Pharmacognosy Journal 5 (2013) 60–65

Contents lists available at SciVerse ScienceDirect

Pharmacognosy Journal

journal homepage: www.elsevier.com/locate/phcgj

Original article

Studies on nitrogen fixing bacteria and their application on the growth of seedling of *Ocimum sanctum*

Kandasamy Dhevendaran [a,*], Ganesan Preetha [b], Bodethala N. Vedha Hari [c]

[a] *Professor of Biotechnology, School of Chemical and Biotechnology, Sastra University, Thanjavur 613401, Tamil Nadu, India*
[b] *Student, School of Chemical and Biotechnology, Sastra University, Thanjavur 613401, Tamil Nadu, India*
[c] *Assistant Professor, School of Chemical and Biotechnology, Sastra University, Thanjavur 613401, Tamil Nadu, India*

ARTICLE INFO

Article history:
Received 19 January 2013
Accepted 9 March 2013
Available online 19 March 2013

Keywords:
Indole acetic acid
Bio-fertilizer
Azotobacter
Azospirillum

ABSTRACT

Introduction: Representatives of the *Azotobacteriaceae* family are regular inhabitants of soils of roots (rhizosphere) and on the root surface of free-living nitrogen fixing bacteria of the genera *Azotobacter* and *Azospirillum*. They are the regular associates of the roots of many tropical grasses and cereals. Their associations with medicinal plants are of paramount importance. The medicinal plants such as *Aloe vera* and *Datura alba* were selected to access the nature of association in the rhizosphere and on the roots by the above bacteria.
Methods: The roots of *A. vera* had the dominance of nitrogen fixing bacteria (132×10^2/g). The characteristics of the selected bacteria revealed the presence of *Azotobacter chroococcum*, *Azotobacter beijerinckii*, *Azotobacter vinelandii* and *Azospirillum lipoferum*. The carbon sources such as sucrose, lactose, glucose, maltose, rhamnose, xylose and mannitol either individually or in combination induced the growth of selected nitrogen fixing bacteria *(A. chroococcum*, *A. beijerinckii*, and *A. vinelandii)*.
Results: The maximum growth was recovered with maltose and glucose (1.36S0.D). The mixed carbon sources such as mannitol, maltose and xylose showed elevated growth of the bacterium *(A. chroococcum* and *A. beijerinckii)*. The production of IAA, a growth promoting hormone by *A. chroococcum*, *A. beijerinckii* and *A. vinelandii* was studied at varying pH ranges from 6 to 9 in the growing medium. The increase in the pH stimulated the growth as well as the synthesis of IAA. Both the growth and the growth hormones are similar in the growing liquid broth. The above observations paved the way for accessing the growth of the seedling of *Ocimum sanctum*. The application of *Azotobacter* and *Azospirillum* species as biofertilizers is a testimony to the effect that IAA on seedlings growth.
Conclusion: With this we conclude that IAA with its growth promoting capacity dominates the Microbial and Agricultural Biotechnology in the years to come.

1. Introduction

Bio-fertilizers are the formulation of living microorganisms which are able to fix atmospheric nitrogen in the available form to plants either by living freely in the soil or being associated symbiotically with plants. They are capable of mobilizing nutritive elements from non-usable form to usable form through biological process.[1] The primary object of this study is on *Azotobacter*, a member of the family *Azotobacteriaceae* and it has been isolated from both soil and rhizosphere environment. *Azotobacter* spp. are mesophilic bacteria whose growth and nitrogen fixing ability are highly dependent on the temperature. The application of free-living N_2 fixing *Azotobacter* and *Azospirillum* as bio-fertilizers is known to result in increased productivity of a variety of field ground crops. *Azospirillum* is a Gram negative soil bacterium living in close association with roots of plants.[2]

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Medicinal plants constitute an important component of flora and are widely distributed in India. The pharmacological evaluation of substances from plants is an established method for the identification of lead compounds which can lead to the development of novel and safe medicinal agents. Medicinal plants are the vital source of medication in developing countries. According to the World Health Organization (WHO) in 1977 "a medicinal plant" can be of any plant, which is one or more of its part contains substances that can be used for the therapeutic purposes or which are precursors for the synthesis of useful drugs. Among 250,000 higher plant species on

* Corresponding author.
E-mail address: dheven.k@gmail.com (K. Dhevendaran).

0975-3575/$ – see front matter
http://dx.doi.org/10.1016/j.phcgj.2013.03.003

earth, more than 80,000 species are reported to have both at least some medicinal value and therapeutic value. They are classified according to the part used, habit, habitat, therapeutic value, etc.

Azotobacter synthesizes some biologically active substances, including some phyto-hormones such as auxin, thereby stimulating plant growth. They also facilitate the mobility of heavy metals in soil and enhance bioremediation of soil from heavy metals such as mercury, lead, etc. Many workers have reported on the production of plant hormones by *Azotobacter*[3,4] and by *Azospirillum*. It's ability to fix molecules of nitrogen, increase soil fertility and stimulate plant growth.[5–7]

The present study deals with the distribution of nitrogen fixing *Azospirillum* spp. in rhizosphere and soil sediments and also the potentiality of indole acetic acid production.

2. Materials and methods

The following medicinal plants were collected for the isolation of N_2 fixing bacteria such as *Aleo vera* (family; Asphodelaceae), and *Datura* family; (Solanoideae) from SASTRA University campus and the plants were authenticated and herbarium was made for further studies.

2.1. Enumeration and isolation of bacteria from rhizosphere and on the root associated soil samples

For measuring bacterial load, serial dilution and pour plate techniques were followed as per Pramer and Schmidt.[8] Bacteria were counted by plate method in which 1 ml of a suitable dilution (10^{-1}) of the original sub- sample of rhizosphere and root associated soil sample was pipetted out into a sterile Petri dish. About 20 ml of the (*Azotobacter* medium) at (40 °C) the ear bearing temperature was added to the sample in the Petri dish and then mixed by rotating it in clockwise and anti-clockwise directions. With regard to the aerobic (*Azotobacte*r spp., and *Azospirillum* spp.) in rhizosphere and root associated soil sample and their enumeration the pour plate method was followed[9,10] and showed that the temperature of the molten agar at the time of pouring was important in relation to the number of the colonies developing on pour plates. The above procedure was done in the laminar flow chamber to prevent contamination from externals. The inoculated petri dishes on solidification of the medium were inverted, to prevent the condensed water from falling on the medium and then kept in an incubator for 7 days at room temperature (28 °C). The bacterial colonies were counted after 7 days of incubation period. For obtaining pure cultures the colonies were streaked on agar plates and then subcultured on the same medium slants. On satisfactory growth of the bacteria on the slants, these were stored in a refrigerator (4 °C) till further use. *Azotobacter* medium was used for the isolation of *Azotobacter* with pH 8.5.

2.2. Preparation of soil extract (for 100 ml)

250 g of soil sample was weighed out and transported to a flask containing 100 ml of sterile water. To it 0.5 g of calcium chloride was added and the flask was then autoclaved. After this, the mud was filtered and the clear solution was again sterilized and stored for further use.

2.3. Plant sources

Isolation and enumeration of N_2 fixers were done in the rhizosphere and root associated soils. Here, 1 g each of rhizosphere soil sample and root were taken from the medicinal plants selected and treated with distilled water for 10 min to isolate *Azotobacter* and *Azospirillum*. The roots and soils were crushed using sterile 9 ml blank (10^{-1} dilution). This was aseptically transferred to the dilution level of 10^{-2}. 1 ml from that dilution was aseptically transferred to each sterile Petri dish. About 15–20 ml of sterilized media (*Azotobacter* medium) were aseptically transferred to each Petri dish respectively. The plates were rotated clockwise and anti-clockwise directions to get uniform mixing of the samples and the medium. They were incubated at room temperature till the appearance of microbial colony forming units for 7–10 days.

2.4. Enumeration and isolation of Azospirillum

Azospirillum was isolated in the same way as *Azotobacter*. For isolation an N/b basal medium was employed. Total number of colonies of *Azotobacter* and *Azospirillum* in each petri dish was counted and recorded. The dry weight of the sample (rhizosphere and root associated soil samples) were noted for both the medicinal plants selected in the present investigation.

2.5. Identification of Azotobacter and Azospirillum by staining techniques (Beijerinck, 1901a ;1925)

Cell morphology of the above bacteria was studied from Gram staining and also the biochemical studies. In this Gram staining technique a thin heat fixed smear of *Azotobacter* was made on a clean glass slide. To this, a few drops of crystal violet (primary stain) staining reagent was added and left for 1 min. The slide was washed in tap water. The smear was flooded with a few drops of gram iodine (mordent) and left for 1 min. The smear was washed gently in tap water and then decolorized with 95% of ethanol. The slide was washed with tap water and counter stained with saffron in "O", washed in tap water and blot dried. The slide was observed under microscope in oil immersion (100×). Gram negative rods were observed for the identification of both *Azotobacter* and *Azospirillum* species.

2.6. Effect of carbon sources

Mannitol, glucose, lactose, rhamnose, xylose, maltose and sucrose were utilized for the growth of *Azotobacter* and *Azospirillum*. For the effect of carbon sources on their growth the *Azotobacter* broth medium was prepared with the ingredients as before without agar. At different ways, the carbon sources were weighed at 1% level to assess the effect on their growth either with individual carbon source or in combinations and added into the 100 ml broth in a conical flask. The flasks were then steamed for 15 min. These were disbursed with 10 ml each in a tube and inoculated with *Azotobacter* isolated from the different samples and incubated for five days at room temperature. The turbidimetric reading of the bacterial culture was observed using UV–Vis Spectrophotometer at 600 nm (Unicam).

2.7. Indole acetic acid estimation

Tryptophan is an essential amino acid that can undergo oxidation by way of the enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products mediated by the ability to tryptophanase. The ability to hydrolyze tryptophan with the production of indole is not the characteristic of all microorganisms and therefore it serves as a biochemical marker. *Azotobacter* is known to have the ability to hydrolyze tryptophan and produce indole acetic acid (IAA). The determination of IAA production was followed according to Tien et al[1] The isolates of *Azotobacter* were grown in 100 ml of the respective medium mentioned with various pH levels (6, 7, 8 and 9) supplemented with tryptophan (100ug/L) along with