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Azospirillum spp. Isolated from Raigarh District of Chhattisgarh

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Summary

Azospirillum is considered the most important rhizobacterial genus for improvement of plar growth or crop yield worldwide. They are potential N_2 fixer, associated closely with roots an rhizospheres of many economically important plants and grasses. By the help of soil dilutio technique and Root tip cuttings we can isolate these bacteria. 25 soil samples were collecte from Raigarh district of Chhattisgarh and 25 different bacterial cultures were isolated out c which 4 *cultures* are *Azospirillum* and rest 20 culture are identified as *Bacillus subtilis* and 1 a *Streptococcus* spp. All 4 are gram negative in nature. These all are growth plant promotin bacteria and play a beneficial role on crop productivity by fixing atmospheric nitroger solubilising unavailable phosphates, decomposing organic wastes and enhance nutrie recycling by producing bioactive substances such as vitamins, hormones, enzymes etc *Azospirillum* can fix nitrogen biologically in to the soil thus are beneficial for environment.

Introduction

The term 'Biofertilizer' denotes all the "nutrient inputs of biological origin for plant growth". With this view, microorganisms are the best alternative. Thus, use of Biofertilizer that is microbial inoculants *(Azospirillum*) as a source of Biofertilizer has become a hope. As far as economical and environmental view points are concerned, biologically fixed nitrogen is such a source which can supply an adequate amount of nitrogen to plants and other nutrients. *Azospirillum* is considered the most important rhizobacterial genus for improvement of plant growth or crop yield worldwide. (Bashan *et al* 2004). They are a potential N_2 fixer, which is closely associated with roots and rhizospheres of many economically important plants and grasses. (Baldani and Dobereiner 1980; Baldani 1996; Baldani *et al* 1996; Baldani *et al* 1997; Hartman *et al* 1995; Olivares et al 1996; Weber *et al* 1995). *Azospirillum* produces some growth hormones like cytokinens, gibberellins, auxins (Hartmann *et al* 1983; Jain and Patriquin 1985; Reynders and Vlassak 1979; Tien et al (1979), Plant growth regulatory substance such as polyamines (Thuler *et al* 2003) particularly cadaverine, which may be correlated with root growth promotion (Nemi *et al* 2002) and sidrophore (Saxena *et al* 1986) it can also solubilize inorganic phosphorus (Seshadri *et al* 2000).

Materials and Methods

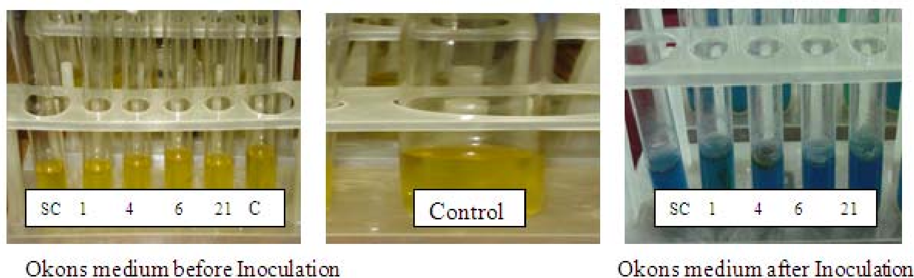
Collection and preservation of soil samples- Total 25 soil samples were collected from Raigarh district of Chhattisgarh. The soil samples were collected to a depth of 6" (15 cm) from soil surface. About 100g of soil was dried in shade, powdered and preserved in polythene bag for chemical analysis. Another 200g soil was kept as such in polythene bag to prevent the moisture loss. These samples were properly tagged, sealed and stored in refrigerator for isolation of *Azospirillum*.

Collected soil samples

Isolation of Azospirillum

By serial dilution technique- Ten grams of rhizospheric soil was suspended in a known volume of sterilized water and serial dilutions were prepared in to sterilized water blanks. Appropriate dilutions were plated on malate medium (Okon *et al*., 1977) containing NH_4Cl (Ammonium chloride) as source of nutrient for initial growth of bacteria

By root bit cuttings-Besides isolation from soil, root pieces of the same plant samples were also used for isolation of bacteria. The root were cut in to small fragments of two-three cm aseptically, its surface were sterilized by 0.1 percent-acidified $HgCl_2$ for three minutes with the help of sterilized gloves and then in 70 percent alcohol for one minute. The roots were then subsequently washed with five to six times of sterilized water to free from these chemicals. Now placed in semi solid nitrogen free broth (Nfb) in sterile test-tubes using sterile forceps and incubated for four to five days in incubator at 28 -30°C. (Okon *et al*, 1977). The slants were then preserved in refrigerator of laboratory for further studies.



Okons medium before Inoculation

Okons medium after Inoculation

Microscopic observations

Gram staining

Gram staining given by Benson (1990) was conducted as per the procedure. Firstly thin smears of bacteria were made on glass slides. Then they were air dried followed by fixing the smear by heat. Then each smear was covered with crystal violet for 30 seconds followed by briefly washing off the strain using a wash bottle of distilled water. Drain off the excess water from the slides and then smear was covered with Gram's iodine solution for 60 seconds. These slides were then washed with 95% ethyl alcohol to wash off the iodine solution. Ethyl alcohol was added drop by drop, until no more colour flowed from the smear. Again the slides were washed with distilled water and drained. Safranin was then applied to the slides for 30 seconds. Then they were washed with distilled water and blot dried with absorbent paper and left for air drying. The bacteria that appeared purple were referred to as gram- positive and those which were appeared pink were described as Gram-negative.

Capsule Staining

Place a single drop of India ink on the left-hand end of a clean microscope slide. Using a sterililized loop and sterilized technique. Take the culture and mix it into the drop of India ink. Be sure there are no large clumps of organism, but try to avoid spreading the drop. Place the end of another clean microscope slide at an angle to the end of the slide containing the organism. Spread out the drop out into a film. This is done by contacting the drop of India ink with the clean microscope slide and using the capillary action of the dye/ slide to spread the India ink across the smear. Allow the film to air dry. Saturate the slide with crystal violet for 1 minute. Rinse the slide gently with water. Allow the slide to air dry. Observe the slide under the microscope, using proper microscope technique.

Endospore Staining

Perform a bacterial smear of culture. Place a small piece of bibulous paper over the smear. Saturate the paper with malachite green. Heat the slide gently over the bunsen burner for 5 minutes. Be sure to keep the bibulous paper saturated with malachite green during heating; if the slide is steaming, you're okay; if it stops steaming, add more malachite green. Rinse the slide gently with water and dispose of the used bibulous paper in the trash. Counter stain with Safranin for 2 minutes. Rinse the slide gently with water. Carefully blot the slide dry with bibulous paper. Observe the slide under the microscope, using proper microscope technique. Endospores will stain green. Parent cells will stain red.

The Hangiing Drop Method

With the coverslip on a flat surface, place a small amount of Vaseline near each corner. Transfer several loopsful of broth culture to the center of the coverslip.Take a depression slide and, with the concave portion over the drop, press the slide onto the coverslip.Invert the slide quickly to keep from disrupting the drop. Begin focusing with the lowest power objective and work up to the oil immersion objective. Be careful to not break or displace the coverslip.

Result

Formation of characteristic colonies by *Azospirillum* isolates, in malate media, was taken as a tool for preliminary identification. Each isolate was picked and streaked on Petri plates containing Nfb malate agar and incubated for seven days at room temperature. Characteristic small white dense colonies were observed. Further, the single isolated colonies were restreaked on potato infusion agar and Congo red medium and incubated for seven days to observe for pink and scarlet colored colonies, respectively (Baldani and Dobereiner, 1980).Some isolates are gram negative, rod in shape and motile in nature. Out of all 25 isolates 4 isolates are *Azospirillum* spp. and rest 20 culture are identified as *Bacillus subtilis* and 1 as *Streptococcus* spp. (From IARI, Plant Pathology Department, Delhi).

Morphological characterization

SN	ISOLATES	CELL SHAPE	CELL ARANGMENT	CAPSULE CELLSIZE	ENDOSPORE	MOTALITY	GRAM REACTION
1	ISO 1	Rod	Chain	-	-	+	-
2	ISO 4	Rod	Single	-	-	+	-
3	ISO 6	Rod	Chain	-	-	+	-
4	ISO 21	Rod	Chain	-	-	+	-
5	SC	Rod	Single	-	-	+	-

SC= Standered cheak (*Azospirillum brasilense*)

Cultural characterstics—On Nutrient Agar Medium

S.N.	ISOLATES	GROWH	MARGIN	COLOUR	FORM
1	ISO 1	Abundant	Entire	Cream	Circular
4	ISO 4	Abundant	Undulate	Light yellow	Irregular
6	ISO 6	Abundant	Entire	Cream	Circular
21	ISO 21	Abundant	Entire	Cream	Circular
22	SC	Abundant	Entire	White	Circular

SC= Standard check (*Azospirillum brasilense*)

Cultural characteristics on Okons Broth Medium

S.N.	ISOLATES	SURFACE GROWTH	CLOUDING	SEDIMENT
1	ISO 1	Blue ring	Heavy	Little
2	ISO 4	Blue ring	Heavy	None
3	ISO 6	Blue ring	Heavy	Little
4	ISO 21	Heavy blue ring	Heavy	None
5	ISO SC	Light blue ring	Heavy	Abundant

SC= Standard check (*Azospirillum brasilense*)

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