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ISOLATION OF *AZOSPIRILLUM* AND COMPATIBILITY TESTING WITH MICRONUTRIENTS, PGPR ACTIVITY AND EFFICACY ON TOMATO

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ABSTRACT
Efficient *Azospirillum* cultures was isolated from soil samples of two different locations practicing organic farming *viz*. Kathwada (Ahmedabad) and Ravipura (Anand) was conceded out. Three strains were selected on the basis of their morphological, biochemical and physiological nature. Isolates As-1, As-2 &As-3 were found curved, motile, rods, gram negative, with optimum pH range 4 to 8 and salt tolerance upto 5 % NaCl concentration. *In vitro* compatibility of *Azospirillum* isolates with micronutrients was 100 ppm concentration by qualitative & quantitative assay with all isolates also checked PGPR characteristics of *Azospirillum* concluded that nitrogen fixing potentiality of isolates were ranged from 11.1 to 25.0 mg N_2 fixed/g of sucrose consumed, phosphate solubilization capacity ranged from 11.7- 20.8 P µg/ml. Among all isolates, As-1 and ASA-1 (standard strain) are able to solubilize zinc oxide, IAA production compared to 3 isolates of *Azospirillum* at 5 DAI, ranged from 0.30 to 25.85 µg/ml IAA in tryptophan supplemented media. *In vitro* effect of *Azospirillum* isolates on seed germination was 74 % observed after 120 h by As-2 isolate followed by As-1. Among all the isolates of *Azospirillum* isolate As-2 was performity best activity *in vitro* condition.

INTRODUCTION

The rhizosphere is a favourable habitat for microorganisms and a challenge for ecological studies. At the interface of root and soil, the growing plant provides macronutrients as well as micronutrients and the soil contributes its structure, mineral composition, physicochemical properties and water regime.

Micronutrients play an important role as essential trace elements in all living systems and are essential component of number of peptidases and phosphohyrolases (Devlin and Withan, 1988; Massoud *et al*., 2005). Plant Growth Promoting Bacteria (PGPB) also plays an important role in mobilizing macro and micro nutrients required by the plants in rhizosphere.

Ribaudo *et al*., 2006 studied tomato seeds, inoculated with the plant growth–promoting rhizobacteria. *A. brasilense* FT326, and changes in parameters associated with plant growth were evaluated 15 days after inoculation. Azospirilla were localized on roots and within xylematic tissue. An increase in shoot and root fresh weight, main root hair length, and root surface indicated that inoculation with *A. brasilense* FT 326 resulted in plant growth improvement. In the present study, an attempt has been made for development of *Azospirillum* liquid bio-inoculants fortified with micronutrients and their assessment on tomato (*Lycopersicon esculentum* L.).

MATERIALS AND METHODS

Isolation of *Azospirillum*

Azospirillum As-1, As-2 & As-3 were isolated from rhizopsheric soil of tomato by serially dilution of soil. Diluted sample from 10^{-6} to 10^{-8} dilutions was taken and 0.1 ml of aliquot was inoculated in test tube containing Nfb (Nitrogen free BTB) semisolid media. All the tubes were incubated at 32ºC for 48 h and observed the growth by the formation of pellicles. The pellicles were streaked on Nfb solid media and incubated at 32ºC for 48h. Morphologically divergent bacterial colonies were picked from the plates and streaked on Nfb solid and incubated at 32ºC for 48h. After attaining growth, all the isolates were preserved in a cold room at 4ºC for further investigations. The isolates were subcultured in fresh Nfb slants once in a month and maintained at 4ºC.

Identification and characterization of *Azospirillum* isolates

Bacterial isolates were further streaked for purification on NFB agar medium. Microscopic observations of all isolates were carried out. Three isolates were recognized as of the genus *Azospirillum* based on the morphological, cultural and some biochemical characteristics, by referring 9th edition of Bergey's Manual of Systematic Bacteriology.

In vitro compatibility of *Azospirillum* isolates with micronutrients

Qualitative

All *Azospirillum* isolates and standard strains compatibility with micronutrient were checked by method of Ganapathy and Savalgi (2006). Nutrient broth was prepared and stock solution of micronutrients *viz*. Zn, Fe, Mn, B, Cu were prepared separately and after sterilization of broth micronutrients were

added at different concentration @ 50, 100 and 150 ppm individually. Isolates were inoculated as per labeled broths @ x 10^6cells/ml. The growth was observed at regular interval upto 7 days.

Quantitative

All *Azospirillum* isolates and standard strains were checked for their micronutrient compatibility by quantitative method. Different concentration of micronutrient *viz*. Recommended dose (1X), ¼X dose, ½ X dose ($FeSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, H_3BO_4) individually and mixture were mixed in NFB medium. After sterilization specific labeled tube were inoculated with 10^5 cells. Colony forming unit (cfu/ml) was recorded at 24 hour interval upto 120 h.

***In vitro* studies on PGPR traits of native *Azospirillum* isolates**

Selected three *Azospirillum* isolates were further characterized for plant growth promoting attributes *viz*. Nitrogen fixation, P solubilization, Zinc solubilizing, Indole Acetic Acid (IAA) Production and ACC deaminase enzyme.

Nitrogen fixation

The plant growth promoting effect shown by rhizopheric diazotrophs is directly attributed to its capacity to fix atmospheric nitrogen into the forms utilized by plants. In this study the rhizopsheric bacterial isolates were inoculated into the Nfb broth medium without any nitrogen source and containing sucrose as carbon source and were incubated at 32 ± 2ºC for 7 days and nitrogen fixation measured by Micro-Kjeldahl method (A.O.A.C., 1965) and sugar utilization was estimated by Fehling's method. The rate of nitrogen fixation was expressed as mg nitrogen fixed / gram of sucrose consumed.

Phosphate solubilization capacity

Sterilized NBRIP agar medium was prepared and plated. Bacterial isolates were spot inoculated on NBRIP agar plates. Plates were incubated at 32 ± 2ºC for seven days. Zones of clearance were observed around the colonies. Then after Erlenmeyer flasks (250 mL) containing 100 mL of the liquid PKVK medium were inoculated with 100 μL of bacterial suspension (approx. 10^8 cfu/ml). For each isolate three replication were inoculated. The flasks were incubated on rotary shaker (150 rpm) at 32 ± 2ºC after 3 and 5 days, phosphate estimation was carried out by Vanado-molybdate method by APHA (1995). The graph of OD versus concentration of phosphate in μg was plotted for the standard and samples were compared to calculate P concentration.

Zinc solubilization

All the bacterial isolates along with standard check were screened for their ability to solubilize zinc on Bunt and Rovira medium amended with insoluble zinc oxide (ZnO). The actively growing cultures were spot inoculated onto the medium, incubated at 32°C and solubilization zone observed after 5 days of incubation.

Indole acetic acid (IAA) production

All the isolates of *Azospirillum* and standard strains were screened for IAA production. Bacterial cultures were inoculated in the Nfb medium supplemented with and without tryptophan at 32 ± 2ºC for 48 hrs. Cultures were centrifuged at 5000 rpm for 30 min. The supernatant liquid was mixed with Salkowski reagent (1:2) and the colour was measured by spectroscopy at 530 nm after 30 min. The levels of IAA production were estimated referring IAA standard graph.

ACC deaminase enzyme production

Qualitative screening of bacterial isolates for ACC deaminase enzyme production was carried out based on their ability to use ACC (1-Aminocyclopropane-1-Carboxylate) substrate as a sole source of nitrogen in the Dworkin and Foster salt minimal medium. Cultures were spot inoculated on petri plates containing DF salt minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC substrate. Plates containing DF minimal medium without ACC served as negative control and with $(NH_4)_2SO_4$ (2.0 gm/L) as a nitrogen source served as positive control. The plates were incubated for 3-4 days at 32 ± 2°C. Growth of isolates on ACC supplemented plates were compared with positive and negative control plates. Isolates growing well on ACC plates were considered as ACC deaminase enzyme producers (Daun *et al*., 2009).

***In vitro* effect of *Azospirillum* isolates on tomato seed germination**

Tomato cv. AT-3 seeds were surface sterilized by washing in 95 % ethanol solution for 5 min, 0.2 % $HgCl_2$ solution for 2 min and rinsed thoroughly with distilled water 3-5 times. Thoroughly washed seeds were kept on previously sterilized filter paper sheet placed in petri plates and incubated at 32 ± 2°C in growth chamber for 5 days, seed germination was observed after 120 h and germination percentage was calculated (Rodrigues *et al*., 2010).

RESULTS AND DISCUSSION

Isolation and characterization of *Azospirillum* from organic as well as conventional soil

Isolation of bacteria

Azospirillum sp., was isolated in NFB semisolid medium. Soil was serially diluted and 0.1 ml aliquot was inoculated in NFB tubes. After 48h incubation, the NFB semi-solid medium showed blue colored pellicle. Appearance of pellicle formation on NFB semi-solid medium indicated presence of microaerophilic *Azospirillum*. The pellicles were transferred into NFB plates. After 48 h white, merged colonies were observed on the medium. Typical white, often wrinkled colonies were picked out and transferred into NFB semi-solid medium and three morphologically distinct *Azospirillum* isolates were screened out and used for further studies. Kanimozhi and Panneerselvam (2011) also used defined selective semi solid media for isolating diazotrophs belonging to the genus *Azospirillum* from rhizospheric soil and its detection by characteristic features of sub-surface white pellicle formation (Jhala *et al*., 2014).

Identification and characterization of isolated bacteria

Growth pattern on NFB plates

The colonies of the three isolates (As-1 to 3) were slightly raised, smooth and round on NFB plate forming very characteristic fine white subsurface pellicle in semisolid NFB medium within 48 hours. The cells of all the three isolates,

Table 2.1: Morphological characteristics of isolates and standard strain

Isolates	Colony morphology on malate medium	Subsurface pellicle on semi solid NFB medium	Gram reaction	Cell shape	Motility	Growth on Rojo congo medium	Growth on PDA
As-1	White Dense small	+	-ve	curved rods	+	Reddish	Grayish & rough
As-2	Small Pale white	+	-ve	curved rods	+	Light Pink	Grayish & rough
As-3	Small pale white transparent	+	-ve	curved rods	+	Reddish	Slight pinkish & rough
ASA-1	White Dense small	+	-ve	curved rods	+	Light Pink	Grayish & rough
MTCC 2306	Small pale white transparent	+	-ve	curved rods	+	Reddish	Slight pinkish & rough

Table 2.2: Tests for specific breakdown products of isolates

Part 1	Tests	As-1	As-2	As-3	ASA-1	MTCC 2306
1.	ONPG	-	-	-	-	-
2.	Lysine utilization	⊥	⊥	-	-	⊥
3.	Ornithine utilization	⊥	⊥	-	-	⊥
4.	Urease	-	-	-	-	-
5.	Phenylalanine deamination	-	-	-	-	-
6.	Nitrate reduction	+	+	-	+	+
7.	H_2S production	-	-	-	-	-
8.	Citrate utilization	+	-	+	+	+
9.	Voges proskauer's	+	+	-	+	-
10.	Methyl red	-	-	-	-	-
11.	Indole	+	+	+	+	+
12.	Malonate utilization	-	-	-	+	-

Part 2	Tests	As-1	As-2	As-3	ASA-1	MTCC 2306
1.	Esculin hydrolysis	+	+	+	+	-
2.	Arabinose	-	-	+	-	-
3.	Xylose	⊥	-	-	-	-
4.	Adonitol	-	-	-	-	-
5.	Rhamnose	-	-	-	-	-
6.	Cellobiose	+	⊥	-	+	-
7.	Melibiose	-	-	+	-	-
8.	Saccharose	+	+	+	+	-
9.	Raffinose	-	-	+	-	-
10.	Trehalose	+	+	+	+	-
11.	Glucose	+	+	+	+	-
12.	Lactose	-	⊥	-	-	-

Note: +: positive; ⊥: medium; -: Negative; Results of biochemical tests for characterization of rhizospheric bacterial isolates As-1 to As-3 were matching to that of genus *Azospirillum* according to Bergey's manual of systematic bacteriology (1983).

Table 2.3: Qualitative micronutrient compatibility profiles of isolates

Sr no.	Concentration	Micronutrient	As-1	As-2	As-3	ASA-1	MTCC 2306
1.	50 ppm	Fe	+ +	+ +	+ +	+ +	+ +
2.		Mn	+ +	+ +	+ +	+ +	+ +
3.		Zn	+ +	+ +	+ +	+ +	+ +
4.		Cu	+ +	+ +	+ +	+ +	+ +
5.		B	+ +	+ +	+ +	+ +	+ +
6.	100 ppm	Fe	+ + +	+ + +	+ + +	+ + +	+ + +
7.		Mn	+ + +	+ + +	+ + +	+ + +	+ + +
8.		Zn	+ + +	+ + +	+ + +	+ + +	+ + +
9.		Cu	+ + +	+ + +	+ + +	+ + +	+ + +
10.		B	+ + +	+ + +	+ + +	+ + +	+ + +
11.	150 ppm	Fe	+ +	+ +	+ +	+ +	+ +
12.		Mn	+ +	+ +	+ +	+ +	+ +
13.		Zn	+ +	+ +	+ +	+ +	+ +
14.		Cu	+ +	+ +	+ +	+ +	+ +
15.		B	+ +	+ +	+ +	+ +	+ +

Note: + +: Medium growth, + + +: Full growth

Table 2.4: *In vitro* phosphate solubilization efficiency of isolates

Isolates	P μg /ml3 DAI	P μg /ml5 DAI
As-1	11.7	17.4
As-2	9.3	1.2
As-3	20.8	17.5
ASA-1	14.7	14.2
MTCC 2306	10.5	16.2

Table 2.5: IAA productions by isolates

Isolates	IAA without Tryptophan	with Tryptophan
	μg/ml	μg/ml
As-1	0.30	1.06
As-2	0.47	0.97
As-3	13.56	25.85
ASA-1	1.99	3.47
MTCC 2306	0.81	1.23

Table 2.6: ACC deaminase activity by isolates

Sr No.	Isolates	Growth of ACC medium
1.	As-1	-
2.	As-2	+ +
3.	As-3	-
4.	ASA-1	+
5.	MTCC 2306	-

Note: -: No growth, +: Growth

Table 2.7: Germination effect of tomato seeds

Treatments	% seed germination		
	72 h	96 h	120 h
As-1	17	49	63
As-2	29	60	74
As-3	19	31	57
ASA-1	14	31	60
MTCC 2306	19	29	51
Control	5	14	26

ASA-1 and MTCC 2306 were Gram negative, curved rods with spirilloid movement but once the medium has turned alkaline (blue color) the bacteria changes into large pleomorphic forms which is characteristic feature of genus *Azospirillum* while all were actively motile and were able to form cyst.

Growth pattern on Rojo Congo

Light-pink and reddish colonies were observed after 48 h. After 72 h, the light-pink colonies became scarlet. Small scarlet colonies were observed in the first streaks, indicating the presence of *Azospirillum* sp. among the contaminants. Colonies that developed in petri dishes of RC medium further incubated at 32ºC for 96 h showed the scarlet color, abundant growth, dry consistency, round or irregular form, undulate edge, ridges radiating from the centre (Table 2.1).

Growth pattern on PDA

Isolates were streaked on Potato Dextrose Agar plates and incubated for 48 h at 32ºC. Colonies first appear smooth and grayish at 48 h and later on it turn pinkish and structured (Table 2.1).

Biochemical characterization

The results of tests for specific breakdown products are presented in Table 2.2. The data revealed that all the isolates were Urease, Phenylalanine deamination, H_2S production, Methyl red and ONPG negative. While isolates As-1, As-2 as well as standard strain ASA-1 and MTCC 2306 showed nitrate positive. Isolate As-1, As-3 and standard strains ASA-1 and MTCC 2306 also showed citrate positive. Voges proskauer's test was positive by isolates As-1, As-2 and standard strains ASA-1. All isolates along with standard check were positive for catalase, indole production and gelatin negative.

The present study also indicated that *A. lipoferum* related strains were able to utilize large group of carbohydrate including glucose which can be catabolized by *Azospirillum* sp. by the action of NAD(P)- glucose 6-P-dehydrogenase, is required for 6-phosphoglucanate dehydrogenase synthesis which is a key enzyme of the ED pathway for glucose catabolism. All the isolates and standard culture ASA-1 can hydrolyse esculin, saccharose, trehalose, and glucose as carbon source. These results are supported by Attitalla *et al.* (2010) who had reviewed occurrence and microbiological characteristics of *Azospirillum* strains associated with leguminous and non-leguminous plants and through biochemical as well as cultural characteristics 15 strains of *A. lipoferum* were identified. Thakuria *et al*. (2004) characterized four bacteria in each group for taxonomic identification, characterization and efficacy tested to promote rice growth. The three isolates of azospirilla group were identified as *A. brasilense* and the fourth isolate was identified as *A. amazonense*.

Results of biochemical tests for characterization of rhizospheric bacterial isolates As-1 to As-3 were matching to that of genus *Azospirillum* according to Bergey's manual of systematic bacteriology (1983).

In vitro compatibility of *Azospirillum* isolates with micronutrients

Qualitative

Bacterial isolates were examined for their growth in different concentration of micronutrients qualitatively. The growth was observed continuously upto 5 day after inoculation. When compared to three concentrations, 100 ppm of all the micronutrients had influence on the growth (Table 2.3).

In vitro studies on PGPR traits of native *Azospirillum* isolates

Selected three potential *Azospirillum* isolates were further

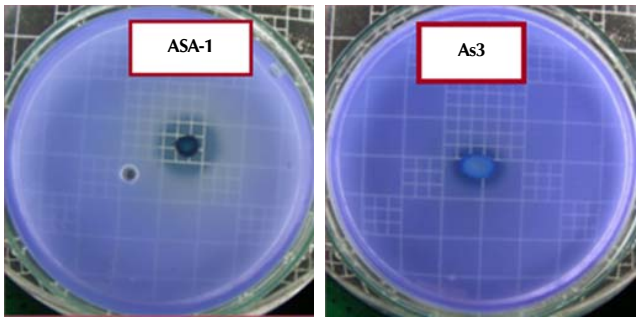

Plate 2.1: *In vitro* organic acid detection on NBRI BPB agar

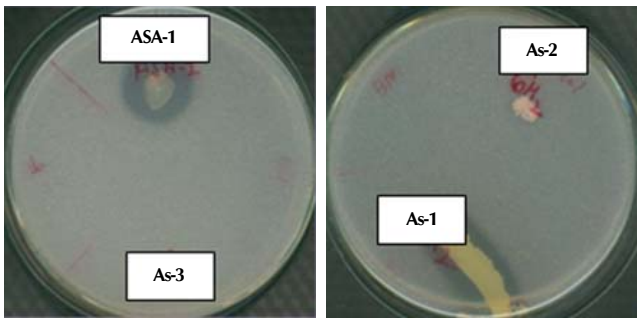

Plate 2.2: Zinc solubilizing efficiency of *Azospirillum* isolates

characterized for plant growth promoting attributes nitrogen fixation, P solubilization, Zinc solubilizing, Production of Indole-3-Acetic Acid (IAA), ACC deaminase enzyme.

Nitrogen fixation

In vitro nitrogen fixation efficiency of isolates was assessed before laying out pot and field trials. The results of this experiment are mentioned in Fig. 2.1. All the isolates were confirmed to have ability of fix atmospheric nitrogen**.** It was revealed from the results that nitrogen fixing potentiality of these isolates were ranged from 11.1-25.0 mg N fixed/ g of sucrose consumed (Micro kjeldhal method) and isolate As-2 was showing the highest nitrogen fixation capacity among all the isolates (25.0 mg N/g of sucrose consumed). Jolly *et al*. (2010) reported nitrogen fixing potential of ten isolates of *Azospirillum* sp. ranged from 2 to 6.16 mg N g^{-1} substrate utilized in semisolid nitrogen free malate medium. Kanimozhi and Panneerselvam (2011) measured nitrogen fixation ability of *Azospirillum* sp. by Micro Kjeldhal method in nitrogen-free semisolid medium with malate. Altogether, 30 isolates tested, only 28 isolates were able to fix nitrogen. Among them, only 10 isolates were able to produce the highest amount of nitrogen (from 11.0 to 15.06 mg N/kg). The two isolates *viz*. KA03 and PA07 were not able to fix nitrogen.

Phosphate solubilization capacity

Plates incubated for seven days for zones of clearance on NBRIP agar medium were observed around the colonies while Isolate As-3 and ASA-1 showed clear zone around colony (Plate 2.1). Data regarding phosphate solubilization activity of isolates are presented in Table 2.4. Estimation of P in the medium revealed that all the strains released P from tri calcium phosphate (TCP). Isolate As-3 recorded maximum soluble phosphorous (20.8 P ìg/ml) at 3 DAI, closely followed by ASA-1 (14.7 P µg/ml) and As-1 (11.7 P µg/ml) and As-2, As-3 and ASA-1 decreased after 5 DAI because of utilization of solubilized phosphate by microorganisms.

The present findings established the phosphate solubilization as an additional benefit of rhizospheric bacterial isolate and thereby, apart from fixing atmospheric nitrogen all the isolates can also improve the availability of phosphorous in crops rhizosphere. Similarly Seshadri *et al*. (2000) revealed phenomenon of P solubilization by nitrogen fixing *A. halopraeferans* to find out the mechanism of action or the involvement of genes. A study on the molecular mechanism would throw light on the *ps* (phosphate solubilizing) genes that could be incorporated in agriculture as *A. halopraeferans* offers traits for nitrogen fixation, phosphate solubilization and salinity tolerance. Rodriguez *et al*. (2000) reported that *A. brasilense*, can reduce quantity of soluble phosphate after incubation for 48 h as auto-consumption of soluble phosphate by the growing bacterial population. In *A. lipoferum*, P solubilization is combined with a decrease in pH. The latter may result from production of gluconic acid and NH_4^+ uptake, which may release protons to the medium.

Zinc solubilization

All the bacterial isolates along with standard check were tested for their ability to solubilize zinc oxide. Of all isolates, As-1 and ASA-1 (standard strain) is zinc solubilizer (**Plate 2.2)**. Desai *et al*. (2012) isolated *Azotobacter* (31)*, Azospirillum* (38)*, Bacillus* (19) and *Pseudomonas* (82) strains from diverse crop production systems and evaluated for solubilization of 'Zn' and 'Pi' *in vitro* from insoluble zinc (ZnO, $ZnCO_3$) and phosphorus [tricalcium phosphate (TCP)], respectively. After 15 days of incubation, 15 strains solubilized zinc and produced solubilization zone on solid media.

Indole acetic acid (IAA) production

All three tested isolates of *Azospirillum* were inoculated in a culture medium with and without tryptophan @ 1 % as nitrogen source for IAA production, after 5 DAI IAA production was detected by the Salkowski reagent under spectrophotometer in the range of 0.30 to 25.85 µg/ml IAA. The highest concentration of IAA was obtained by

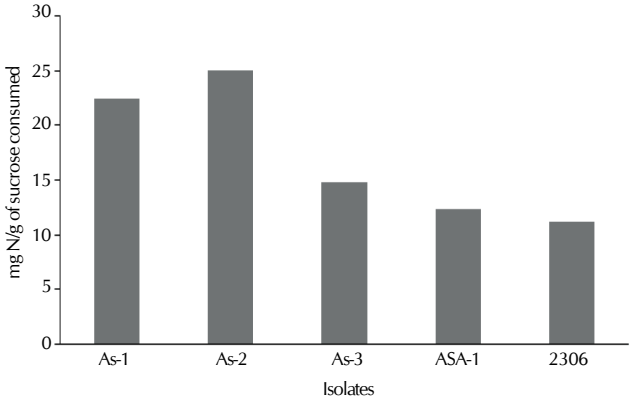

Figure 2.1: *In vitro* nitrogen fixation capacity of isolate

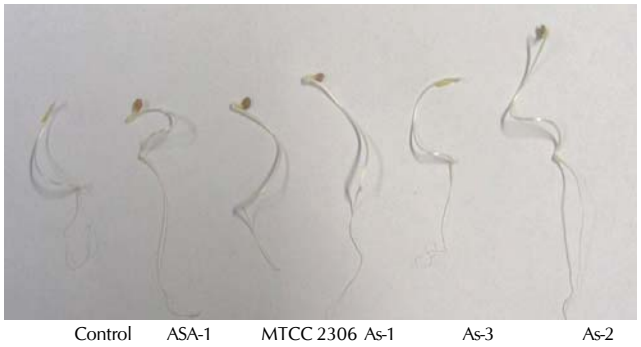

Plate 2.3: *In vitro* growth promotion effect of *Azospirillum* isolates on tomato

Azospirillum As-3 isolate (13.56 μg/ml IAA) without tryptophan and with tryptophan showed 25.85 µg/ml IAA (Table 2.5). Similar experiment was carried out by Akbari *et al*. (2007) for 25 *Azospirillum* sp. in a culture medium containing DL-Tryptophan as N source to produce IAA and detected by the Salkowski reagent by colorimetric which ranged from 29 to 761 ppm. Similarly, Mahalakshmi and Reetha (2009) screened 18 *Azospirillum* isolates amongst which 87.8% of isolates were positive for IAA production ranging from 1.23 to 3.6 μg/ml.

ACC deaminase enzyme production

Isolate As-2 and ASA-1 found positive for ACC deaminase enzyme production, utilizing ACC as source of nitrogen (Table 2.6). All the test isolates showed very poor growth on control plate having only minimal salt, whereas showed luxurious growth on plate containing $(NH_4)_2SO_4$ as nitrogen source. Plates having ACC as sole source of nitrogen, the isolates As-2 and standard ASA-1 were grown well, showing their ability to utilize ACC via production of ACC deaminase enzyme which ultimately leads to reduce the ethylene level in roots under stress conditions and helps plant to tolerate stress. The results are in corroboration with results of Shahzad *et al*., (2010) who have screened *Azospirillum, Agrobacterium, Achromobacter, Burkholderia, Enterobacter, Pseudomonas* and *Ralstonia* for ACC-deaminase which can facilitate plant growth to overcome the deleterious effects.

***In vitro* effect of *Azospirillum* isolates on tomato seed germination**

In vitro effect of *Azospirillum* isolates on seed germination was checked in laboratory. The highest germination 74 % was observed after 120 h by As-2 isolate followed by As-1 whereas As-3, ASA-1 2306 showed 50-60% influence (Table 2.7).

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