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Studies on the Response of Bacterial Biofertilizers on Growth Characteristics of *Jatropha Curcas*

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Abstract: **Biofertilizer producing microorganisms *Azospirillum brasilense* and *Azotobactoer vinelandii were isolated* from coastal area agricultural field plant rhizosphere soil in Puthumayakulam at Ramnad District. In these two bacterial species were used to determine the effect of their IAA on *Jatropha curcas* plant by growth parameter measurement. The biochemical constituents such as protein, proline and soluble sugar in plant leaf samples were analyzed. The physio-chemical parameters from bacterial biofertilizers inoculated soil samples were determined.**

Keywords: **Azospirillum Brasilense, Azotobactoer Vinelandii, Indole Acetic Acid, Jatropha Curcas.**

1. INTRODUCTION

The introduction of chemical fertilizers in the last century, farmers were happy of getting increased yield in agriculture in the begining. But slowly chemical fertilizers started displaying their ill-effects, such as leaching out, and polluting water basins, destroying micro-organisms and friendly insects, making the crop more susceptible to the attack of diseases, reducing the soil fertility and thus causing irreparable damage to the overall system. A number of intellectuals throughout the world started working on the alternatives and found that biofertilizers can help in increasing the yield without causing the damage associated with chemical fertilizers.[1] Bacteria of genus *Azospirillum* consist of seven species i.e *Azospirillum brasilense*, *Azospirillum lipoferum*, *Azospirillum amazonense*, *Azospirillum irakenes*, *Azospirillum halopraeferens*, *Azospirillum dobereinerae*, *Azospirillum largimobile*. *Azospirillum* species belong to the facultative endophytic diazotrophs group, microaerophilic, N_2 fixing and gram –ve rods. Some microorganisms of soil, like *Azospirillum* sp, *Azotobacter* sp, *Enterobacter* sp, etc., have shown to encourage plant growth, by promoting the outbreak of secondary roots. Bacteria of the genus *Azospirillum* have been isolated from the rhizosphere and roots of a variety of plants including cereals and grasses. *Azotobacter paspali* which was first described by Dobereiner and Pedrosa has been isolated from the rhizosphere of *Paspalumnotatum*, a tetraploid subtropical grass and is highly host specific [2],[3]. *Azospirillum* can produce *in vitro* phytohormones IAA, Gibberellins, Cytokines and ethylene. Some times, external application of synthetic hormones or hormones purified from bacterial culture imitated the positive effects of *Azospirillum* on root development and morphology [4].*Azotobacter* naturally fix atmospheric nitrogen in the rhizosphere. some strains have higher nitrogen fixing ability than others. Besides, nitrogen fixation, *Azotobacter* also produce Thiamine, Riboflavin, Nicotine, Indole acetic acid and Gibberellin. When *Azotobacter* is applied to seeds, seed germination is improved to a considerable extent [5]. In this present investigation bacterial Biofertilizers isolated from rhizosphere of coastal agricultural plants subjected for their production of IAA and screened for their Biofertilizer effect of Jatropha plant.

2. MATERIALS AND METHODS

Isolation of Azospirillumsp and Azotobactersp:

The root and rhizosphere soil samples were collected from coastal area agricultural field in Puthumayakulam at Ramnad District and washed to remove adhering soil particles and it was cut into small pits of 1 to 2 cm size. The root bits were first surface sterilized using mercuric chloride (0.1%) for one minute and followed by 80% alcohol for one minute. The root bits were washed in sterile distilled water to remove the excess of chemicals. The bits were taken aseptically transferred to test tubes (1-2 root bits / test tubes) containing 5ml of nitrogen free semi solid malic acid medium (NFB) and incubated for 3-5 days at 30ºC. The positive tubes were observed for the development of small white subsurface pellicle in the medium with colour change from yellowish green to blue. For *Azotobacter*sp the Soil samples were added into a bottle having medium containing glucose (N_2-free glucose medium) as a carbon source and incubated at 30ºC for seven days. After incubation, the wet mount of slide was prepared from the surface. The visible organisms were prepared in agar plate of the same medium and streak the agar surface with surface growing culture. Plates were incubated for another seven days. The wet mount of the culture were prepared and observe under microscope.The colonies growth on Nitrogen free glucose agar medium also was observed.

Extraction and Analysis of IAA from Azospirillum brasilense and Azotobacter vinelandii:

Both bacterial cultures (200ml) were centrifuged at 7,700rpm for 30 minutes. The supernatant was reduced to 50ml by evaporation under vacuum by using rotary vaccum evaporator (Digital Diagonal) and extracted into ethyl acetate and n-butanol fraction. Then n-butanol fractions were separated and evaporate under vacuum. Then redissolve in absolute methanol. The extracts were filtered though 0.45µm membrane filter. This filterate was

used to thin layer chromatography analysis and high pressure lipid chromatography.

TLC and HPLC Analysis of IAA:

The extracts of both cultures were run on silica gel plates (0.05 mm thick). Solvent systems was chloroform : Ethyl acetate: Formic acid (50:40:10 [vol/vol]) to separate indole compounds. After sample run it was allowed to dry. Then plates were sprayed by Salkowski reagent for the detection of indole compounds. The development of bulish pink colour spot and Rf value between 0.1-1.0 indicates the presence of IAA. Further the HPLC chromatogram was produced by injecting 5 to 10µl of the filtered extracts of both bacterial cultures into a 10µl reverse phase column. (Water associates µ bondapak C18 4 nm by 30 cm) in a water associates liquid chromatography equipped with a differential ultra violet detector absorbing at 254 nm. The solvent systems was used as water:acetonitrile: acetic acid (85:15:1 [vol/vol]), flow rate was 1.5 ml/min and operating pressure was 1,400 lb/in2 (95atm). The presence of IAA was identified by retention times for peaks and peak hights. HPLC analysis was done at science instrumentation centre ANJA College, Sivakasi, Tamil Nadu.

Effect of Microbial Biofertilizers on morphological parameters of Jatropha curcas:

Jatropha curcas plant seeds were collected at Government Agriculture College Melur, Madurai, Tamil Nadu and surface sterilized. The seeds were soaked in broth cultures of *Azospirillum brasilense* and *Azotobacter vinelandii* and coculture of both as separately allow to germination. The germinated seeds were sown in 1 liter pots cotaining 750 g sterilized sandy loam soil. Each pots were inoculated with bacterial suspension in the order of *Azospirillum brasilense, Azotobacter vinelandii* and coculture of both in separate pots. The number of cells suspension amounted to 10^8 – 10^9 (c.f.u) per ml were added for every one week. The growth parameters such as plant height, stem height, leaf length, and leaf count was taken for every week.[6]

3. RESULTS

Isolation of Azospirillum brasilense and Azotobacter vinelandii:

The nitrogen free semi solid malic acid medium was inoculated with coastal area agricultural field plant rhizosphere soil and root sample and isolated colonies were observed with watery drop like, white subsurface pellicles as *Azospirillum brasilense* .When the nitrogen free glucose medium was inoculated with rhizosphare soil, the moist mucoid colonies were observed as *Azotobacter vinelandi* .

Indole acetic acid analysis in Azospirillum and Azoto- bacter:

The test cultures were undergone extraction process and then the indole acetic acid (IAA) production in both cultures were identified by proceeding TLC and HPLC analysis.

Estimation of IAA production:

The *Azospirillum brasilense* and *Azotobacter vinelandii* cultures were separately inoculated into 1ml of Salkowski reagent to determine the IAA production. After incubation in darkness, a red colour was observed. The intensity of red colour was read in spectrophotometer at 530 nm. The *Azospirillum brasilense* inoculated broth has given OD values as 0.033 and *Azotobacter vinelandii* inoculated broth has shown optical density values as 0.013.

Biofertilizer effect of Azospirillum brasilense and Azotobactor vinelandii on Jatropha curcas:

In general, all the growth measurement showed greater values in *Jatropha curcas* co-inoculated with *Azospirillum brasilense* and *Azotobacter vinelandii* when compared to individual inoculation of *Azospirillum brasilense* and *Azotobacter vinelandii*. This results indicates that the growth characteristics were improved by co-culture of *Azospirillum brasilense* and *Azotobacter vinelandii*. Similarly LAR and LWR, RSR, SLA also showed positive relationship with co-inoculated plant growth (Table 1, 2, 3, 4 and 5). And Biochemical components of protein, soluble starch and proline of *Jatropha curcas* were estimated (Table 6).

Table1. Effect of Microbial Biofertilizers on the Total Biomass of Jatropha Curcasat 15 Weeks of Treatment

Biomass	Uninoculated	*A.vinelandii* Inoculated	*A.brasilense* Inoculated	*A.v +A.b* Co-inoculated
Stem dry weight (g)	1.038 ± 0.047	1.599 ± 0.241	2.022 ± 0.036	3.073 ± 0.118
Leaf dry weight (g)	0.586 ± 0.130	0.636 ± 0.065	0.762 ± 0.007	0.740 ± 0.174
Root dry weight (g)	1.025 ± 0.007	1.296 ± 0.055	1.426 ± 0.170	2.293 ± 0.177
Total dry weight (g)	4.242 ± 0.226	6.769 ± 0.183	8.555 ± 0.154	11.246 ± 0.255

± - Standard error of triplicates, *A.v +A.b*: *Azotobacter vinelandii* and *Azospirillum brasilense* co-inoculated

Table2.Various Growth Parameter Ratio of Uninoculated Jatropha Curcas

Growth parameter ratio	Days of Treatment				
	20 days	40 days	60 days	80 days	100 days
LAR	14.340 ± 0.297	29.063 ± 0.060	42.520 ± 0.192	58.164 ± 0.170	62.220 ± 0.191
RSR	0.158 ± 0.066	0.273 ± 0.038	0.428 ± 0.025	0.238 ± 0.041	0.418 ± 0.016
SLW	0.005 ± 0.001	0.007± 0.001	0.012 ± 0.000	0.016 ± 0.001	0.023 ± 0.005
LWR	0.085 ± 0.008	0.173 ± 0.017	0.226 ± 0.045	0.342 ± 0.037	0.468 ± 0.027
SLA	14.176 ± 0.305	29.206 ± 0.296	43.463 ± 0.203	58.135 ± 0.296	62.492 ± 0.436
SHWR	3.919 ± 0.033	11.728 ± 0.327	17.365 ± 0.448	23.550 ± 0.260	26.370 ± 0.360

± - Standard error of triplicates, *A.v +A.b* : *Azotobacter vinelandii* and *Azospirillum brasilense* co-inoculated LAR – Leaf area ratio, RSR – Root shoot ratio, SLW – Specific leaf weight, LWR – Leaf weight ratio, SLA – Specific leaf area, SHWR – Stem-height weight ratio.

Table3.Various Growth Parameter Ratio of Azotobacter Vinelandii Inoculated Jatropha Curcas

Growth parameter Ratio	Days of Treatment				
	20 days	40 days	60 days	80 days	100 days
LAR	16.360 ± 0.405	31.083 ± 0.130	48.350 ± 0.434	65.081 ± 0.072	81.359 ± 0.060
RSR	0.138 ±0.006	0.250 ± 0.020	0.401 ± 0.002	0.526 ± 0.023	0.659 ± 0.023
SLW	0.002 ± 0.001	0.004 ± 0.000	0.104 ± 0.007	0.012 ± 0.002	0.016 ± 0.040
LWR	0.046 ± 0.005	0.095 ± 0.002	0.141 ± 0.001	0.175 ± 0.014	0.267 ± 0.039
SLA	16.160 ± 0.144	32.350 ± 0.307	47.001 ± 0.002	65.181 ± 0.217	81.227 ± 0.226
SHWR	4.143 ± 0.125	9.541 ± 0.052	14.313 ± 0.099	19.007 ± 0.013	23.540 ± 0.468

± - Standard error of triplicates, *A.v+A.b*: *Azotobacter vinelandii* and *Azospirillum brasilense* co-inoculated LAR – Leaf area ratio, RSR – Root shoot ratio, SLW – Specific leaf weight, LWR – Leaf weight ratio, SLA -Specific leaf area, SHWR – Stem-height weight ratio.

Table4.Various Growth Parameter Ratio of Azospirillum Brasilense Inoculated Jatropha Curcas

Growth parameter ratio	Days of treatment				
	20 days	40 days	60 days	80 days	100 days
LAR	16.543 ± 0.013	23.181 ± 0.222	49.410 ± 0.3602	66.333 ± 0.178	80.222 ± 0.216
RSR	0.101 ± 0.089	0.223 ± 0.040	0.702 ± 0.002	0.796 ± 0.045	0.915 ± 0.013
SLW	0.002 ± 0.000	0.005 ± 0.001	0.014 ± 0.004	0.012 ± 0.002	0.036 ± 0.007
LWR	0.011 ± 0.002	0.036 ± 0.007	0.144 ± 0.017	0.178 ± 0.003	0.199 ± 0.001
SLA	16.660 ± 0.118	33.188 ± 0.233	49.331 ± 0.3350	66.370 ± 0.170	82.402 ± 0.355
SHWR	4.747 ± 0.050	9.313 ± 0.272	14.155 ± 0.130	18.106 ± 0.115	22.544 ± 0.505

± - Standard error of triplicates, *A.v+A.b*: *Azotobacter vinelandii* and *Azospirillum brasilense* co-inoculated LAR – Leaf area ratio, RSR – Root shoot ratio, SLW – Specific leaf weight, LWR – Leaf weight ratio, SLA – Specific leaf area, SHWR – Stem-height weight ratio.

Table5.Various Growth Parameter Ratio of Azospirillum Brasilense and Azotobacter Vinelandii Co-Inoculated Jatropha Curcas

Growth parameter ratio	Days of treatment				
	20 days	40 days	60 days	80 days	100 days
LAR	19.310 ± 0.121	38.176 ± 0.210	57.409 ± 0.548	76.766 ± 0.408	82.560 ± 0.112
RSR	0.160 ± 0.012	0.334 ± 0.010	0.034 ± 0.016	0.601 ± 0.020	0.921 ± 0.020
SLW	0.033 ± 0.011	0.006 ± 0.001	0.008 ± 0.002	0.016 ± 0.005	0.080 ± 0.005
LWR	0.036 ± 0.006	0.073 ± 0.005	0.137 ± 0.059	0.156 ± 0.020	0.177 ± 0.038
SLA	19.310 ± 1.625	38.350 ± 0.106	57.316 ± 0.269	76.606 ± 0.533	82.541 ± 0.505
SHWR	3.576 ± 0.146	7.235 ± 0.237	11.322 ± 0.191	14.626 ± 0.560	17.706 ± 0.221

± - Standard error of triplicates, *A.v+A.b*: *Azotobacter vinelandii* and *Azospirillum 0brasilense* co-inoculated LAR – Leaf area ratio, RSR – Root shoot ratio, SLW – Specific leaf weight, LWR – Leaf weight ratio, SLA – Specific leaf area, SHWR – Stem-height weight ratio.

Table6.Estimation of Protein, Soluble Starch and Proline of Jatropha Curcas

Biochemical components (mg/ml)	Uninoculated	Azotobacter vinelandii Inoculated	Azospirrillum brasilense Inoculated	*A.v+ A.b* co-inoculated
Protein	0.282 ± 0.053	0.743 ± 0.086	0.660 ± 0.020	0.940 ± 0.070
Sugar	0.063 ± 0.005	0.116 ± 0.011	0.076 ± 0.005	0.210 ± 0.017
Proline	0.075 ± 0.007	0.136 ± 0.063	0.081 ± 0.010	0.126 ± 0.030

± - Standard error of triplicates, *A.v+A.b- Azotobacter vinelandii* and *Azospirillum brasilense* co-inoculated A.v +A.b- *Azotobacter vinelandii* and *Azospirillum brasilense* co-inoculated

The leaf area ratio (LAR) of *Azospirillum* and *Azotobacter*co-inoculated *Jatrophacurcas* plant showed significant greater values (82.560 ± 0.112 for 100 days) (Table 5) when compared to *Azotobacter* and *Azospirillum* separately inoculated plant. Root shoot ratio (RSR) of *Azotobacter* co-inocuated plant showed greater values (0.921±0.020 for 100 days) (Table 5). Specific leaf weight (SLW) of *Jatrophacurcas* plant showed greater values (0.023±0.005 for 100 days) (Table 2) in the uninoculated plant. Leaf weight ratio (LWR) of *Jatropha curcas* plant showed greater value (0.468±0.027 for 100 days) (Table 2) in uninoculated plant. Specific leaf area (SLA) values (82.541±0.505 for 100 days) (Table 5) were greater in co-inoculated one followed by *Azospirillum* inoculated and *Azotobacter* individually inoculated and low in uninoculated plant. Stem-height weight ratio (SHWR) had a greater value (22.544±0.505 for 100 days) (Table 4) in the *Azospirillum brasilense* inoculated plant followed by uninoculated and *Azotobacter* inoculated and the low in co-culture inoculated plant.

4. DISCUSSION

Biofertilizers are natural fertilizers or microbial inoculants of bacteria, algae, fungi alone or in combination that augment the availability of nutrients to the plants. Their preference to chemical fertilizers offers economic and ecological benefits by the way of soil health and fertility to farmers. The bacteria *Azospirillium brasilense*and *Azotobacter vinelandii* has biofertilizer activities and these facilitate the uptake of plant nutrients, increase the nitrogen content of plants through symbiosis

and improve the plant growth by providing plant growth promoting substance. [7]. In the present study *Azospirillum brasilense* and *Azotobacter vinelandii* were isolated from costal area agricultural field rhizosphere soil and root sample in Puthumayakulam at Ramand District and maintain in nitrogen free malic acid medium and nitrogen free glucose medium.

Azospirillum has been isolated from leaves of marine mangrove plants and roots of several field-grown graminaceous plants like *Eleusinecoracona* and *Setariaitalica*. Martyniuk, 2003, isolated *Azotobacter*s pp. from different regions soils of Poland with the help of nitrogen –free glucose agar medium. [8]

Though the present study in TLC analysis results of R_f value 0.85 and pinkish blue colour spot and in HPLC analysis results of 8.5 minute retention time were confirmed as Indole acetic acid were produced by both *Azospirillum brasilense* and *Azotobacter vinelandii*. The indole acetic acid production by *Azospirilum brasilense* are 0.033 OD value and for *Azotobacter vinelandii* are 0.013 OD value were measured by UV-spectrophotometer.

Azospirillum brasilense and *Azotobacter vinelandii* produce Indole acetic acid from tryptophan. The IAA productions were confirmed by TLC and HPLC and bioassay. They TLC results with pinkish blue colour spot and R_f value about 0.8 and the peak at 8.5 min of retention time in HPLC analysis. [9] [10].

In this present study *Azospirillum brasilense* and *Azotobacter vinelandii* co-inoculated *Jatropha curcas* plant has shown increased growth parameters such as plant height, stem height, root length, leaf length, number of leaf when compared to plant which inoculated with *Azospir-illum* and *Azotobacter* separately. Leaf area ratio (LAR), Leaf weight ratio (LWR), Root shoot ration (RSR), Shoot length area (SLA), leaf area ratio (LAR) showed positive relationship in co-inoculated field plant growth.

Biofertilizer activity of *Azotobacter* and effective microorganisms and humus in *Jatropha curcas* plant. They reported that the grown activities of plant height, number of leaf , shoot base, diameter, fresh mater weight of root and shoot and dry matter weight significant fresh mater weight were recovered in *Azotobacter* and humus. [11]

Generally, *Azospirillum, Azotobacter* and other biofertilizers which improve soil properties. Further *Azospirillum* increase availability nitrogen about 2 $kaha^{-1}$ through fixation and favourably influenced the soil physical and chemical and biological fertility over the inorganic which in turn paved way for better crop yield and quality [12]. Both *Azospirillum* and *Azotobacter* has the good biofertile capacity. Hence when co-inoculation it leads good growth of *Jatrophacurcas* plant and improve the soil properties.

5. Conclusion

From this study it was concluded that, we can imrove the biodiesel producing *Jatropha curcas* growth and yield by using coinoculation of *Azospirillum brasilense* and *Azotobacter vinelandii*. However, further detailed studies are warranted to understand the nitrogenase activity and ecological significance of bacterial fertilizer on germination and physiological characteristics of *Jatropha curcas*.

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