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Assessment of Plant Growth Promoting Parameters of *Azotobacter* for their Contribution to Yield of Chili (*Capsicum annum*) through Path Coefficient Analysis

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ABSTRACT

The plant growth promoting (PGP) traits of *Azotobacter* were evaluated in the present study for their direct influence on crop yield and indirect influence through interaction with yield attributing parameters like root & shoot biomass, chlorophyll content of leaves, fruit weight, and fruit number per plant of chili (*Capsicum* annum). Significant variations were found among 10 studied isolates of *Azotobacter* when PGP traits like Acetylene Reduction Assay -ARA (for nitrogenase activity), Indole-3-acetic acid (IAA) production, phosphate solubilization, and siderophore production, were estimated. Among them, isolates VIBAZOTO08 and VIBAZOTO01 exhibited the highest nitrogenase activity, while VIBAZOTO03 recorded the highest IAA production. Siderophore production was notably high in VIBAZOTO08 and VIBAZOTO10, and several isolates showed effective phosphate solubilizing ability. Inoculation of chili seedlings with these isolates significantly enhanced growth parameters, chlorophyll content, and yield, with VIBAZOTO08 and VIBAZOTO03 outperforming others. Correlation and regression analyses indicated strong positive relationships between yield and attributes such as number of fruits per plant, root biomass, and chlorophyll-a content. Path coefficient analysis revealed phosphate solubilization and IAA production as the major contributors to yield, both directly and through indirect pathways involving key physiological traits. The findings highlight the potential of select *Azotobacter* strains as effective bioinoculants to promote sustainable chili cultivation through multifaceted growth promotion mechanisms.

Introduction

Sustainable agriculture is most relevant subject for present-day agricultural, ecological and economical scenario. According to FAO (2014), sustainable agriculture may be defined as a system of farming that meets current food and fibre needs without compromising the ability of future generations to meet their own needs. It integrates agriculture, environment health, economic profitability and social equity. This practice, with special emphasis on organic amendment, Integrated Nutrient Management, Integrated Pest Management helps in soil fertility and enhances biodiversity (Pretty *et al.,* 2018).

Reduction of environmental footprint of farming and promotion of ecosystem resilience may be achieved through this practice. Small farm holders may be able to minimize the cost of production and improve profitability (Altieri, 2002). Lal (2004) was believed that sustainable agriculture may mitigate greenhouse gas emission through cover cropping, agroforestry, and reduced tillage. Diversified cropping system may enhance adaptive capacity of agro-ecosystem with changing climate and environmental stress (Vermeulen *et al.,* 2012). Social equity may be promoted through sustainable agriculture by empowering farming communities with knowledges and skills.

For sustainable agriculture, *Azotobacter* may demand its special importance for its primary role in biological nitrogen fixation in free-living conditions thus reducing our dependence on synthetic nitrogen fertilizers in agriculture (Mrkovacki and Milic, 2001). For this reason, *Azotobacter* is used as a partial chemical nitrogen substitute for different crops (Sumbul *et al.,* 2020; Bastakoti *et al.,* 2024). Chennappa *et al.,* (2017) reviewed its application in sustainable agriculture. This diazotrophic bacteria is designated as Plant Growth Promoting Rhizobacteria (PGPR) due to its additional plant growth-promoting activities like the production of phytohormones (Wani *et al.,* 2010; Spaepen *et al.,* 2007), phosphate solubilization (Rodríguez and Fraga, 1999) and siderophore production (Ahmed and Holmström, 2014). Siderophore, an iron-chelating compound, promotes iron acquisition in iron-limiting conditions and inhibits the growth of soil-born plant pathogenic microbes. Reports are available for plant growth promoting activities of this diazotrophic bacteria through production of antifungal compounds and enhancing then plant to develop natural resistant against stress condition (Hernandez *et al.,* 2000). These properties of *Azotobacter* make it especially important to use as a biofertilizer for enhancing nutrient uptake, stimulating of plant vigour, and improving crop productivity (Kundu *et al.,* 2009). Selection of the proper *Azotobacter* strain for the formulation of biofertilizer is an important task and for this selection, the estimation of relative contribution of nitrogen fixation, IAA production, phosphate solubilization and siderophore production is essential so that we can emphasize the characters accordingly.

In agricultural statistics Path coefficient, originally developed by Wright (1921), analysis not merely indicates that which variable is closely linked to yield, it, in addition, also indicates casual relationships among the different variables. Actually, it visualizes that a particular trait how much influences the economic trait directly and also indirectly through other traits (Dewey and Lu, 1959). This method is generally applied to estimate the direct and indirect effects of yield-attributing parameters of the crop on yield. It was shown by Kang *et al.,* (1983) that traits, like ear weight and number of kernels per row had significant direct and indirect effects on the grain yield in maize. It was also shown that in wheat, traits like the number of grains per spike and 1000 grain weight imparted a significant positive effect on grain yield (Ali *et al.,* 2008).

The crop performance from the interaction of different biochemical, physiological, and morphological traits of a cropping system may be determined through this analysis (Singh *et al.,* 2014; Tiwari *et al.,* 2019). Thus, the direct influence of plant growth-promoting traits of a biofertilizer bacteria, if applied to soil or root system, on the yield of a particular crop and indirect influence through various yield-attributing traits of the crop plant may be estimated through this path coefficient analysis. Such an effort is undertaken in this endeavour.

Materials and Methods

Ten *Azotobacter* isolates, VIBAZOTO01, VIBAZOTO02, VIBAZOTO03, VIBAZOTO04, VIBAZOTO05, VIBAZOTO06, VIBAZOTO07, VIBAZOTO08, VIBAZOTO09 and VIBAZOTO10, were taken as experimental materials. These isolates were collected from the natural soil of different locations of Sundarban, West Bengal, and germplasms are maintained in the Institute laboratory. Four plant growth-promoting parameters, nitrogenase activity, Phosphate solubilization capacity, Indole-3- Acetic acid (IAA) production capacity, and capacity for production of siderophore were studied in the laboratory.

Studies on Plant Growth Promoting (PGP) parameters of *Azotobacter*

Nitrogenase activity was assessed through Acetylene Reduction Assay (ARA) following the method of Hardy *et al.,* (1968). Culture of log-phase (72 h) in Jensen's nitrogen-free medium (10 ml) was taken in a 20 ml airtight rubber cap tube. The tubes were injected with 10% (v/v) acetylene, and incubated for 24 h, and ethylene evolution was measured in a Gas Chromatograph (Young Lin YL6100GC, South Korea) and expressed as nmols/ml of culture with 10^8 cfu per ml in 24 h. For the assessment of siderophore production capacity, microbial isolates were grown in LGI medium for 72 h and then centrifuged at 15000 rpm for 5 min, about 0.5 ml of supernatant was mixed with 0.5 ml CAS reagent (Pyne *at al.,* 1994) as described by Das *et al.,* (2021). The absorbance of the mixture was measured at 630 nm against reference blank where uninoculated medium was taken instead of microbial culture. Production of siderophore was calculated as Siderophore unit (%) = (Ar – As) /Ar x 100 (where Ar= absorbance of reference, As= absorbance of sample).

LB medium containing L-Tryptophan (0.5 mg/L) as described by Rahman *et al.,* (2010) was used for the estimation of Indole-3-acetic acid (IAA). The culture was grown in a shaker (125 rpm) for 48 h, at 28ºC, and centrifuged at 15000 rpm. About 2 ml of Salkowski's reagent was added to 1 ml of supernatant and incubated at dark in room temperature for 20 min for the development of pink colour. IAA production was estimated by taking the absorbance of the mixture at 530 nm against uninoculated medium and reagent as blank and comparing the redding with the standard curve as described by Das *et al.,* (2021). For the estimation of Phosphate solubilizing efficiency, the culture was grown in PK Medium for 72 h and the soluble phosphate was estimated by using Phospho molybdate blue colour method as described by Sanjotha and Manawadi (2016).

Pot experiment with *Azotobacter* isolates

For the pot experiment Chili seed (*Capsicum annum*, Var. *Tejoswini*) was washed with sterilized distilled water and treated with *Trichoderma* and *Pseudomonas* Culture (@ 10g each /1 L water) for one h. The roots of the seedling were treated with Different *Azotobacter* Culture with 10^8 CFU /ml (@ 10 g/L) for 1 h and transplanted in the pot containing 1 Kg sun-treated soil with the characteristic features of pH- 6.8, EC (dsm^{-1})-0.77. OC(%)-0.52 N (kg/ha)-203, P. (kg/ha)- 8.65 and K (Kg/ha)-328. Each treatment along with untreated control was in triplicate and kept in polyhouse in Randomized Block Design. Leaves from the same position for each plant were taken after 36 days and chlorophyll content was estimated following the typical acetone extraction spectrophotometric method. Periodically, fruits were harvested and quantified. All other parameters like, fresh & dry shoot weight, fresh & dry root weight, number of fruits per plant, 10 fruit weight, and yield per plant were quantified after final harvesting (60 days after sowing).

Statistical Analysis

For statistical analysis, the significance level of the data was analysed following ANOVA as suggested by Gomez and Gomez (1984).

Correlation coefficient was analyzed following the formula:

$$r = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2}\sqrt{\sum(y_i - \bar{y})^2}}$$

Where:

- x_i and y_i are the individual data points for variables X and Y,
- x¯ and y¯ are the means of X and Y,
- n is the number of data points.

Regression coefficient was analyzed following the formula:

$$b_1 = r \times \frac{s_y}{s_x}$$

Where:

- r = Pearson's correlation coefficient between x and y,
- s_x = standard dethroughtion of x,
- s_y = standard dethroughtion of y.

Path coefficient analysis was done following the method as developed by Wright (1921) with sequential steps like determination of Standard Dethroughtion, Correlation analysis, multiple regression analysis, and path coefficient analysis.

Direct effect and Indirect effect were calculated as-

Direct effect of x on y (x-y) = Regression coefficient multiplied by (Standard dethroughtion of x / standard dethroughtion of y)

(Where x is in dependent variable and y is dependent variable)

Indirect effect of x on y through p (x-p-y) = (Effect of x on p) multiplied by (Effect on p on y)

Total effect= Direct effect + all the indirect effects.

Results and Discussion

Plant Growth promoting trains of *Azotobacter* isolates

As shown in Table.1, different isolates of *Azotobacter* exhibited considerable variability in plant growth-promoting characteristics. The highest nitrogen fixating ability was shown by isolates, VIBAZOTO01 and VIBAZOTO08 with acetylene reduction assay (ARA) value 19.24 nmol/ml/h, and 19.36 nnml/ml/h, respectively. A higher ARA value is suggestive to have strong potentiality of the bacteria to contribute available nitrogen to the soil through biological nitrogen fixation (Bhattacharya *et al.,* 2012). Three isolates with highest Indole-3-acetic (IAA) production was seen by isolate VIBAZOTO03 (5.18 mg/L) followed by VIBAZOTO01 (5.18 mg/L) and VIBAZOTO09 (4.51) which indicates that these two isolates have their effect on root elongation and branching (Spaepen *et al.,* 2007).

Although all the isolates are shown to produce a considerable amount of siderophore, some variability was found with the highest value by VIBAZOTO08 (56.98%), followed by VIBAZO10(51.36). This finding indicates that these bacteria could enhance iron acquisition in iron-limiting conditions and could suppress the growth of pathogenic bacteria as was shown by Ahmed and Holmstrom (2014). Some of the isolates can contribute available phosphate in soil as was indicated by their phosphate solubilization properties.

Effect of *Azotobacter* inoculation on Chili plant performance

Significant improvement of yield and yield attributing parameters in chili plant was noted by root inoculation of seedlings (Table.2) although there was a significant variability in their performance. The isolate VIBAZOTO08 showed its contributions on shoot fresh weight (85.67 g), root fresh weight (22.56 g), and yield per plant (93.97g). VIBAZOTO01 and VIBAZOTO03 also showed considerable performance in the same parameter.

This isolate enhances photosynthetic potential as reflected by its contribution to Chlorophyll- a: 3.24 mg/g and Chlorophyll-b 3.14 mg/g). Its multifaced role in the growth performance of plant is due to its superior efficiency for ARA, siderophore production, and IAA production. VIBAZOTO03 also demonstrated substantial improvement in yield parameters (90.53 g/plant) and chlorophyll content, consistent with its high IAA and siderophore output.

In contrast, isolates like VIBAZOTO02, despite having high siderophore levels, showed limited yield improvement (52.11 g/plant), possibly due to lower IAA and ARA levels, underscoring the need for a balance among multiple PGP traits for optimal plant response.

Correlation, Regression and Path Coefficient Analysis

Correlation analysis showed a strong positive association between yield-attributing parameters and yield (Table.3). From the analysis the traits number of fruit per plant (r=0.99), Chlorophyll-a content (r=0.94), and fresh root weight (r=0.94) were qualified as strong yield determinants. This result strongly keeps pace with the findings of Kloepper *et al.,* (2004) who suggested that improved physiological traits such as chlorophyll content and root biomass can contribute significantly to crop yield.

Among the biochemical traits of *Azotobacter* Phosphate solubilization showed a dominant influence on yield (R=70.77) followed by IAA production (b_1=3.53) as indicated by the analysis of the regression coefficient (Table.4).

This observation suggests the inductive role of phosphate solubilization and hormone production by bacteria on the yield. Yield also showed substantial dependence on some yield-attributing parameters on plant like root dry biomass (b_1=8.92) and chlorophyll-a content (b_1=23.07) suggesting that these two traits may be strong mediator for the effect of biochemical traits of *Azotobacter* on yield.

Table.1 Plant growth promoting characters of different *Azotobacter* isolates

Isolate No	Acetylene Reduction Assay (ARA) (nanomole/ml)	Soluble Phosphate (%)	Siderophore (%)	IAA (mg/L)
VIBAZOTO01	19.24	0.35	0.96	5.18
VIBAZOTO02	0.14	0.06	42.61	1.54
VIBAZOTO03	6.98	0.35	49.89	12.09
VIBAZOTO04	2.68	0.01	49.25	1.51
VIBAZOTO05	1.48	0.01	42.68	1.25
VIBAZOTO06	0.59	0.01	42.20	1.69
VIBAZOTO07	0.50	0.41	42.48	2.08
VIBAZOTO08	19.36	0.41	56.99	3.2
VIBAZOTO09	7.36	0.33	40.15	4.51
VIBAZOTO10	0.75	0.14	51.37	1.2
SD	7.36	0.18	19.26	3.34

Table.2 Effect of seed inoculation with different *Azotobacter* isolates on yield and yield attributing parameters of Chili

Treatments	Fresh Weight of Shoot (g)	Dry Weight of Shoot (g)	Fresh Weight of Root (g)	Dry weight of Root (g)	Chlorophyll-a (mg/g)	Chlorophyll-b (mg/g)	No. of Fruits ?plant	10 Fruit weight (g)	Yield per plant (g)
Control	60.67	17.73	11.16	5.21	1.25	0.85	24.00	17.48	41.97
VIBAZOTO01	92.67	27.25	19.40	9.25	3.29	3.74	39.67	20.32	80.23
VIBAZOTO02	64.67	19.83	12.82	6.88	1.82	0.63	27.67	18.80	52.11
VIBAZOTO03	83.67	24.82	22.52	9.71	3.30	2.86	43.67	20.73	90.53
VIBAZOTO04	72.00	20.25	12.94	7.01	1.77	1.21	26.33	17.09	43.93
VIBAZOTO05	77.33	23.62	18.82	7.93	2.45	2.14	34.67	20.54	70.90
VIBAZOTO06	73.00	20.62	13.86	6.09	1.51	0.60	28.67	18.58	53.23
VIBAZOTO07	73.00	20.86	13.01	7.67	2.12	1.11	31.67	20.41	64.80
VIBAZOTO08	85.67	23.60	22.56	10.65	3.24	3.14	45.00	20.87	93.97
VIBAZOTO09	77.67	24.01	11.50	5.80	2.30	0.83	28.33	19.38	54.85
VIBAZOTO10	75.33	23.78	15.70	5.42	1.93	1.39	30.00	19.37	58.03
SEm	5	2	2	1	0	0	2	1	5
CD05	14	5	5	2	1	1	7	3	15
CD01	20	6	6	3	1	1	9	4	20
SD	9.12	2.74	4.26	1.82	0.73	1.11	7.14	1.31	17.83

Table.3 Correlation coefficients of different combinations of yield attribution variables of Chili plant

	Fresh Weight of Shoot (g)	Dry Weight of Shoot (g)	Fresh Weight of Root (g)	Dry weight of Root (g)	Chlorophyll-a (mg/g)	Chlorophyll-b (mg/g)	No. of Fruits /plant	10 Fruit weight (g)	Yield per plant (g)
Fresh Weight of Shoot (g)		0.93	0.78	0.74	0.91	0.87	0.84	0.72	0.83
Dry Weight of Shoot (g)			0.70	0.56	0.85	0.73	0.73	0.73	0.73
Fresh Weight of Root (g)				0.87	0.87	0.90	0.96	0.75	0.94
Dry weight of Root (g)					0.89	0.86	0.93	0.71	0.91
Chlorophyll-a (mg/g)						0.91	0.94	0.82	0.94
Chlorophyll-b (mg/g)							0.90	0.67	0.87
No. of Fruits /plant								0.84	0.99
10 Fruit weight (g)									0.89

Table.4 Regression Coefficients of different dependable variables with different independent variables

Independent variable	**Dependent variables**								
	Fresh Weight of Shoot (g)	**Dry Weight of Shoot (g)**	**Fresh Weight of Root (g)**	**Dry weight of Root (g)**	**Chlorophyll-a (mg/g)**	**Chlorophyll-b (mg/g)**	**No. of Fruits /plant**	**10 Fruit weight (g)**	**Yield per plant (g)**
IAA Production	1.46	0.43	0.69	0.31	0.11	0.17	1.34	0.21	3.53
ARA	1.05	0.32	0.34	0.18	0.09	0.09	0.07	0.15	0.51
Phosphate solubilization	34.44	9.21	10.98	6.26	3.04	3.43	27.15	5.33	70.77
Siderophore production	0.03	0.01	0.05	0.02	0.01	0	0.08	0.02	0.20
Fresh Weight of Shoot (g)									1.61
Dry Weight of Shoot (g)									4.77
Fresh Weight of Root (g)									3.93
Dry weight of Root (g)									8.92
Chlorophyll-a (mg/g)									23.07
Chlorophyll-b (mg/g)									14.03
No. of Fruits /plant									2.48
10 Fruit weight (g)									12.12

Table.5 Path coefficients analysis to find direct effects of plant growth promoting characters of *Azotobacter* on yield.

Independent Variables	Direct effect on yield	Indirect effect on yield through								
		Fresh Weight of Shoot (g)	Dry Weight of Shoot (g)	Fresh Weight of Root (g)	Dry weight of Root (g)	Chlorophyll-a (mg/g)	Chlorophyll-b (mg/g)	No. of Fruits /plant	10 Fruit weight (g)	TOTAL Effect
ARA	0.21	0.70	0.63	0.55	0.66	0.86	0.52	0.07	0.75	**4.95**
IAA	0.66	0.44	0.38	0.51	0.52	0.48	0.45	0.62	0.48	**4.54**
PO4	0.71	0.56	0.44	0.44	0.56	0.71	0.49	0.68	0.65	**5.24**
Siderophore	0.22	0.05	0.05	0.21	0.19	0.25	0.01	0.21	0.26	**1.45**

The direct and indirect contribution of Plant Growth promoting traits of *Azotobacter* on chili yield was estimated through path coefficient analysis (Table.5). Phosphate solubilization trait was qualified the highest contributor directly on yield (p=0.71) in total through other traits of plant (p=5.24).

IAA production also showed a significant total effect on yield (p=4.54) mediated through enhancement in root development (p=0.50 & P=0.51, for fresh root weight and dry root weight, respectively) and number of fruit per plant (p= 0.62). The trait ARA has a very low direct effect on yield (p=0.21) while showing a considerable total effect (p=4.95) through indirect effect through shoot / root biomass development and chlorophyll content

The present study reflects a strong positive association between yield and key physiological traits in chili, especially root biomass, chlorophyll content and fruiting intensity in plant as revealed from correlation coefficient analysis.

This result keep pace with the finding of Kloepper *et al.,* (2004) who demonstrated that yield in crop plant can be improved through improving root biomass and photosynthetic efficiency of plant.

Phosphate solubilizing property of *Azotobacter* came out as the dominant yield influencing trait with high regression coefficient while production of IAA stood second. This result clearly suggest that microbial strains having good phosphate solubilizing and IAA producing property may play significant role on crop performance.

This is well known that phosphate solubilization is important factor for converting insoluble phosphate into plant available form Rodríguez & Fraga (1999) and IAA production is known to stimulate root elongation and branching increasing absorption area (Glick, 2012; Bhattacharyya & Jha, 2012).

Path coefficient analysis further elucidated the direct and indirect contributions of PGP traits to yield. Phosphate solubilization showed the highest direct effect on yield and a total effect when indirect effects through plant traits were considered.

IAA production also contributed significantly mainly through its impact on fresh and dry root biomass and fruit number per plant. Interestingly, although *Azotobacter* is traditionally valued for its nitrogen-fixing ability, the trait ARA exhibited a relatively low direct effect on yield, but a notable total effect predominantly through improvements in biomass and chlorophyll content. This highlights that nitrogen fixation, while important, may exert its influence on yield through indirect physiological enhancements rather than direct contribution alone.

These findings collectively suggest that the beneficial impact of *Azotobacter* on chili yield is not the result of a single mechanism but a complex interplay of multiple PGP traits.

Similar conclusions have been drawn in earlier studies, advocating for a holistic evaluation of microbial traits when selecting efficient bioinoculants (Saharan & Nehra, 2011; Glick, 2012). Relying solely on nitrogen-fixing capacity may lead to underestimation of the potential of certain isolates, especially those excelling in phosphate solubilization and phytohormone production.

Thus, the present study confirms that the studied *Azotobacter* isolates possesses different plant growth promoting (PGP) traits. It is also clear that synergistic interaction of multiple PGP traits drives effective plant growth promotion rather than dependence on single trait. So, for the selection of *Azotobacter* isolate / strain one must consider all the PGP traits, not solely depending on nitrogen fixing activity.

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Author Contributions

Sadhan Kumar Das: Investigation, formal analysis, writing—original draft. Tarun Kanti Das: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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