Research Article

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DEVELOPMENT AND CHARACTERISATION OF ABIOTIC STRESS TOLERENT AZOSPIRILLUM

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Abstract: Abiotic stress is one of the most important environmental constraints that limit survival and productivity of staple crops like wheat and maize particularly in arid and semi-arid regions. Plant growth promoting bacteria (*Azospirillum*) are beneficial bacteria present in soil and forming associations with roots of plants. This investigation deals with the isolation and characterization of *Azospirillum* from maize roots. *Azospirillum* strain was identified by physiological, morphological and biochemical characters. These strains was identified based on morphological characters such as shape, and biochemical characters such as IMViC, urease were studied. Factors affecting growth of *Azospirillum* like temperature, salt stress, effect of pesticides and antibiotic tolerance were studied. The pot culture experiment was carried out to study the *Azospirillum* strain used on the growth of wheat plants. Salt stress has become an ever increasing threat to food production, irrigation being a major problem of agricultural fields due to gradual salinization.

Keywords: *Azospirillum*, NaCl, Pesticides, Antibiotics

INTRODUCTION

Azospirillum is a free living plant growth promoting bacterium (PGPB), capable of affecting growth and yield of numerous plant species, many of agronomic and ecological significance. It was first isolated from nitrogen-poor sandy soil in the Netherlands (Beijerinck 1925)[2]. *Azospirillum* is considered the most important rhizobacterial genus involved in improvement of plant growth or crop yield worldwide (Bashan et al., 2004). Bacteria of the genus *Azospirillum* are associative nitrogen (N_2)-fixing rhizobacteria that are found in close association with plant roots.

Azospirilla are Gram-negative free-living nitrogen-fixing rhizosphere bacteria. They display a versatile C- and N metabolism, which makes them well adapted to establish in the competitive environment of the rhizosphere. [2][8] Free-living diazotrophs repeatedly detected in association with plant roots, include *Acetobacter diazotrophicus, Herbaspirillum seropedicae, Azoarcus spp.* and *Azotobacter*. Four aspects of the *Azospirillum*-plant root interaction are highlighted: natural habitat, plant root interaction, nitrogen fixation and biosynthesis of plant growth hormones. [2]

MATERIALS AND METHODS

***Preparation of media for Isolation of Azospirillum*:** Semi solid sodium malate agar is used. $NaNO_3$-5g**;** K_2HPO_4 -0.1g**;** KH_2PO_4-0.4g**;** $MgSO_4.7H_2O$-0.2g**;** NaCl-0.1g**;** $CaCl_2$ -0.02g**;** $FeCl_3$-0.01g**;** Na_2MoO_4-0.002g**;** Bromothymol blue-5ml**;** Distilled water-1000ml**;** Agar-20g**;** p^H-6.8**;** 70% alcohol**;** Phosphate buffer.

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Isolation of Azospirillum

Azospirillum species may be isolated from rhizosphere biofilms associated with the roots of various grasses, cereals, and tuber plants. Azospirilla are generally gram-negative rods which are motile by means of a single flagellum. Azospirilla are also capable of fixing nitrogen. Their isolation is based on the premise that these organisms can grow in concentrations of nitrogen too low to support growth of most microorganisms [1][8]. Prepare petriplate with sodium malate agar medium. Maize roots were collected and with sterile scalpel they were cut into root bits. These root bits were sterilized in 70% alcohol. Then these roots bits were transferred into phosphate buffer for 3 washes. Now the root bits were transfer onto the petriplate containing medium. Now these plates were kept at 37° incubation for 48-72 hours. [3][4][7].

Biochemical characterization

IMViC:

Indole test: Preparation of peptone broth, loopful of culture was taken into broth and kept for incubation

Methyl red test: preparation of MR-VP medium culture was inoculated and kept for incubation.

Vogesproskauer test: preparation of MR-VP medium, culture was inoculated and kept for incubation.

Citrate test: simmon's agar slants was prepared and culture was added and kept for incubation.

Urease test: slants were prepared by adding 20 g of urea, 9.5 g of Na_2HPO_4 9.1 g of KH_2PO_4, 0.1g of yeast extract 0.01 g of phenol red and agar. The pH is made to 6.8±0.2 at 25°c. Mix thoroughly and dispense aseptically in sterile tubes. Cool the tubed medium in a slanted position so that deep butts are formed. Using a sterile technique, inoculate each experimental organism into its appropriately labeled tube by means of loop inoculation. Incubate cultures 24-48 hours at 37°C.

Salt tolerance

NaCl salt used of different concentrations. Semi solid sodium malate broth is prepared and different concentrations of salt such as 1%, 2%, 3%, 5%, and 10% of NaCl is prepared and kept for autoclave. Then culture is added and kept for incubation for 48hrs. Now O.D is checked at 520nm. [3][8]

Factors affecting growth of Azospirillum

Temperature: 6 flasks of 100ml sodium malate broth was prepared. Culture was added into each flask and incubated at different temperatures such as 4°c, 10°c, 25°c, 37°c, 42°c [3][4][8]. These flasks were kept for incubation for 48hours. O.D is checked at 520nm.

Effect of pesticides

2 different pesticides have used namely Xylocaine, Chloropoid. 2 petriplates were prepared with sodium malate agar. 0.1ml of the culture was spread on to the media [11][12]. 3 wells were prepared on the media of different concentration such as 0.1mg/ml, 0.2mg/ml, and 0.5mg/ml. These plates were incubated at 37°c for 24-48 hours.

Pot culture studies for plant growth promotion

Pot culture experiment was conducted for a period of 10 days. 4 pots were taken, in the 1st it is control seeds were added, in the 2nd pot it is inoculum added to seeds and sowed. In the 3rd pot inoculum is added to seeds and the seeds are dried and then sowed. In the 4th pot inoculum with seeds are added and the inoculum is added for every 5 days.[11]

Antibiotic resistance

4 different antibiotics have used namely streptomycin, tetracycline, penicillin, ampicillin. 4 petriplates were prepared with sodium malate agar[6][10]. 0.1ml of culture was spread on to the media. 2 wells were prepared on the media of different concentrations such as 50µg/ml and 100µg/ml. These plates were incubated at 37°c for 24-48 hours.

RESULTS AND DISCUSSION

Isolation of Azospirillum:

Pellicle was observed around the root bit (Fig. 1). On gram staining it shows gram negative characters. Plump, slightly-curved and straight rods (Fig. 2)

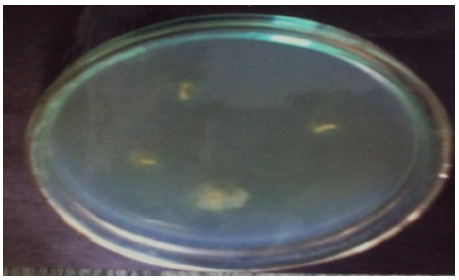


Fig.1: pellicle around the root

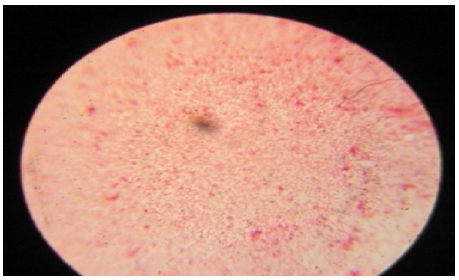
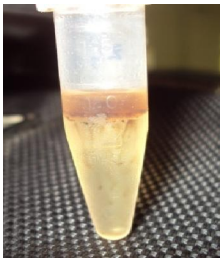


Fig.2: Gram's staining

IMViC test

Test	Result
Indole	Negative
Methyl red	Positive
Vogesproskauer	Negative
Citrate	Positive
Urease	Positive

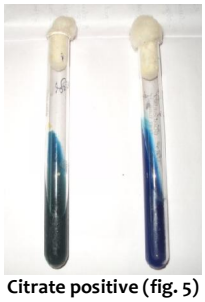


Indole test negative

Methyl red positive (fig 3)

Voges proskauer negative

Urease positive (fig. 4)



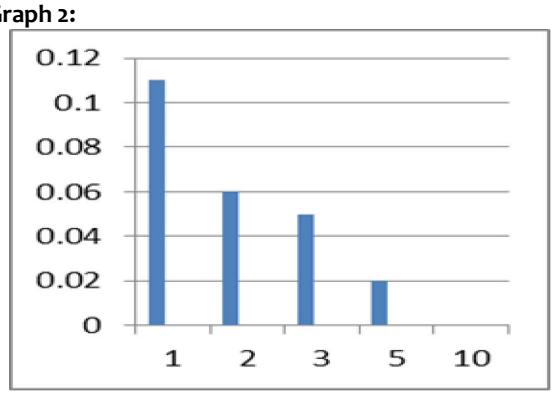
Citrate positive (fig. 5)

Table 2: Salt tolerance

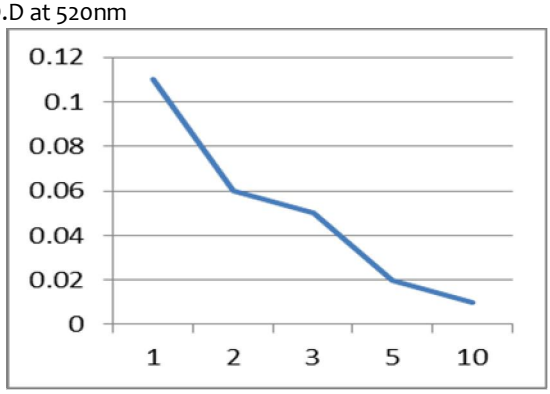
% of NaCl	O.D at 520nm
Control	0.00
1%	0.11
2%	0.06
3%	0.05
5%	0.02
10%	0.01

- Growth was observed.
- Turbidity
- Salt concentration which is optimum for *Azospirillum* is 1%

Graph 2:



O.D at 520nm



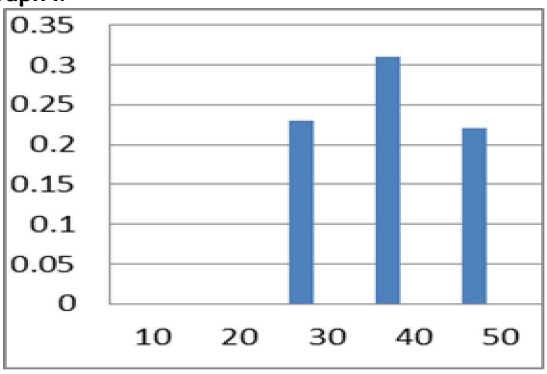
Concentration of NaCl ⟶ concentration of NaCl

Table 1: Temperature

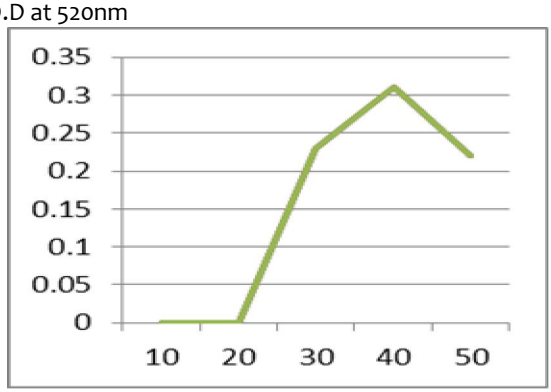
Temperature	O.D at 520nm
Control	0.0
4°c	0.0
10°c	0.0
25°c	0.23
37°c	0.31
42°c	0.22

Optimum temperature for growth of *Azospirillum* is 37°c

Graph 1:



O.D at 520nm



Temperature °c

Effect of Pesticides

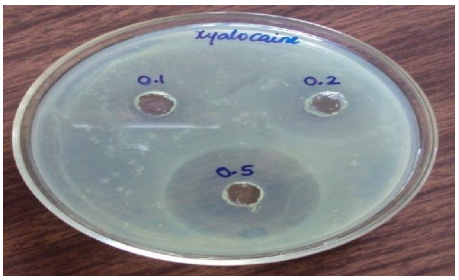


Fig.6: Xylocaine is sensitive.

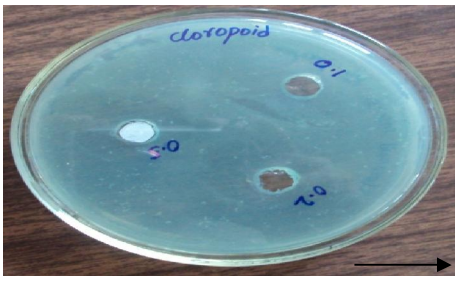


Fig.6: Chloropoid is resistant.

Pot culture studies for plant growth promotion

Length of the shoot was measured for every 5 days of the 4 pots

Table 3:

Wheat seeds	3rd day	5th day	10th day
Control	3cm	7cm	17cm
Seeds with inoculum	4cm	9cm	19cm
Inoculum added to seeds and dried	5.5cm	9.5cm	24cm
Inoculum added to seeds for every 5days	8.5cm	15cm	22cm

Fig.9: 3rd day

Control inoculum+ seeds inoculum+ dried seeds inoculum is added for every 5 days

Fig 9: 5th day

Control inoculum + seeds inoculum + dried seeds inoculum is added for every 5 days

10th day

Control inoculum + seeds inoculum + dried seeds inoculum is added for every 5 days

Table 4: Antibiotic resistance

Antibiotic	50µg/ml (diameter)	100µg/ml (diameter)
Streptomycin	31mm	39mm
Ampicillin	21mm	23mm
Penicillin	18mm	24mm
Tetracyclin	33mm	35mm

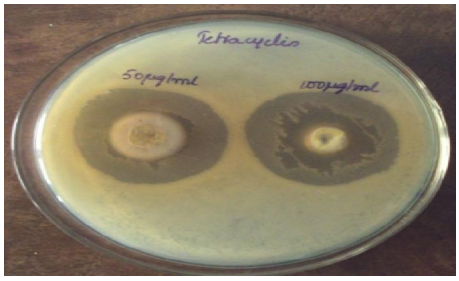


Fig.7: Tetracyclin

Fig.7: Streptomycin

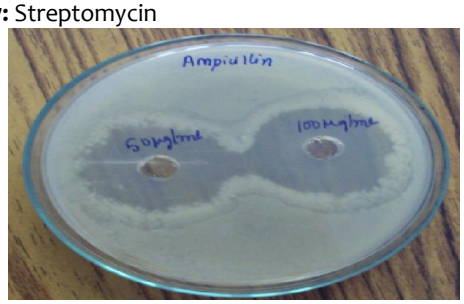


Fig.8: Ampicillin

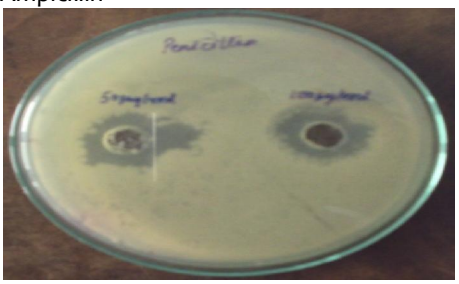


Fig.8: Penicillin

DISCUSSION

Azospirillum strain was obtained from maize roots, it is observed by its colony characters. Dobereiner and Day (1976) reported microaerophilic growth in semisolid agar stagnant conditions were helpful for the inoculation of the organism. Since *Spirillum lipoferum* grows in a typical pellicle 1 to 4mm below the surface, this method was particularly useful for studying the substrates and growth conditions for nitrogen fixation. The isolated strains were rod and vibroid in shape. This result was confirmed by Doberenier and Baldani (1979) and Krieg *et al.*, (1984) and also reported that microscopic examination revealed polymorphism, but the dominant forms on a solid malate medium are characteristic curved rods of various sizes with predominant refractive fat droplets.

Indole test is negative which produce tryptophanase, an enzyme that cleaves tryptophan, producing indole and other products. Methyl red test is positive; (fig 3) Enterics that subsequently metabolize pyruvic acid to other acids lower the pH of the medium to 4.2. At this pH, methyl red turns red. Vogesproskauer test is negative because it does not produce acetyl methyl carbinol. Citrate test positive Bacteria with the enzyme citrase metabolise citrate to produce alkaline end-products that raise the p^H of the

medium to 7.6, causing the bromothymol blue to turn blue. (Fig. 5) Urease test is positive due to Rapid urea hydrolysis; strong urease production. (Fig. 4)

Growth of the bacteria in the presence of different NaCl concentrations showed that the strain could tolerate upto 10% NaCl. And the highest growth is observed at 1% NaCl. (Table 2) (Graph 2)

Growth of *Azospirillum* was checked at various temperatures and maximum growth is seen at 37°c. Growth is also observed at temperatures like 25°c and 42°c which supports the fact that they can grow over a range of 25°c to 42°c. (Table 1) (Graph 1)

Two types of pesticides have been used namely Chloropoid, Xylocaine to check the resistance towards *Azospirillum* strain. When strain is added with these pesticides to the media, *Azospirillum* showed sensitive to Xylocaine and resistant to Chloropoid. (Fig. 6)

Azospirillum strain added onto wheat plants. All the inoculants enhanced plant height over control. Growth was observed in all the pots. Growth of the plants in the pots containing inoculum with seeds, inoculum with dried seeds, and the inoculum added at every 5 days shown more growth when compared to that of control, which suggest the fact that *Azospirillum* can be used as biofertilizer. (Fig. 9) (Table 3)

Antibiotic resistance has showed that the antibiotics which are used such as streptomycin, tetracycline, (fig 7) penicillin, ampicillin at different concentration showed that *Azospirillum* strain sensitive towards above antibiotics. (Fig. 8) (Table 4)

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