Phaeosphaeria* sp., whereas IAA is synthesized by *Pseudomonas* sp., *Bacillus* sp., *Aspergillus niger, Rhizopus, Acetobacter diazotrophicus, Herbaspirillum seropedicae, Rhizobium* sp., *Azospirillum* sp., and *Erwinia* sp.

Physiological evidence for different Trp-dependent pathways for synthesis in *Azospirillum brasilense* has been reported (Carreno-Lopez *et al*., 2000). In Trp dependant pathway, tryptophan is converted to indole-3-acetamide (IAM) by tryptophan-2-monooxigenase and IAM is metabolized to IAA by IAM-hydrolase (Matsukawa *et al*., 2007).

The physiologically most active auxin in plants is Indole acetic acid (IAA) which is known to stimulate rapid cell elongation

and cell divison and differentiation in plant (Hayat *et al*. 2010). Inoculation with IAA producing PGPR has been used to stimulate seed germination, accelerate root growth and modify the architecture of the root system and increase the root biomass (Martinez-viveros *et al*. 2010).

The present study focused on production, purification of plant growth hormones (IAA and Gibberellic acid) were produced in chemically defined medium using *Bacillus Licheniformis.* The optimum day, pH, purified using acetone (95%) followed by dialysis and column chromatography temperature and carbon sources were also studied. The compound was further partially. The hormones were identified using UV-visible spectrophotometer, paper, TLC when compared with standard. The compound father identified using HPLC followed by effect of hormones as plant. The effect of hormones on plant have also been studied, viz, CHO, protein, chlorophylls with negative control.

Bacillus pumilus and *Bacillus licheniformis*, isolated from the rhizosphere of *Alnus glutinosa* L. Gaertn., both have strong growth-promoting activity. Gutiérrez-Mañero *et al*. (2001) showed that the dwarf phenotype induced in *A. glutinosa* seedlings by Paclobutrazol (an inhibitor of gibberellin biosynthesis) was effectively reversed by applications of extracts from medium incubated with both bacteria and also by exogenous GA3. GC-MS analysis of extracts of these media showed the presence of GA1, GA3, GA4 and GA20. Probanza *et al*. (2002) also reported that inoculation with *Bacillus licheniformis* and *B. pumilus* enhanced growth of Pinus pinea plants, presumably by bacterial gibberellin production.

Cytokines are small polypeptides, proteins or glycoproteins involved in the regulation of processes as diverse as proliferation, differentiation and mobility of cells. Cytokines are important components of the immune system.

2. MATERIALS AND METHODS

2.1. Sources of Inoculums

2.1.1. Production of Indole Acetic Acid (IAA)

For the capacity of the bacterial isolates to synthesize indole acetic acid (IAA), bacterial cultures were grown for 48 h in tryptic soy broth (TSB) at 28±2^{0}C. A bacterial suspension (100 µl each) of fully grown bacterial culture was inoculated in 5 ml Luria Broth (LB) medium in absence and presence of 500 µg/ml of tryptophan and, placed for 48 h in an incubating shaker at 28±2^{0}C. Centrifugation of bacterial culture was done at 3000 rpm for 15 minutes and the supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent (50ml, 35% of perchloric acid, 1ml 0.5M FeCl3 solution). The development of a pink color indicated IAA production, and the optical density (O.D) 530 nm was read using a spectrophotometer.

2.1.2. Preparation of standard graph of IAA

Standard graph of IAA was prepared as mentioned by Different IAA concentrationsare prepared as aqueous solution of IAA ranging from 10 microgram/ml to 100 micrograms/ml. To each 1 ml of the standard, 2ml of 2% 0.5 M FeCl3 in 35% perchloric acid i.e. Salkowaski reagent is added and readings are taken after 25 minutes at 530 nm by UV-Visible spectrophotometer SL Elico 159. Standard graph is prepared by plotting concentration of IAA in micrograms/ml Vs Optical Density at 530 nm.

2.2. Confirmation of IAA by using TLC

Selected 4 rhizobial cultures were inoculated in YEMB amended with 5mM tryptophan. 1% inoculum of O.D. 600 1.0 was used for inoculation. The inoculated broth was incubated at 28^{0}C for 24 hrs. After 24 hrs of incubation, broth was centrifuged at 7000 rpm for 10 minutes. pH of broth brought to 3.0. 4:1 aliquots of liquid portion of centrifuged sample were extracted three times with ethyl acetate. The organic phase was concentrated to dryness and then diluted with 0.5 ml methanol. This solution along with the standard IAA was applied on silica gel G plate and TLC was run by using a solvent system chloroform:Ethyl acetate:Formic acid in 5:3:2 proportion and developed by using Salkowaski reagent. Red colour spots were developed. Rf value of the standard and IAA produced by the selected isolates was calculated.

2.3. Effect of tryptophan concentration

To check the effect of tryptophan on IAA production, YEMB amended with 1- 5mg/ml, as well as 5mM were inoculated with the selected isolates as 1% inoculum of O.D.600 1.0 and incubated at 28^{0}C for 24 hrs. After incubation the broth was centrifuged at 7000 rpm for 10 minutes. Supernatant was collected. To 1ml supernatant, 2ml of Salkowaski reagent was added and extent of red colour i.e. IAA produced was measured spectrophotometrically at 530 nm.

2.4. Effect of Carbon sources on IAA production

IAA production was studied by replacing mannitol from YEMB by glucose, sucrose, lactose, arabinose, xylose and mannitol 1% w/v. supplemented with 2.5 mg/ml of tryptophan. IAA production was studied by using Salkowaski reagent after 24, 48 and 72 hrs. Cultures selected for the study are RG and RH i.e. *Bacillus licheniformis* isolated from groundnut and chickpea respectively.

2.5. Effect of pH on IAA production

To study the extent of IAA produced by the different isolates at different pH, YEMB with 2.5 mg/ml of tryptophan is adjusted to different pH as 5, 6, 7, 8 and 9. Media were inoculated with 1% inoculum of O.D. 600 and incubated at 28^{0}C for 24 hrs. IAA production was studied by using Salkowaski reagent after 24hrs.

2.6. Characterization of IAA production

To determine the amounts of IAA produced by each isolate, a colorimetric technique was performed with Van Urk Salkowski reagent using the Salkowski's method (Ehmann, 1977). The isolates were grown in yeast malt dextrose broth (YMD broth) (Himedia, India) and incubated at 28°C for 4 days. The broth was centrifuged after incubation. Supernatant was reserved and 1ml was mixed with 2ml of Salkowski's reagent (2% 0.5 FeCl in 35% HCLO solution) and kept in the dark. The optical density (OD) was recorded at 530 nm after 30 min and 120 min.

IAA production was compared in YMD and LB media. YMD medium was compared with and without tryptophan.

2.7. Extraction and purification of IAA

Isolates were cultivated in YMD broth and it was centrifuged by Beckman centrifuge SW 40 Ti rotor with 17738 × g for 15 min. The supernatant was collected and mixed with ethyl acetate (1: 2). After vigorous shaking it was allowed to stand for 10 min. IAA was extracted within solvent layer. The procedure was repeated 3 to 4 times.

2.8. Thin layer chromatography

TLC slide was prepared with silica gel G and calcium carbonate. Propanol: Water (8:2) was used as solvent system. The extracted sample and standard IAA (10mg/100ml) were spotted on TLC plate. Chromatogram was developed with the Salkowski's reagent (Kuang-Ren *et al*., 2003).

2.9. FTIR

Infrared spectrum of the purified compound was obtained using Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu FTIR 8400) and the spectra was recorded in the nujol mull using KBr cells and expressed in wave number (cm-1).

2.10. Method of seed treatments:

Bio control agents were grown in respective medium on rotating shaker (150rpm) for 2 days and centrifuged at 10,000 rpm for 5min the pellet was mixed with sterile carboxyl methyl cellulose (CMC) suspension. *Oryza sativa* surface sterilized with sodium hypochlorite solution were placed in CMC cell suspension and air dried inside laminar air flow chamber (jagadeesh, 2000).The bio coated seeds were transplanted into the field .

2.11. Shoot length and root length (cm)

Three plants were randomly selected for recording the root length and shoot length of *oryza sativa* plants. They were measured by using centimeter scale.

2.12. Fresh and dry weight (mg/g dry wt.)

Three plant samples were randomly selected at experimental pot. They were separated into root and shoot. Their fresh weight was taken by using an electrical single pan balance. The fresh plant materials were kept in a hot air oven at 80^{O}C for 24 hr and then their dry weight were also determined.

3. RESULT & DISCUSSION

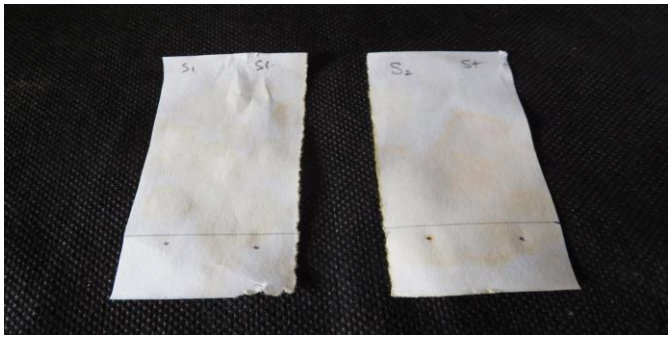

Figure 1. Paper Chromatography was measured in sample movements. IAA standred = 0.63; Sample 1 = 0.22; Sample 2 = 0.55

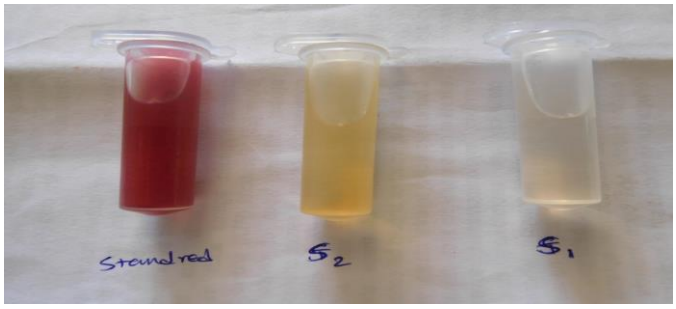

Figure 2. IAA colour changed for red colour formed. This colour changed in present for chlorophyll. The gibberellic acid standard vaiue = 0.129

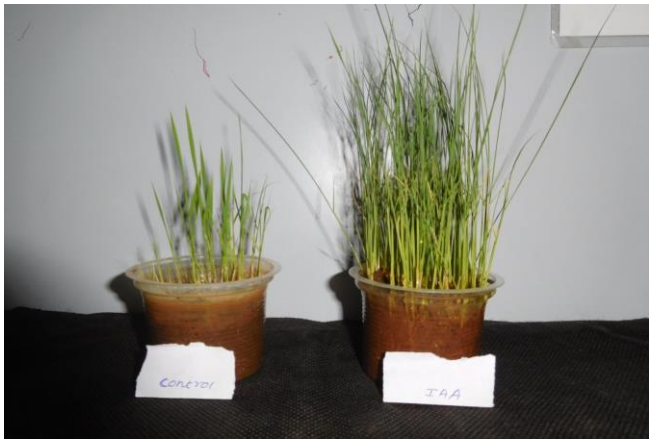

Figure 3. The IAA spray plant in promoting the hormones good condition for present in chlorophyll.

4. DISCUSSION

PGPR mediated phosphate solubilisation through biosynthesis of organic acids that create acidification conditions in the media. PGPPR solubilised more inorganic phosphate through gene modification followed by their expression in specific bacterial strain. phytase genes have been cloned from number of PGPR. In our study significance drop in pH of broth culture IAA which was observed comparatively low without tryptophan.

5. SUMMARY

Modern biotechnology has resulted in a resurgence of interest in the production of new therapeutic agents using botanical sources. With nearly 500 biotechnology products approved or in develop- ment globally and with production capacity limited, the need for efficient means of therapeutic protein production is apparent. Through genetic engineering, plants can now be used to produce pharmacologically active proteins, including mam- malian antibodies, blood product substitutes, vac- cines, hormones, cytokines and a variety of other therapeutic agents. Efficient biopharmaceutical

Growth hormone production in plants involves the proper selection of host plant and gene expression system, including a decision as to whether a food crop or a non-food crop is more appropriate. Product safety issues relevant to patients, pharmaceutical workers, and the general public must be addressed, and proper regulation and regulatory oversight must be in place prior to commercial plant-based biopharmaceutical production. Plant production of pharmaceuticals holds great potential, and may become an important production system for a variety of new biopharmaceutical products.

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