International Journal of Agriculture Sciences
ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 11, Issue 13, 2019, pp.-8692-8696.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

INFLUENCE OF *AZOTOBACTER* ON ENHANCING SEEDLING VIGOUR IN *ORYZA SATIVA*

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Received: June 25, 2019; Revised: July 11, 2019; Accepted: July 12, 2019; Published: July 15, 2019

Abstract: *Azotobacter* plays an important role in increasing the development and physical conditions of plants. It improves seed germination and has beneficial response on Crop Growth Rate (CGR) and helps to increase nutrient uptake and ultimately boost up BNF. In this study 16 rhizosphere soil samples were collected from Cuddalore and Madurai districts of Tamil Nadu. The soil physico chemical properties were analysed and totally 16 isolates were obtained. The isolates were characterized for fermentation of carbohydrates, starch hydrolysis, catalase and citrate utilization. They were screened for plant growth promoting traits such as IAA, PO_4 solubilization, gibberlic acid production and siderophore production. Among the isolates, MAZO 13 produced higher amount of IAA (6.1±0.08µg/ml);higher degree of PO_4 solubilization (8.12±0.10g/l) and gibberlic acid production (19.11±0.39µg/ml). All the isolates showed positive for siderophore production. The isolate MAZO13 significantly increased the seed germination, plant growth and vigour of the rice seedlings (with 65.8% increase) over uninoculated control. Hence this can be considered for inoculation to enhance the germination and early vigour of the rice seedlings, which will improve the survival and establishment in the main field.

Keywords: *Azotobacter*, Seed Germination, Plant Growth Promoting Traits, Vigour

Citation: Vanitha V., *et al*., (2019) Influence of *Azotobacter* on Enhancing Seedling Vigour in *Oryza sativa*. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 11, Issue 13, pp.- 8692-8696.

Academic Editor / Reviewer: Dr N Umashankar Kumar

Introduction

Feeding increasing human population with available limited resources is a major challenge for future decades. Developing better plants with the characters of high yield, growth, nutritional quality, pathogen immunity or stress tolerance is an essential approach as a solution. However genetic or agronomical improvement is a complicated process, with many regulations. As a result, crop growth -promoting microbes have received interest in sustainable agriculture in latest years as a cheap alternative to resource- consuming agrochemicals. These PGP microbes are known to produce hormones like abscic acid, gibberlins, cytokinins, auxins and enzymes like 1-aminocyclopropane 1-carboxylate (ACC) deaminase to increase the germination and to enhance the seedling vigour in rice. PGP nitrogen fixing bacteria especially *Rhizobium, Azotobacter, Azospirillum* are known to increase the plant growth by the production of phytohormones [1]. Among which *Azotobacter* sp., a heterotrophic free-living bacteria is well known to improve productivity of the non-leguminous crops *viz*., rice, maize *etc*. Rice soils are mainly N deficient [2] which has led to decrease in production of rice by 20.6%. *Azotobacter* spp. having the potency of providing 19-47% of the total N required for rice field [3,4]. Similarly, it is considered as a key component for in situ Nitrogen fortification and able to fix 10 kg of N/acre [5,6]. Besides, from being a growth promoter & N2 fixer, it had showed antagonistic activity against plant pathogens. Different species *viz*., *Azotobacter chroococcum* followed by *A.agilis, A.vinelandii, A. beijerinckii, A.insignis, A.macrocytogenes* and *A.paspali* are mainly found in the soil. The presence of *Azotobacter* sp. in soils has beneficial effects on plants, but the abundance of these bacteria is related to many factors *viz*., soil physico-chemical (e.g. organic matter, pH, temperature, soil moisture) and microbiological properties. Therefore, present study is focused mainly on isolating seedling vigour enhancing *Azotobacter* spp. From soils of different districts of Tamil Nadu and their influence on enhancing seedling vigour of rice.

Materials and Methods

Collection of soil samples

Totally 16 rhizosphere soil samples were collected from different locations of Cuddalore and Madurai districts and analysed for different soil parameters such as pH, EC, organic carbon, N,P and K content [Table-1].

Analysis of soil physico-chemical properties

Soil samples were air dried and stored at 28±2˚C. They were made ready by sieving through a 150µm size mesh for analysis. Selected physico-chemical properties were analysed using standard protocols. pH and EC estimation (Rowell, 1996) was done in 1:1 (w/v) soil: water suspension. Total nitrogen content was analysed by Kjeldahl method [7], and available P &K were estimated by Olsen and sommers, 1982; Rowell, 1996 respectively. The total organic carbon was analysed by wet oxidation method having standard as $K_2Cr_2O_7$.

Isolation of *Azotobacter* sp.

Isolation of *Azotobacter* was done from collected soil samples in Waxman 77 agar medium by serial dilution and plating method.

Morphological characterization of *Azotobacter* isolates

Morphological characterization *viz*., colony characters, pigmentation and microscopic examination of cell shape,colour [8] and Gram reaction [9] were analysed using standard protocols.

Biochemical and physiological characterization

All the isolates were subjected to different biochemical tests *viz*., fermentation of carbohydrates, starch hydrolysis, catalase test and citrate utilization test. Those isolates showing characteristic growth, pigmentation and biochemical reactions of *Azotobacter* species as described in Bergey's manual of systemic bacteriology were purified & characterized.

Fermentation of carbohydrates

Fermentation of carbohydrates was performed by using different carbon sources such as glucose, lactose and sucrose. Fermentation broth was inoculated in 5ml quantities in test tubes and the release of gas was detected in Durham´s tube after 48-72h. The appearance of colour change from red to yellow indicates positive for acid production and no colour change indicates negative.

Starch hydrolysis test

Bacterial isolates were spotted on the plate containing starch agar medium and incubated for 2 days. After 2 days of incubation the plates were tested using iodine solution as indicator[10]. Development of yellow colour surrounding the growth indicates positive reaction and blue colour indicates negative reaction

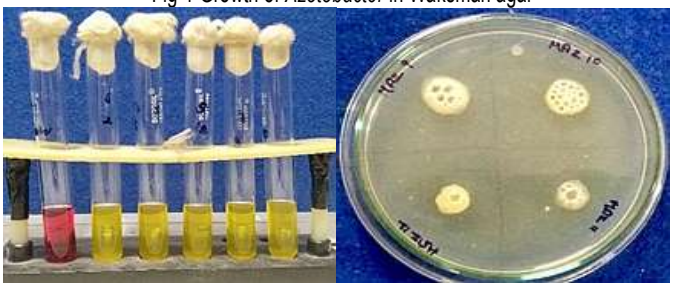
Catalase test

The bacterial isolates were grown on Waksman 77 agar plates and after 2 days of incubation a drop of 3% H_2O_2 was added over the colonies. Prompt effervescence indicates the catalase positive reaction and no effervescence indicates the negative result [11].

Citrate utilization test

Citrate utilization test was performed by streaking the isolates on Simmon´s citrate agar plates. The plates were incubated at 28±2˚C for 48h and observed for the colour change in the medium. Change of the colour from green to blue indicates positive result and no growth with green colour indicates negative result [12]

Fig-1 Growth of *Azotobacter* in Waksman agar



2A Test for fermentation of Carbohydrates 2B Catalase test

Fig-2 Biochemical Characterization

Screening of isolates for plant growth promoting (PGP) traits

The isolates were screened for several PGP traits as detailed below.

Indole acetic acid production

The cultures were grown in Waksman broth supplemented with 0.1% tryptophan and incubated at 28±2◦ C for 3 days. After incubation the cultures were centrifuged at 10000rpm for 15 min and then 2 ml of supernatant was taken to which 2 to 3 drops of 0.1 mM of Orthophosphoric acid and 4 ml of Salkowaski reagent were added and incubated for 30 min to develop colour. Development of pink colour was observed and optical density was taken at 530nm with help of spectrophotometer [13].

Phosphate solubilization

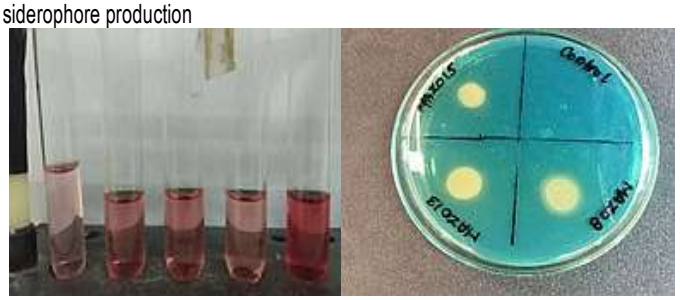
Phosphate solubilization was done according to Murphy, et al., (1962) [14]. The cultures were grown in Pikovaskaya´s broth supplemented with 0.5% tricalcium phosphate and incubated. After 7 days of incubation the broth was centrifuged for 5 min and 1 ml of the supernatant was taken followed by addition of 1 ml distilled water. After that, 2 ml of colour reagent was added and the volume was made to 6 ml by addition of distilled water and incubated for 15 min to develop colour. The intensity of blue colour developed was measured in spectrophotometer at 882nm. KH_2PO_4 was used as standard and the amount of phosphate solubilization was expressed as total p release mgl-1.

Gibberlic acid production

Gibberlic acid estimation was done according to Desai, (2017) [15]. The isolates were grown in Waksman broth and incubated for 7 days. After incubation the cultures were centrifuged at 10000 rpm for 15 mins.2 ml of supernatant was added with 2 ml of ethyl acetate and kept in shaker for 10 min. After 15 min the ethyl acetate layer was allowed to evaporate at room temperature and the remaining contents were dissolved in 1 ml alcohol. From the above content 2 ml of suspension was taken and 1 ml of DNPH reagent was added and incubated for 5 min. After 5 min,5 ml of 10% KOH was added. Development of red wine colour intensity was measured in spectrophotometer at 430nm.Gibberlic acid was used as standard and the amount of gibberlic acid production was expressed as µg /ml.

Siderophore production

Siderophore production was estimated qualitatively. Bacterial isolates were spotted on the plates containing CAS medium and incubated at 28±1◦ C for 2-3 days. Appearance of orange/reddish brown colour indicates positive for siderophore production



IAA Production Siderophore production

Fig-3 Plant Growth promoting traits by *Azotobacter* isolates

Seedling vigour test

Seedling vigour test was done by roll towel method. The Rice variety ADT 46 seeds were soaked in water for 24 h before surface sterilization and surface sterilization was done with 5% sodium hypochlorite and 70% ethanol and washed 4-5 times with sterile water. The surface sterilized seeds were soaked in *Azotobacter* broths (containing ≈ 109 cells/ml) for 4h. After incubation they were transferred to germination towel and kept undisturbed for 15 days. Simultaneously control seeds were soaked in Waxman 77 broth for 4 h and transferred to germination towel. Percent germination, total root length and shoot length of all the seedlings were measured on 15th day and the seedlling vigour index was calculated by the following formula [16]:

Vigour index= Total plant height × germination percent Results and Discussion

Physico-chemical properties of soil

The physico-chemical properties of soil samples *viz*., pH, EC, available N,P,K& organic carbon were analysed and the results obtained were tabulated . Organic carbon ranged from 0.32-0.65g/kg, minimum pH range is 5.4 whereas the maximum recorded 7.4,EC ranged from 0.08 to 0.28dSm-1,N content was

Table-1 Physico chemical properties of collected soil samples

Sampling location	Crop	Designation of the isolates	Org C g/kg	pH	EC dSm^{-1}	N kg/ha	P kg/ha	K kg/ha
Cuddalore-Ramapuram	Chilli	MAZO1	0.56	6.5	0.12	223	24	210
Cuddalore-Naduveerapattu	Sugarcane	MAZO2	0.52	6.8	0.09	216	28	216
Cuddalore-Baghur	Groundnut	MAZO3	0.38	7.2	0.15	256	32	218
Cuddalore-Nathapattu	Rice	MAZO4	0.43	6.4	0.13	196	28	212
Cuddalore-M Puthur	Sugarcane	MAZO5	0.53	6.5	0.13	188	26	119
Cuddalore-Kurinchipadi	Banana	MAZO6	0.32	5.4	0.14	182	27	122
Cuddalore-Alapakkam	Sugarcane	MAZO7	0.41	7.3	0.08	235	18	187
Madurai-Tirumangalam	Casuarina	MAZO8	0.46	7.1	0.21	220	32	310
Madurai-Kullanchavadi	Rice	MAZO9	0.65	6.9	0.25	243	17	221
Cuddalore-Uchimedu	Tapioca	MAZO10	0.43	5.8	0.23	235	13	198
Cuddalore-Nellikuppam	Groundnut	MAZO11	0.48	7.4	0.28	254	12	189
Cuddalore-Pattampakkam	Rice	MAZO12	0.46	7.2	0.28	258	14	192
Madurai-Vaadipatti	Rice	MAZO13	0.48	7.2	0.22	230	35	295
Madurai-Mangulam	Papaya	MAZO14	0.42	6.8	0.20	240	34	320
Madurai-Solavanthan	Rice	MAZO15	0.38	6.9	0.23	250	36	330
Madurai-Samayanallur	Groundnut	MAZO16	0.41	6.5	0.22	220	31	315

Table-2 Biochemical characterization of Azotobacter isolates

Isolates	Fermentation of carbohydrates			Starch hydrolysis test	Catalase test	Citrate utilization test
	Glucose	Lactose	Sucrose			
MAZO1	+	+	+	+	+	+
MAZO2	+	+	+	+	+	+
MAZO3	+	+	-	-	-	-
MAZO4	+	+	+	+	+	+
MAZO5	+	+	+	-	-	-
MAZO6	-	+	+	+	-	-
MAZO7	+	+	+	-	-	-
MAZO8	+	+	+	+	+	+
MAZO9	-	+	+	-	+	-
MAZO10	+	-	-	-	+	+
MAZO11	+	+	+	+	+	-
MAZO12	+	+	+	+	+	+
MAZO13	+	+	+	+	+	-
MAZO14	+	-	+	+	-	+
MAZO15	+	+	+	+	+	+
MAZO16	+	+	+	-	-	+

+ positive growth- Negative growth

Table-3 Testing of PGP properties of Azotobacter isolates

S	Isolates	IAA production µg /ml	Phosphate solubilization (P release g/l)	Gibberlic acid production (µg /ml)	Siderophore production
1	MAZO1	3.5±0.08^{e}	5.38±0.10^{f}	14.01±0.30^{f}	+
2	MAZO2	4.2±0.11^{c}	6.29±0.08^{d}	16.91±0.26^{c}	++
3	MAZO3	2.1±0.05^{i}	3.06±0.02^{i}	16.24±0.21^{d}	+
4	MAZO4	4.5±0.09^{b}	4.06±0.10^{h}	15.66±0.05^{d}	+
5	MAZO5	2.3±0.02^{h}	7.56±0.07^{b}	14.63±0.30^{f}	+
6	MAZO6	2.0±0.04^{i}	7.46±0.09^{b}	17.14±0.15^{c}	+
7	MAZO7	3.3±0.06^{f}	2.30±0.04^{j}	13.51±0.23^{g}	+
8	MAZO8	4.6±0.03^{b}	7.05±0.03^{c}	14.14±0.04^{f}	+
9	MAZO9	3.7±0.03^{d}	8.12±0.16^{a}	17.90±0.07^{b}	+
10	MAZO10	3.1±0.01^{g}	4.81±0.12^{g}	18.68±0.18^{a}	+
11	MAZO11	2.0±0.02^{i}	8.12±0.07^{a}	16.91±0.32^{c}	+
12	MAZO12	2.4±0.04^{h}	2.84±0.04^{i}	15.37±0.28^{e}	+
13	MAZO13	6.1±0.08^{a}	8.12±0.10^{a}	19.11±0.39^{a}	+
14	MAZO14	3.2±0.04^{f}	6.01±0.04^{e}	10.74±0.25^{h}	+
15	MAZO15	1.6±0.02^{j}	8.12±0.01^{a}	8.91±0.09^{i}	+
16	MAZO16	4.2±0.09^{c}	5.38±0.10^{f}	8.59±0.21^{i}	+
	SE(d)±	0.0837	0.116	0.327	
	CD	0.17	0.237	0.666	

+ positive growth, Values are mean (± standard error) (n=3) and column values followed by different letters are significantly different from each other at 5% LSD

recorded medium (182-256 kg/ha); P content ranged from 12-36 kg/ha and K content ranged from 119-310 kg/ha.

Isolation and identification of *Azotobacter* sp.

After 7-10 days of incubation, *Azotobacter* colonies were observed in plates, the colonies appeared light to dark brown, viscous, raised and transleucent [Fig-1]. These colonies from different plates were picked, grown in Waksman77 slants and subjected to characterization. Totally 16 isolates were recovered and designated. All isolates appeared Gram negative rods.

Biochemical and physiological characterization

Fermentation of carbohydrates

Inoculated tubes containing different carbon sources were observed for colour change and gas production. Most of the isolates *viz*., MAZO1, MAZO2, MAZO4, MAZO7, MAZO8, MAZO11, MAZO12, MAZO13, MAZO15, MAZO16 were positive for all three carbon sources tested [Fig-2a] whereas MAZO3 was negative for

Table-4 Influence of Azotobacter isolates on seedling vigour of rice (ADT 46)-15 DAS

Isolates	Root length Cm	Shoot length Cm	Germination percentage	Plant height Cm	Vigour index
MAZO1	19.6±0.50^{a}	16.0±0.12^{c}	93.3±2.1^{a}	35.6±0.31^{e}	3324±32.9^{b}
MAZO2	18.9±0.18^{e}	18.0±0.14^{d}	96.7±0.3^{a}	33.8±0.35^{f}	3573±3.7^{a}
MAZO3	17.2±0.44^{f}	12.6±0.28^{i}	96.7±0.8^{a}	29.8±0.09^{h}	2890±18^{e}
MAZO4	21.2±0.44^{c}	15.0±0.32^{e}	96.7±1.1^{a}	36.2±0.60^{c}	3501±87.4^{a}
MAZO5	18.4±0.47^{d}	15.4±0.18^{a}	93.3±0.1^{a}	36.9±0.05^{b}	3161±60.9^{c}
MAZO6	21.5±0.03^{b}	12.3±0.14^{i}	96.7±2.2^{a}	33.8±0.49^{f}	3269±62.9^{b}
MAZO7	24.2±0.09^{a}	9.6±0.02^{j}	96.7±0.2^{a}	34.3±0.55^{e}	3285±71.8^{b}
MAZO8	22.8±0.24^{a}	11.5±0.17^{l}	96.7±1.9^{a}	33.9±0.22^{f}	3320±53.6^{b}
MAZO9	16.5±0.21^{f}	12.5±0.32^{i}	96.7±1.8^{a}	29.0±0.10^{h}	2869±0^{e}
MAZO10	19.6±0.20^{d}	12.4±0.15^{i}	96.7±1.3^{a}	32.0±0.64^{g}	3097±30.6^{d}
MAZO11	16.5±0.38^{f}	13.3±0.05^{h}	96.7±0.4^{a}	29.8±0.12^{h}	2890±67.7^{e}
MAZO12	21.6±0.11^{b}	10.6±0.09^{k}	93.3±1.4^{a}	32.2±0.77^{g}	3007±67.3^{d}
MAZO13	22.6±0.16^{a}	16.5±0.39^{b}	93.3±1.0^{a}	39.1±0.16^{a}	3653±66.6^{a}
MAZO14	19.8±0.47^{d}	14.3±0.24^{g}	70.0±0.1^{b}	34.1±0.17^{f}	2390±36.1^{g}
MAZO15	21.5±0.46^{b}	14.1±0.04^{g}	73.3±0.8^{b}	35.6±0.09^{e}	2613±36.7^{f}
MAZO16	23.5±0.39^{a}	14.7±0.31^{f}	60.0±0.2^{c}	38.3±0.51^{a}	2295±58.5^{h}
Control	19.0±0.15^{d}	16.9±0.12^{b}	60.0±0.6^{c}	36.7±0.93^{c}	2204±32.9^{h}
SEd	0.56	0.31	1.71	0.714	78.8
CD	1.15	0.63	3.48	1.45	160

Values are mean (± standard error) (n=3) and column values followed by different letters are significantly different from each other at 5% LSD

sucrose utilization as a sole carbon source; MAZO6 and MAZO9 was found negative for glucose as a carbon source; Similarly, MAZO10 was found negative for lactose and sucrose and MAZO14 was found negative for lactose as a carbon source.

Starch hydrolysis test

Development of yellow colour was observed after the addition of Iodine solution in most of the plates. The isolates MAZO1, MAZO2, MAZO4, MAZO6, MAZO8, MAZO11, MAZO12, MAZO13, MAZO14, MAZO15 showed positive for starch hydrolysis and the isolates MAZO3, MAZO5, MAZO7, MAZO9, MAZO10, MAZO16 remained negative.

Catalase test

Catalase production was positive in MAZO1, MAZO2, MAZO4, MAZO8, MAZO9,MAZO10,MAZO11,MAZO12, MAZO13, MAZO15 with the formation of effervescence and the remaining isolates MAZO3,MAZO5, MAZO6, MAZO7,MAZO14,MAZO16 showed no effervescence which indicated negative result.50% isolates may not be of *Azotobacter*, since showed the negative result for catalase production.

Citrate-utilization test

After 48 hours of incubation the isolates MAZO1,2,4,8,10,12,14,15,16 were positive which showed the change of medium from green to blue. Rest of the isolates showed negative for citrate utilization [Table-2].

Plant growth promoting traits

Plant growth promoting traits *viz*., production of Indole acetic acid, siderophore and phosphate solubilization were observed in most of the isolates [Table-3]. IAA production was observed in the range of 2±0.04-6.1±0.08µg/ml with the addition of 0.1% tryptophan. Most of the isolates exhibited significant variation in IAA production, and the isolate MAZO13 produced more quantity of IAA (6.1±0.08µg/ml), which is significantly higher than other isolates [Fig-3a]. IAA production by the *Azotobacter* isolates is an inherent property of the organism and this is in agreement with the reports of other researchers who reported the production of varying quantity of IAA by *Azotobacter* with the addition of the precursor tryptophan [13,14]. Similarly, these isolates recorded GA production at varying levels(8.59µg/ml to 19.11µg/ml) and significant variation among the cultures were noted. The isolate MAZO13 recorded significantly higher production (19.11±0.39µg/ml) of GA, followed by MAZO10(18.68±0.18µg/ml) and the lesser production was noticed in MAZO16(8.59±0.21µg/ml). The higher production of Gibberlic acid by *Azotobacter* (71.42%) isolates, which is known to stimulate cell division in vegetative growth. IAA and GA production may contribute for enhanced growth of the plants is reported [17]. Phosphate solubilization potential of the isolates were evaluated and the results indicated a significant variation among the isolates to solubilize different quantities of PO_4 and release the P into the broth. Maximum P release was noticed with the inoculation of MAZO13 and MAZO15(8.12±0.04 g/l),followed by MAZO6.Minimum was reported with the inoculation of MAZO7,(2.3±0.04 g/l).Potential PGPR was able to solubilize phosphorous, and level of phosphate solubilization vary from 3.2-26.9µg/ml and the isolate AZ11 and AZ12 solubilize maximum phosphates(26.9µg/ml) [18].This PO_4 solubilization potential may be due to the production of organic acids and enzymes. Siderophores are iron chelating compounds, and this may be useful tool for assessing the potential of the isolates to increase the plant growth by augmenting iron availability as well as reduction of plant pathogens in the rhizosphere. In the present study, all the isolates showed positive for siderophore production [Fig-3b] which is corroborated with the earlier reports [19,20].

Uninoculated control MAZO13 Inoculated

Fig-4 Influence of *Azotobacter* isolates vigour of rice seedlings

Seedling vigour test

The results of the in vitro plant infection study showed that inoculation of *Azotobacter* isolates could significantly increase the growth of rice seedlings. Shoot length, root length, and vigour index were significantly boosted by the inoculation of the *Azotobacter* isolates over uninoculated control at varying levels [Table-4].Significant variation among the cultures were noted on enhancing the shoot length and root length. Root length of the seedling was ranged between 17.2-23.5 cm/plant;shoot length ranged between 11.5-18 cm/plant with the germination of 60-96.7 percent in inoculated plants. This showed the variation of inherent potential of *Azotobacter* isolates. *Azotobacter* inoculation enhanced the root length to the tune of 30%, shoot length by 6.5% and germination by 36.7% over uninoculatted control [Fig-4].

Though not much increase in shoot length was observed, a significant enhancement in seedling vigour was observed. MAZO13 showed higher vigour index of (3653), followed by MAZO1(3324),MAZO8(3320) than other isolates as well as uninoculated control (2204).*Azotobacter* inoculated plants registered upto 65% increase in seedling vigour over control plants. Enhancement in vigour index as well as germination of chilli was reported earlier with the inoculation of *Azotobacter* to a considerable extent [20]. Among the isolates tested, MAZO13 produced more vigorous seedlings than the other isolates, which may be due to the production of more quantity of IAA and GA. Auxin production increases early germination and seedling growth. Vigour of seedlings is considered important for the better establishment in the main field. Hence to improve early vigour and better establishment in rice, the *Azotobacter* isolate MAZO13 showed good scope and potential.

Application of Research: This study primarily concerns on isolates of *Azotobacter* inoculation in rice seedlings to improve early seed vigour.

Research Category: Microbiology

Abbreviations: IAA: Indole Acetic Acid, CAS: Chrome azurol S, DNPH: 2,4-Dinitrophenylhydrazine

Acknowledgement / Funding: Authors are thankful to Agricultural College and Research Institute, Madurai, 625 104, Tamil Nadu Agricultural University, Coimbatore, 641003, Tamil Nadu, India

***Research Guide or Chairperson of research: Dr K Kumutha**
University: Tamil Nadu Agricultural University, Coimbatore, 641 003, Tamil Nadu
Research project name or number: MSc Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Madurai and Cuddalore district

Cultivar / Variety / Breed name: Rice, Maize

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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