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Original Research Article

Impact of Liquid Bio-Fertilizers on Okra (*Abelmoschus esculentus L. Moench.*) Plant Growth Indices using *Azotobacteria* and *Azospirillum* Consortia

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Abstract: This study was to make bio-fertilizers with *Azotobacteria* and *Azospirilum* consortiums and to test the influence of bio-fertilizers on Okra plant growth. This investigation was conducted using the soil from the Biological Garden of the University of Cross River State, Calabar. Pure culture and mass production was performed in batch fermentation at optimum condition by means of specific medium for *Azospirilum* and *Azotobacter*. Organisms were isolated from the soil samples and confirmed using biochemical tests. The bio-fertilizer effect on the growth parameters of Okra plants was carried out on Day 21. The post germination, phenotypic and observations showed significant differences in performance between plant length, mass, root numbers, root length, total plant length, breadth of leaves, shoot length, root mass and biological yield. The biochemical indices exibited a significant rise ($P < 0.05$) in chlorophyll content (mg/g.fr. wt) as compared to Control (0.010±0.000), *Azospirilum* (0.016±0.000), Azotobacter (0.016±0.000) and consortium (0.018±0.000). Significant rise ($P<0.05$) in carbohydrate content (%) control (51.976±0.768), *Azospirilum* (69.690±3), Azotobacter (69).656+2), and consortium 70).190± (5). Protein content control (0.028±0.001), *Azospirilum* (0.040±0.000), *Azotobacter* (0.060±0.001) and consortium (0.061±0.000) all significantly increased [$P< 0.05$]. Growth is significantly affected by single injection and control when compared to plants being treated with *Azotobacter*, *Azospirilum* or both ($P < 0.05$). According to the findings, plant mineral fixation through nitrogen fixation and phosphorous solubilization seems to be improved by bio-fertilizer inoculation as well as their mineral nutrition's improvement.

Keywords: Soil enhancement, crop yield, bio-fertilizer, biotechnology, consortium.

INTRODUCTION

Grown for its tasty green seed pods, okra (*Abelmoschus esculentus L. Moench*.) is a flowering plant in the *Malvaceae* family. Okra is used for its edible green fruits as well as young leaves. It occurs pantropically in tropical to warm temperate environments. It is sometimes referred to as ladyfinger due to the shape of its capsule. Okra fruit possesses phenolic compounds that function as natural antioxidants. Fresh okra can be eaten in salads along with other vitamin supplements. Extracts from okra seeds are used as antitumor agents. Its high fiber content aids in weight loss. The phyto-nutrient constituents of okra enable it to stabilize blood sugar levels (Eva *et al.*, 2019).

In India, okra covers an area of 0.542 million hectares yearly and yields about 50 million tons every year. On a global scale, this accounts for only 5% and 7% of the total production and coverage respectively (Sarwar *et al*., 2020). Based on climate, soil conditions, fertilizer application, expression of variety, duration of crop, environmental-vegetative, and management practices, yield variation in okra has been estimated between 600 kg/ha and10,000 kg/ha (Kumar *et al*., 2024). Factors inherent in some varieties make Nigeria's okra variety low performing. The three main limiting elements that cause growth retardation and low yield in all vegetative crops, including grain cereals like maize, are deficits in nitrogen (N), phosphorus (P), and potassium (K). However, one of the most significant

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ones that, if not sufficiently provided, would result in low output because of the high requirements of crops like okro (agushie).

Food produced without the use of synthetic chemicals, such as pesticides, fertilizers, antibiotics, or genetically modified organisms, is referred to as organic food (Vigar *et al*., 2019). Because they think it's safer, healthier, and more ecologically friendly than conventional food, some consumers choose organic food (Kia *et al*., 2019). However, organic food is also more expensive and less available than conventional food (Gumber *et al.,* 2021). The reasons some people advocate for cultivating natural food include reduced soil erosion, water pollution, and greenhouse gas emissions by using natural methods of fertilization, pest control, and crop rotation (Ashoka *et al*., 2023). Organic farming can increase biodiversity, soil quality, and crop diversity by avoiding monocultures, GMOs, and chemical inputs (Bibi *et al*., 2022). Organic food can have higher levels of antioxidants, vitamins, minerals, and phytochemicals than conventional food, which may have positive effects on human health (Bibo *et al*., 2023). Organic food can have lower levels of pesticide residues, nitrates, heavy metals, hormones, and antibiotics than conventional food, which may reduce the risk of chronic diseases, allergies, and antibiotic resistance (Yura *et al.*, 2021).

Azotobacter is a bacterium that can fix atmospheric nitrogen and produce plant growth-promoting substances (Aasfar *et al.,* 2021). One of the genes involved in processing plant growth is nifH, which encodes for the nitrogenase enzyme that catalyses the reduction of N_2 to NH_3 (Choudhary and Varma 2017). *Azotobacter* can also synthesize hormones such as auxins, cytokinins, and gibberellins, which regulate plant development, such as cell division, root formation, and flowering (Stefen *et al*., 2022). Additionally, *Azotobacter* can produce siderophores, antibiotics, and phytohormones that protect plants from pathogens and stress. It is for this reason that *Azotobacter* is considered in this research as a potential biofertilizer for soil and plant growth. *Azotobacter* and *Azospirillum* are fascinating microorganisms that play crucial roles in enhancing soil fertility (Aisha *et al*., 2020).

Azospirillum is a well-studied genus of plant growth-promoting rhizobacteria (PGPR) (Fukami *et al*., 2018). It significantly enhances plant growth, development, and productivity under field conditions. Besides nitrogen fixation, *Azospirillum* produces indole-3-acetic acid (IAA), which positively influences plant growth (Tang *et al*., 2023). Its mode of action involves additive and selective effects (Hassan and Bernard, 2024). *Azospirillas* are used as inoculants in millions of doses. They are applied to various crops, including cereals and legumes. Combined inoculation of legumes with both rhizobia and *Azospirillum* can further enhance plant performance due to complementary biological processes. In general, harnessing these biofertilizers as consortia in liquid form can contribute to sustainable agriculture by improving soil fertility and promoting plant yield.

Genes Conferring Advantages to *Azotobacter* and *Azospirillum* for Biofertilizers

Azotobacter and *Azospirillum* possess several key genes that make them ideal candidates for biofertilizer production. These genes contribute to their nitrogen-fixing ability, plant growth promotion, and survival in soil environments. According to research some of the genes in *Azospirillum* and *Azotobacter* involved in soil and plant fertility are presented in Table 1 for a detailed illustration.

All the genes and functions listed in Table 1 **(***nif, amt, acz, acdS, siderophore production, stress tolerance)* are found in both *Azotobacter* and *Azospirillum*. These two bacteria share many beneficial traits for biofertilizer development, and both possess the genetic machinery for nitrogen fixation, production of plant growth hormone, and nutrient acquisition. While there may be some variations among strains within each genus, they generally share these genetic capabilities (Lamichhane *et al*., 2024). Carbon sources and growth conditions significantly influence the growth and nitrogen fixation (N-fixation) efficiency of *Azotobacter* and *Azospirillum*, impacting their suitability for biofertilizer development. Nitrogen fixation is an energy-intensive process. These bacteria prioritize utilizing readily available carbon sources for cellular maintenance and growth before allocating resources towards N-fixation.

Simple sugars like glucose and sucrose are generally preferred carbon sources as they provide a readily accessible energy source (Eggleston *et al.*, 2018). Complex carbon sources like cellulose or organic acids require more processing by the bacteria, potentially reducing the energy available for N-fixation (Lamichhane *et al*., 2024). Therefore, finding the optimal balance is crucial. A moderate level of available carbon allows for sufficient bacterial growth while not completely suppressing N-fixation. N-fixation is an anaerobic process, meaning it occurs in the absence of oxygen. *Azotobacter* is a microaerophilic bacterium that tolerates low oxygen levels. It can fix nitrogen in environments with limited oxygen availability. *Azospirillum* is a strict aerobe, requiring oxygen for growth. However, some *Azospirillum* strains can perform a process called associative N-fixation in close association with plant roots where oxygen levels are lower. pH**:** Both bacteria prefer a slightly acidic to neutral pH range (around 6.0-7.5) for optimal growth and N-fixation (Sumbul *et al.,* 2020). Moderate temperatures (around 25-30°C) are generally favorable. However, some strains may have adapted to function in cooler or warmer environments. Thus, the Presence of combined nitrogen (e.g., ammonium or nitrate)**,** these readily available forms of nitrogen repress N-fixation in many

diazotrophs, including *Azotobacter* and *Azospirillum*. Therefore, the formulations and application of Biofertilizers would minimize the presence of combined nitrogen to avoid inhibition. The presence of other beneficial microbes in the biofertilizer or soil environment can influence N-fixation through competition or synergistic interactions (Kamal *et al*., 2019).

Therefore, optimizing this biofertilizer and understanding inhibition factors will help us to design biofertilizer formulations that provide the ideal balance of carbon source and growth conditions to promote bacterial growth while maximizing N-fixation. This may involve selecting readily available carbon sources that do not completely suppress N-fixation, using carrier materials that create a microaerophilic environment for *Azotobacter*, and formulating the biofertilizer to minimize the presence of combined nitrogen sources.

MATERIALS AND METHODS

For the pilot study, forty (40) seeds in total were used. The seeds were separated into four groups, each containing ten seeds, and then planted in soil that had been inoculated with bacteria that fix nitrogen and solubilize phosphate. Using the soil as indicated in Table 2, the following treatments were applied.

Table 1: Experimental Design and Preparation of Microorganisms

Treatment	Groups	Prepared Media (broth)
Soil + MSB	Control (Mineral Salt Broth)	1000ml MSB
Soil + PSB+ MSB	Phosphate solubilizing bacteria	1000ml Pikovskaya
Soil + NFB+ MSB	Nitrogen-fixing bacteria	1000ml Ashby
Soil + PSB+NFB+ MSB	Consortium of bacteria	500ml Pikovskaya + 500ml Asby

MSB: Mineral Salt Broth, **PSB:** Phosphate solubilizing bacteria, **NFB:** Nitrogen-fixing bacteria

The phenotypic and physiological parameters of Okra seed were examined twenty-one (21) days after sowing. Analysis was done on the phenotypic parameters. The selective media were prepared using the Subba (1977) method, and the biochemical parameters were evaluated.

Identification of Microbes

In accordance with Bergey's Manual of Systematic Bacteriology (Williams and Wilkins 1989), *Azotobacter* and *Azospirilum* were identified. Pikovskaya's and Asbhy's agar are the media used for the isolation of *Azospirilum* and *Azotobacter*.

Research on Physiology of the Chosen Strains

The procedures outlined by Collee and Miles (1989) were used to test the physiological activity of the chosen strains. The process outlined by Sagar (2023) was employed to ascertain the bacterial growth curve. The process for producing bio-fertilizer was followed by Kumar *et al*., (2024). Pikovskaya's broth was made in one thousand milliliters (1000 mL) using the following ingredients (gL-1): calcium phosphate, 5.00, ammonium sulphate, 0.50, potassium chloride, 0.20, magnesium sulfate, 0.10, manganese sulphate, 0.0001, ferrous sulphate, 0.0001, agar, 15.00, and pH 7. The following ingredients were added to one thousand milliliters (1000 mL) of Ashby's broth (gL-1): mannitol (20.00); dipotassium phosphate (0.20); magnesium sulfate (0.20); sodium chloride (0.20); potassium sulfate (0.10); calcium carbonate (5.00); agar (15.00); pH 7. The broth was then sterilized. 500 milliliters (500 mL) of Ashby's broth and 500 milliliters (500 mL) of prepared Pikovskaya's broth were mixed. The media were infected individually with a consortium of *Azospirillum* and *Azotobacter* or a pure culture of each, and they were then cultured for 14 to 21 days at 300C in a shaker incubator.

Analysis of Biochemical Indices

Total chlorophyll content was estimated using Arnon's (1949) method; the carbohydrate content was determined using Hedge's (1962) method; the carbon content in liquid samples was determined using the wet oxidation method in the manner described by Walkey and Black (1934); the concentration of nitrate was estimated using the methods of Jackson (1973), Trivedy and Goel (1986), and Horita (1988); The content of nitrite was estimated using the diazotization reaction method as explained by Horita (1988); the phosphate concentration was calculated using the approach outlined by Pearson (1976).

Data Analysis

The data was analyzed statistically using version 16 of the Statistic Package for Social Sciences (SPSS). The mean plus standard deviation of the results is displayed. Analysis of Variance (ANOVA) was used to compare the groups. An interval of confidence of 95%, $p < 0.05$ was designated as the acceptable significance threshold.

RESULTS AND DISCUSSION

Soil samples were examined to determine whether phosphate-solubilizing and nitrogen-fixing bacteria were present. The ability of phosphate-solubilizing microorganisms to create organic acids in the surrounding media and solubilize recalcitrant phosphates was demonstrated by the creation of transparent zones around their colonies (Aisha *et al*., 2020). The emergence of pale, opalescent gel-like formations in the petri dishes was an indicator of bacteria that fix nitrogen. According to the findings, 32 bacteria that fix nitrogen were isolated on Ashby's medium and 45 phosphatase-solubilizing colonies of bacteria were

isolated on Pikovskaya's agar media with insoluble tri-calcium phosphate (TCP) from the soil used for farming. From the third to the twenty-first day, the phosphorus level of the combination treatment biofertilizer (CTB) increased significantly ($P < 0.05$), showing the highest amounts of phosphorus when compared to the other treatments. This could be explained by the bacteria's combinatorial activity, which increases the bacteria' survivability. As shown in Tables 3 and 4, with a confidence interval of 95%, the usage of different biofertilizers on the growth of the Okra plant resulted in substantial differences in plant length, the number of leaves per plant, whole plant mass, number of roots, root length, total plant length, leaf width, shoot length, leaf length, and root mass, respectively.

Table 2: Effects of various bio-fertilizers on Okra growth indices

Treatments	Plant length (cm)	Number of leaves per plant	Mass of whole plant (gm)	Number of roots	Length of roots (cm)	The total length of the plant (cm)
CONTROL	20.30±0.08	3.00±0.00	4.16±0.12	13.66±0.47	12.26±0.20	32.56±0.19
PSB	23.43±0.47*	3.00±0.00	4.80±0.08#	14.00±0.82#	13.20±0.20#	36.65±0.20*
NFB	24.40±0.37*	3.00±0.00	5.06±0.16*	14.33±0.47*	14.06±0.17*	38.46±0.20*
PSB+NFB	27.43±0.09*	3.00±0.00	5.20±0.08*	15.00±0.00*	14.23±0.25*	41.66±0.25*

PSB = phosphate solubilizing bacteria, **NFB** = Nitrogen fixing bacteria, **PSB+NFB**= Consortium treatment. Values are means SD. * = Significant increase ($P \leq 0.05$), ** = Significant decrease ($P \leq 0.05$), # = non-significant increase ($P > 0.05$), ## =non-significant decrease ($P > 0.05$) according to Duncan post hoc test.

Table 3: Percentage (%) increase in plant length, total length and leave length

Treatment	Plant length (cm)	% increase	The total length of the plant (cm)	% increase	Leave Length (cm)	% increase
Control	20.30±0.08	-	32.56±0.19	-	11.80±0.08	-
PSB	23.43±0.47	15.41%	36.65±0.20	12.56%	13.00±0.22	10.17%
NFB	24.40±0.37	20.19%	38.46±0.20	18.12%	13.20±0.08	11.86%
PSB+NFB	27.43±0.09	35.12%	41.66±0.25	27.94%	14.90±0.14	26.27%

All results are mean ± SD for 3 determinations

In liquid media, synergistic activity might improve phosphorus mobilization. The microorganisms used carbon and nitrogen as resources. From the third to the twenty-first day, CTB revealed a significant ($P < 0.05$) rise in the concentrations of nitrate, nitrite, ammonium, and organic matter. The nitrogen proportion showed a substantial drop ($P \leq 0.05$) from the third to the sixth day, then a rise on the ninth day, a decline from the twelfth to the fifteenth day, and finally a substantial rise from the eighteenth to the twenty-first day. From the third to the twenty-first day, there was a substantial ($P < 0.05$) rise in the concentration of overall organic matter. Microbes break down organic matter to release fermentation through heat during the process of making biofertilizer (Shenoy *et al*., 2024). The synthesis of phytohormones, which the bacteria produce when they settle in the roots of the host plants, is another theory that has a direct impact on root development (Sumbul *et al*., 2020). When comparing the breadth of leaves for Azospirilum (1.60 + 0.00), Azotobacter (1.60 ± 0.00), and the consortium treatment (1.60±0.00) to the control (1.60+0.00), there was a statistically non-significant increase ($P > 0.05$). As indicated in Tables 2 and 3, there was a statistically significant increase ($P < 0.05$) in shoot length for the consortium treatment (6.73+0.01), Azotobacter (5.43±0.26), Azospirilum (5.33 ± 0.26), and the control (4.80+0.22).

Table 4: Percentage (%) increase in plants' shoot length, number of roots, and root length

Treatment	Shoot length (cm)	% increase	Number of roots	% increase	Root length (cm)	% increase
Control	4.80±0.22	-	13.66±0.47	-	12.26±0.20	-
PSB	5.33±0.26	11.04%	14.00±0.82	2.49%	13.20±0.20	7.83%
NFB	5.43±0.26	13.13%	14.33±0.47	4.90%	14.06±0.17	14.68%
PSB+NFB	6.73±0.01	40.21%	15.00±0.00	9.8%	14.23±0.25	16.06%

All results are mean ± SD for 3 determinations

A statistically significant increase in leaf length for the Control group (11.80±0.08), *Azospirilum* (13.00±0.22), *Azotobacter* (13.20±0.08), and consortium treatment (14.90±0.14) groups, was observed. Many previous studies have also reported an increase in plant growth characteristics when inoculated with biofertilizers, such as in barley (Renoud *et al.*, 2022), okra (Prisa *et al.*, 2023), bean and wheat (Amanpreet *et al*., 2024), and okra again. The plants treated with single or dual inoculation exhibited a deeper green color compared to the green color observed in the control plants. Biofertilizers have been found to improve photosynthetic performance, thereby enhancing the plant's tolerance to stress (Areeshi *et al.*, 2022).

Khan *et al*., (2023) highlighted the critical role that phosphorus plays in numerous enzymatic reactions, particularly those involving phosphorylation, energy conservation, and a wide array of biochemical processes. The researchers examined various biochemical factors, including chlorophyll, carbohydrate, and protein content, to assess the impacts of single and dual inoculations of Okra plants with *Azotobacter* and *Azospirilum*, in comparison to control plants. The results revealed that plants treated with Azotobacter, *Azospirilum*, or their combination exhibited the highest chlorophyll, carbohydrate, and protein levels after 21 days of planting. Specifically, a statistically significant ($P < 0.05$) rise in chlorophyll content was seen in the control group (0.010±0. 000mg/g.fr.wt), *Azospirilum* (0.016±0. 000mg/g.fr.wt), *Azotobacter* (0.016±0. 000mg/g.fr.wt), and combination treatment (0.018±0.000 mg/g.fr. wt) groups, with the combination treatment showing the highest concentration. Corresponding results were reported by (Saikat *et al*., 2024), they found out that biofertilizers significantly improved chlorophyll levels in chilli plants. This article underscores the need for in-depth research to comprehensively understand the long-term impacts and potential limitations of using Azotobacter and *Azospirilum* consortia as biofertilizers. Further exploration of their wide-ranging implications, economic feasibility, scalability prospects, and interactions with other agricultural inputs is crucial for a more comprehensive understanding.

CONCLUSION

Microbial inoculants, also known as bacterial bio-fertilizers, can convert unusable soil nutrients into forms that plants can readily absorb. These bio-fertilizers can enhance nutrient uptake by plants, protect them from harmful root microorganisms, and increase crop yields by 20-30%. The techniques used to isolate and mass-produce these bio-fertilizers have been a boon for agriculture and the restoration of degraded ecosystems. These studies have shown that combining multiple bio-fertilizer inoculants has a more significant influence on the growing of okra plants compared to using a single inoculant. This is because these bio-fertilizers play a crucial role in mending soil richness and sustaining its productive potential. Therefore, the large-scale production of these bio-fertilizers can contribute to increased agricultural productivity and promote sustainable, eco-friendly farming practices.

Recommendation and Knowledge take home Information

The study presented in this article highlights the need for further investigation into the long-term effects and potential drawbacks of using *Azotobacter* and *Azospirillum* consortium as liquid biofertilizers for crop production. Although this study shows promising results in plant growth and biochemical parameters of okra, more extensive research is clearly needed to understand the implications of the widespread usage of these biofertilizers on different crops, soil, and environmental. In addition, the evaluation of economic execution potential and the scalability of these biofertilizer agents for agricultural purposes are essential for actual implementation. In addition, a survey of interactions with biofertilizers with other agricultural inputs such as pesticides and conventional fertilizers will enrich the understanding of potential advantages and restrictions.

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