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Research Article

ISOLATION OF REGION SPECIFIC BACTERIAL STRAIN AND ITS APPLICATION ON SOME IMPORTANT CROP PLANTS IN KUMBHALMER (NORTH GUJARAT)

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Abstract- Two strains of bacteria were isolated from the local niche of kumbhalmer (NG) by the enrichment of medium. Both were identified as *Azatobactor and Rhizobium*. Both bacteria were grown in large scale in their selective medium. Bacteria were applied to the seeds by the seed inoculation techniques. Four crops were selected like Tuvar, Chana, Mustard, and Methi. Physiological character like number of leaves, shoot length, root length, dry weight and fresh weight of shoots and roots, were measured in one-week interval up to six weeks. Seeds inoculated with *Azotobactor* were comparatively more vigorous and healthy, than controlled and seeds inoculated with *Rhizobium.* Isolated strains production.

Keywords- Bacterial strain, *Azatobactor, Rhizobium,* IAA production, PGPR, Biofertilizer.

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Introduction

Under intensive farming systems, most tropical soils exhibit rapid depletion of organic matter and consequently soil nutrients. Such soils will need nutrient replenishment for optimum crop yields [1]. Intensive application of chemical fertilizers in agriculture has caused damage to the ecological state of the agricultural systems. The use of biofertilizers is an alternative to improve the conditions of agricultural fields worldwide. Biological fertilizers do not contaminate the soil and atmosphere and help to produce healthy foods [2]. Microbiological fertilizers, the alternate sources to meet the nutrient requirement, are important part of environment friendly sustainable for crops and to bridge the future gaps [3]. Seed dressing techniques with bacteria called bacterization is one of the most applied techniques for biofertilizer application [4]. Biofertilizer denotes all the nutrients inputs of biological origin for plant growth [5]. Several biofertilizer (bacterial fertilizer) are used worldwide, Phosphobacterin (contain Bacillus megaterium *var.* phosphaticum) and azotobakterin (contain *Azotobacter chroococcum*) in Este uropien countries and U.S.S.R respectively [4]. In India seed-dressing techniques have been tested on several crops, and increasedtheir yields of several crops like wheat, barley, maize, sugar beet, carrot, and potato [5]. But same kind of response was not obtained in all over India. The poor performance of biofertilizer is linked to in appropriate strains and inefficient production technology. As agro climate conditions and soil characteristics vary widely, a large range of stains of each biofertilizer need to be isolated for each area [6]. The objective of this research was to solve the problem of farmers from Kumbhalmer-Gujarat, In Kumbhalmer, farmers turn towards other crops (chana, Tuvar, mustard, and methi), but these crops gave less yield than the other villages in spite of using several biofertilizers. And to address this problem, the author isolated two regional bacterial strains and tested the same in Agricultural Biotechnology Lab, Dantiwada. The outcome of the outstanding performance of isolated bacterial strains is presented in this paper.

Materials and methods

Enrichment and isolation of Rizobium

Rhizobium (R_1) was isolated from root nodules of plant methi by the enrichment method. Medium composition: Di-Potassium Hydrogen Phosphate (K_2HPO_4): 0.5gm, Magnesium Sulphate Hepta Hydrate ($MgSO_4.7H_2O$): 0.2gm, Sodium Chloride (NaCl):0.1, Mannitol: 10gm, Yeast Extract: 1gm, Congored1% solution 2.5ml, Agar-Agar: 2%, Distilled Water: 1000ml. Care should take during the preparation to dissolve K_2HPO_4 separately in d/w, and Congored solution also be autoclaved separately and added to the medium at the time of pouring in the Petri plates (YEMA).

Raising of plant materials

Uproot, root of methi plants was brought to laboratory from agricultural field near Dantiwada, Gujarat, India. The roots were washed in running water to remove soil. Healthy pink unbroken and firm nodules were selected and were immersed in 0.1% $HgCl_2$ solution for 5 min. The nodules were washed repeatedly several times with sterile distilled water. The nodules were crushed by sterile glass rod in 1ml of sterile distilled water, and. then 1mlof suspension was placed on YEMA plates, and the plates were incubated at 26^0C for 10 days.

Enrichment and isolation of Rhizobium

The soil selected was garden soil, green house soil, dry farm soil, and wet farm soil from Kumbhalmer village. 1 gm of soil was inoculated in 100ml of nitrogen free Burk's media contained in a 250 ml flask. The flask was incubated at 30°C with vigorous agitation for 7 days. After seven days the loop full of suspension of enriched medium onto Burk's Medium and the plate was incubated at 30°C.The Isolated colony was developed as pure strain and identified by Bergey's Manual [25].

Enrichment and isolation of Azatobactor

Azatobactor was isolated by the liquid enrichment. Nitrogen free manitol broth (K_2HPO_4 – 0.5 g, NaCl – 0.2g, $FeCl_3$ – 0.003g, $MnSO_4.4H_2O$ – 0.02g, $MgSO_4.7H_2O$ – 0.2g, in 1000ml distilled water was added to20 g $CaCO_3$ before autoclaving) and was incubated with 1 gm of soil from different soil types (garden, dry field, wet field). The flasks were incubated in incubator for one week. After one week, loop full of culture from enriched medium was streaked on nitrogen free manitol agar, and incubated in incubator. From this, isolated colonies were grown on basal medium with different carbohydrate source (rhamnose, manitol, starch, sucrose and glucose) and characterized according to Krieg & Holt, (1984) [25].

Seeds collection & seed dressing

Seeds of four crops, namely Tuvar, Mustard, Methi, and Chana were collected from the Dantiwada Agricultural University, Dantiwada – Gujarat, India. Then the seeds' viability was checked by the Copeland, (1976) & Germ, (1954) [10,23]. Bacteria were pellet down by the centrifugation at 2000 rpm for 10 min. Pellets were dissolved in the minimum quantity of distilled water. And in the meanwhile, 10% of jaggery was boiled for some time. Cooled content was mixed in the distilled water containing bacterial cells, this is known as inoculum slurry and was dried in shade. A same method devoid of bacterial culture was used for the control seeds. After some time, seeds were sown in poly cups, containing sterilized garden soil and watered by distilled water. Plants were watered twice a week. The whole practical was conducted in controlled net house condition.

Screening of isolates for IAA production

Test strains of *Azotobactor* and *Rhizobium* spp. were screened for IAA production [26]. Briefly, test bacterial culture was inoculated in the respective medium (Jensen's/nutrient broth) with tryptophan (1, 2, and 5 mg/ml) or without tryptophan incubated at 28 ± 2 0C for 15 days for *Azotobactor* and 1 week for *Rhizobium* Spp. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant were mixed with 2 drops of orthophosphoric acid and 4 ml of Solawaski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 $FeCl_3$). Development of a pink colour indicates IAA.

Extraction of crude IAA

Single bacterial colonies of isolates of *Rhizobium* spp. and strains of *Azotobactor* was inoculated in 200 ml of nutrient broth amended with 1 and 5 mg/ml of tryptophan and incubated at 28 ± 2°C for 1 week on a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate at double the volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotatory evaporator at 40 °C. The extract was dissolved in 300 ml of methanol and kept at –20 °C.

Thin layer chromatography

Ethyl acetate fractions (10-20 ml) were placed on TLC plates (Silica gel G f254, thickness 0.25 mm) and developed in ethyl acetate: chloroform: formic acid (55:35:10). Spots with Rf values identical to authentic IAA were identified under UV light (254 nm) by spraying the plates with Ehmann's reagent [15].

Efficacy measurement

The efficacy of isolated bacterial strains was determined by monitoring the morphological character like, number of leaves, height of shoots, length of root, dry weight and fresh weight of shoot and root, every week for a period of six weeks.

Result and discussions

The two isolates were tested in the terms of the physiological changes for their effect on the plant growth, out of the two isolates A_1 was identified as the *Azotobacter chroococcum*, and R_1 was *Rhizobium*. Physiological change on crop (chana, Tuvar, mustard, and methi) was studied for number of leaves [Table-1]. It was seen that in all selected crop, plants inoculated with bacterial culture grew faster than control one. Root length [Fig-1] and shoot length [Fig-2] was also showed significant difference among bacterial inoculated and control. Shoot length of Tuvar and chana plants inoculated with A1 were taller than plants inoculated with R1. But in the case of mustard, R1 gave better result than the A1. Root length of chana, mustard, and Tuvar were showed better growth compared to the plants inoculated with R1. But in contrast, methi plants had longer root than the plants inoculated with A1. This might be because of the host specific strain response, because R1 was isolated from methi plant. One significant observation was noticed in our experiment was that no nodulation was observed in case of methi inoculated with R1, the probable reason for this was better discussed by R. C. Duby. The concentration of inorganic nutrients in soil, soil temperature, light and shading condition to plant, and CO_2 concentration in atmosphere [4].

Table-1 *Physiological change on crop (chana, Tuvar, mustard, and methi) was studied of number of weeks*

Name of Plant	Treatment	Number of weeks					
		1	2	3	4	5	6
Tuvar	A_1	3.3	6	8.6	12.3	13.6	13.6
	R_1	4	6.6	8.6	12.3	13.6	14.3
	Control	2.6	4.6	5.6	7.3	8.6	8.6
Methi	A_1	2	6.3	17.3	24	28.3	28.3
	R_1	2	9	17.6	23.3	27	27
	Control	2	2	9.6	15	17.3	17.3
Mustard	A_1	2	2.3	4.6	7.3	8.0	8.0
	R_1	2	3.6	5.6	8.6	8.6	8.6
	Control	2	2	4.3	6.3	6.3	6.3
Chana	A_1	4	8.6	13.6	19.3	25	27.3
	R_1	4.6	9	14	19.6	24.3	24.6
	Control	3	6	10	15.3	19.6	19.6

Table-2 *Root fresh weight and dry weight of Tuvar*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.3	0.02	0.2	0.01	0.08	0.01
2	0.85	0.06	0.5	.005	0.2	0.02
3	1.2	0.09	0.75	0.06	0.27	0.03
4	1.3	0.15	0.82	0.09	0.33	0.03
5	1.3	0.17	0.88	0.10	0.36	0.04
6	1.4	0.17	0.9	0.10	0.37	0.04

The fresh weight of the shoot and dry weight was also showed significant difference among inoculated and uninoculated plants of all four selected crops. [Table-2 & 3] represents the root and shoot dry weight and shoot dry weight, respectively. A Tuvar plant inoculated with A1 was growing very fast compared to the plants inoculated with R1. [Table-4 & 5] represent the result of isolated inoculum strains for the mustard. [Table-6 & 7] shows outcome of experiment with methi plants, and in this case R1 proved to be superior to A1, and control Chana plants inoculated with R1 showed higher fresh weight of roots [Table-8] and higher fresh weight of shoots [Table-9], than the plants inoculated with A1. But in case of dry weight result was quiet opposite, dry matter increased with the plants inoculated with A1.

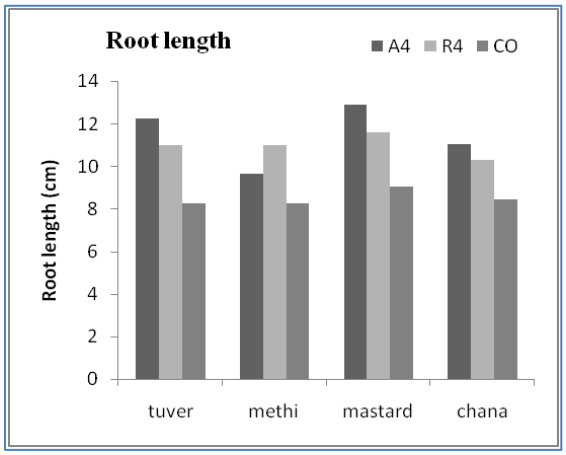

Fig-1 Root length

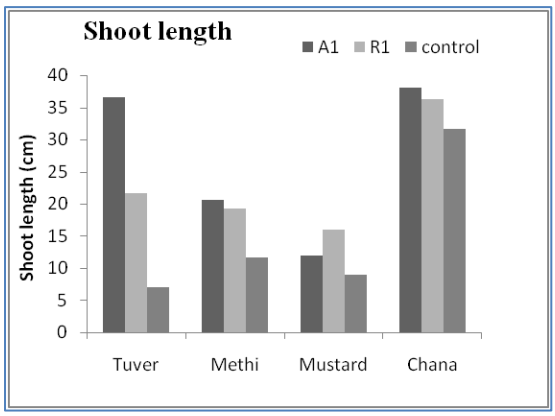

Fig-2 Shoot length

Table-3 *Shoot fresh weight and dry weight of Tuvar*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.29	0.08	0.30	0.07	0.21	0.04
2	0.60	0.11	0.60	0.14	0.37	0.06
3	0.76	0.22	0.77	0.23	0.42	0.09
4	0.85	0.31	0.90	0.30	0.43	0.12
5	0.85	0.34	0.91	0.32	0.45	0.14
6	0.87	0.35	0.91	0.33	0.45	0.14

Table-4 *Root fresh weight and dry weight of mustard*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.14	0.01	0.11	0.01	0.08	0.009
2	0.33	0.09	0.27	0.11	0.25	0.09
3	0.62	0.18	0.43	0.16	0.36	0.12
4	0.89	0.2	0.56	0.19	0.36	0.13
5	1.04	0.22	0.78	0.20	0.41	0.14
6	1.07	0.25	0.83	0.21	0.42	0.14

Several authors have tried such a kind of experiment in the field as well as laboratory level and they found that plant inoculated with appropriate strain will definitely give good response. Das, HK reported a yield increase in wheat, rice, maize, sorghum, potato, tomato, cauliflower, carrot, sugarcane and cotton with application of Azotobacter [31]. Shende & Apte reported increase in the yield of cotton, maize, and sour gum, inoculated with *Azotobacter chroococcum* [32] and is in agreement with our isolated A1 strains which showed healthy development of plants. Similarly, *Rhizobium* spp. was also studied by several authors and found positive increase in the yield, Rewari reported yield increase on *Cajanus cajan*, *Cicer aritinum*, *Lens culinaris*, and *Vigna munga* [29]. Suba Rao and Tilak also reported the efficiency of *Rhizobium* culture as the biofertilizer, on *Triticum aestivum*, *Oryza sativa*, in different locations of India [5].

Table-5 *Shoot fresh weight and dry weight of mustard*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.2	0.02	0.23	0.01	0.19	0.01
2	0.5	0.05	0.59	0.04	0.32	0.02
3	0.8	0.07	0.72	0.06	0.46	0.04
4	1.1	0.11	0.88	0.08	0.52	0.05
5	1.2	0.18	0.91	0.10	0.56	0.06
6	1.2	0.19	0.91	0.11	0.56	0.06

Table-6 *Root fresh weight and dry weight of methi*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.04	0.008	0.08	0.01	0.03	0.006
2	0.09	0.01	0.2	0.01	0.07	0.008
3	0.16	0.022	0.3	0.03	0.15	0.01
4	0.22	0.03	0.37	0.04	0.20	0.02
5	0.33	0.03	0.53	0.05	0.26	0.03
6	0.38	0.04	0.29	0.05	0.28	0.03

Table-7 *Shoot fresh weight and dry weight of methi*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.07	0.006	0.08	0.01	0.05	0.006
2	0.13	0.02	0.17	0.03	0.13	0.02
3	0.33	0.06	0.41	0.08	0.25	0.05
4	0.57	0.08	0.69	0.12	0.37	0.06
5	0.64	0.10	0.79	0.14	0.44	0.07
6	0.67	0.11	0.80	0.13	0.45	0.08

Table-8 *Root fresh weight and dry weight of chana*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.06	0.01	0.1	0.01	0.03	0.01
2	0.11	0.02	0.27	0.02	0.08	0.01
3	0.23	0.05	0.49	0.05	0.12	0.02
4	0.43	0.07	0.64	0.06	0.20	0.03
5	0.50	0.08	0.73	0.07	0.25	0.03
6	0.54	0.08	0.8	0.07	0.29	0.03

Table-9 *Shoot fresh weight and dry weight of chana*

Number of week	Treatment					
	A_1		R_1		Control	
	FW	DW	FW	DW	FW	DW
1	0.32	0.03	1.02	0.07	0.18	0.02
2	0.48	0.06	1.43	0.11	0.27	0.07
3	0.95	0.10	1.90	0.19	0.31	0.10
4	1.11	0.17	2.22	0.33	0.43	0.14
5	1.20	0.18	2.24	0.41	0.46	0.17
6	1.20	0.24	2.26	0.42	0.50	0.21

Both isolated bacterial strain *Rhizobium* and *Azotobacter chroococcum*, performed better over the control experiment. These tremendous differences between inoculated and un-inoculated plants focused on some biochemical secretion from bacterial strain which is responsible for such result. Therefore, both these strains were studied for the IAA production. Out of the two isolated strains A_1 showed positive result for the IAA production, which is responsible for the plant growth and development. Farah Ahmad et al reported that indigenous Azotobacter produced IAA [17], R_1 was also for the IAA detection. *Azotobacter* [8] and *Rhizobium* [33] also reported about the production of gibberellin, one of the important plant growth hormones responsible for several physiological and developmental processes in plants [11,12], like seed germination, seedling emergence, stem and leaf growth, floral induction and flower and fruit growth [24,28,30]. Gibberellins are also implicated in promotion of root growth, root hair abundance [7]. These secreted chemicals help in the plant growth and development, which was absent in the controlled condition. Applied seed dressing techniques were previously used in the field at IARI with *A.chroococcum* on cotton and sorghum and yield was increased by 38 percent and 27 percent respectively [4]. For *Rhizobium* also seed-dressing techniques proved to be best, under the all India Co-ordinated pulse improvement research program at IARI [4]. In our experiment seed dressing with isolated bacteria performed well. *Azotobacter* and *Rhizobium* as a free-living nitrogen fixer & symbiotic nitrogen fixer respectively, all agricultural scientists have been studying this for a long time to look for effective strain. Galiana and colleagues studied effective strain of *Rhizobium* for the acacia. They have isolated some highly effective strains that showed their superiority in glasshouse test [19-22]. Friedericks, J B *et al*., also isolated effective *Rhizobium* strain form clover species [18]. *Azotobacter* also studied long back for its application in different soil condition [9,13,14,16,27]. Like that in present data this bacterial strain also showed good response in green house conditions, which is a good sign for the further field trials.

The present work will set up a strong foundation for future application of these isolates and its application in the field of Kumbhalmer village. For both the isolated strains, growth medium for mass cultivation was standardized and in a very short time it will be ready for the application for the tested four crops. Besides, our isolated strains may meet the farmer's need and thus reduced the need for chemical fertilizer. This then will be a great service to the environment.

Application of research: Results of the present work is highly beneficial for the farmers who want to increase fertility of their soil and

Research Category: Bacterial strain, Biofertilizer.

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