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Effect of Seven Major Plant Micronutrients on the Commercial Growth of the Bioinoculant *Azotobacter chroococcum*

Rahul Bansod[1] and Ravi Upadhyay[2*]

[1]*Biofertilizer Plant, C-sector, Indrpuri Raisen Road, Bhopal, India*
[2]*Department of Botany, Government Narmada College, Hoshangabad, India*

**Corresponding author*

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A B S T R A C T

Azotobacter chroococcum has established its significance in crop production, plant nutrition, and soil fertility. As a free-living N_2-fixing diazotroph, *A. chroococcum* markedly enhances crop production through biological nitrogen fixation and the secretion of growth-promoting substances. Being a beneficial soil bacterium, this genus synthesizes phytohormones and plant growth regulators—specifically auxins, gibberellins, and cytokinins—which possess stimulatory effects on plant development. In addition, it stimulates rhizospheric microbes, protects plants from phytopathogens, and improves overall nutrient uptake. Currently, Indian agricultural soil is increasingly deficient in micronutrients, largely due to the widespread reliance on chemical fertilizers for major nutrient supplementation and the inadequate conversion of agricultural waste into farm compost. To address this, the present study investigates the optimal industrial production of *Azotobacter* bio-inoculants in the presence of critical plant micronutrients. This research ultimately aims to guide the specific production of *Azotobacter* bio-inoculants targeted for micronutrient-deficient agricultural soils, aligning with soil health card recommendations and the Fertilizer Control Order (1985) amendments.

Introduction

The agricultural and natural plant kingdoms depend primarily on soil; thus, the difference between survival and extinction for most land-based life is characterized by the thin layer of soil covering the Earth's surface (Doran & Zeiss, 2000).

Soil is a living mixture of minerals and organisms that provides vital nutrients and a healthy environment to nurture producers as they grow. Consequently, the soil ecosystem is divided into biotic (living) and abiotic (non-living) components.

The biotic fraction contains microorganisms, many of which are now scientifically proven to function as bio-inoculants, or more appropriately, "microbial inoculants." These agriculturally important microorganisms enhance plant growth through biological nitrogen fixation and the solubilization or mobilization of phosphates, potassium,

and sulfur (Gomare *et al.,* 2013; Lugtenberg & Kamilova, 2009). The aseptic cultivation and packaging of these efficient microorganisms constitute the commercial bio-inoculant industry.

The abiotic fraction of the soil contains elements in free or compound forms required by both microorganisms and plants for optimal growth. These are categorized into major nutrients (nitrogen, phosphorus, potassium, sulfur) and minor/micronutrients (zinc, molybdenum, boron, chlorine, iron, manganese, copper). Studies indicate that specific compounds, such as $ZnSO_4$, NH_4MoO_4, H_3BO_3, KCl, $FeSO_4$, MnO, and $CuSO_4$, provide these trace elements in forms that are readily absorbed by plant roots (Grewal & Trehan, 1990). The judicious presence of these necessary abiotic factors contributes significantly to the health and yield of agricultural crops.

The optimal availability and utilization of both biotic and abiotic factors, including N_2-fixation, are profoundly influenced by agriculturally important microorganisms (Jonas *et al.,* 2011; Wani *et al.,* 2013).

The synchronous association between these two components creates an ideal microcosm that favors nutrient absorption and metabolic activity.

Plant Growth-Promoting Rhizobacteria (PGPR) are cultivated in cost-effective media and mixed with suitable carriers to produce bio-inoculants that provide natural nutritional support across agriculture, floriculture, forestry, and horticulture (Gomare *et al.,* 2013; Subba Rao, 1993). The present study was conducted to determine the specific effects of seven major plant micronutrients on the growth of *Azotobacter chroococcum*, thereby ensuring the optimal availability of these trace elements alongside the natural fixation of nitrogen for commercial crop applications.

Materials and Methods

Sample Collection and Bacterial Strain

Azotobacter species are native soil bacteria possessing free-living nitrogen-fixing abilities. The specific bacterium used in this study is *Azotobacter chroococcum*, which serves as the primary production strain for the Biofertilizer Production Plant of the M.P. State Agro Industries Development Corporation Limited, Bhopal, India.

Studies on the Effect of Micronutrients on the Bacterial Strain

A well-identified culture of *A. chroococcum* was inoculated into Jensen's broth and incubated at room temperature for 48 to 72 hours until the cell concentration exceeded an optical density (OD) of 1 at 620 nm, yielding a viable cell count of 1.0×10^{10} per ml of matured broth.

This matured broth was subsequently used to inoculate experimental media supplemented with various micronutrients.

Evaluating Various Concentrations of Micronutrients

Jensen's broth was modified by adding varying percentage concentrations (wt/vol) of seven specific micronutrient compounds. A 5% volume of the modified, micronutrient-enriched Jensen's broth was inoculated with the matured *A. chroococcum* broth. The inoculated media were incubated for 48 hours, after which viable cells were enumerated using the viable plate count method at a 10^{10} dilution.

Screening for Optimum Micronutrient Concentration

Multiple experiments were set up independently for each micronutrient to pinpoint the optimal concentration that yielded the maximum growth of *A. chroococcum*. These experiments were refined by testing narrower concentration gradients slightly above and below the values that showed the highest readable growth in the initial trials.

Bacterial Growth in Optimized Multi-Micronutrient Media

Finally, a 5% inoculum of *A. chroococcum* in Jensen's broth was added to a new broth containing *all seven* micronutrient compounds at their previously determined optimum concentrations.

Following a 48-hour incubation, viable cells were counted at a 10^9 dilution. Overall growth was directly compared against a control consisting of plain Jensen's nutrient suspension.

Results and Discussion

Effect of Various Concentrations of Micronutrients on Growth

Maximum bacterial growth (measured per 0.1 ml of inoculum) was observed in experimental media containing MnO, KCl, $CuSO_4$, NH_4MoO_4, $ZnSO_4$, $FeSO_4$, and H_3BO_3 at concentrations of 0.5%, 0.9%, 0.9%, 0.9%, 1.0%, 0.9%, and 1.1%, respectively (Table 1).

Trace elements function as essential co-factors in enzymatic reactions, stabilizing the structure of the enzymes themselves (Lin *et al.,* 2009). Zinc is a multi-functional element found in nearly 300 enzymes and is involved in broad catalytic and structural functions (Tubek *et al.,* 2008).

Potassium maintains osmoregulation, electrolytic balance, and membrane transport systems, while copper and manganese are vital components of biological redox reactions (Lin *et al.,* 2009).

Boron is an indispensable micronutrient for microbes and higher plants, essential for cell wall synthesis, sugar transport, carbohydrate metabolism, and respiration (Blevins & Lukaszewski, 1994; Camacho-Cristóbal *et al.,* 2008), although it can become toxic to microbes above specific thresholds (Ahmed & Fujiwara, 2010).

Screening of Optimum Micronutrient Concentration for Bacterial Growth

Both prokaryotic and eukaryotic organisms require elements such as iron, zinc, manganese, copper, boron, potassium, and molybdenum in highly specific concentrations for vital biological processes (Lin *et al.,* 2009). Following the initial broad screening, narrow-gradient experiments revealed the exact optimum concentrations for maximum bacterial growth compared to a standard yeast extract mannitol (YEM) control medium.

As detailed in Table 2, the optimal concentrations were identified as MnO (0.4%), KCl (1.0%), $CuSO_4$ (1.0%), NH_4MoO_4 (0.9%), $ZnSO_4$ (1.0%), $FeSO_4$ (1.0%), and H_3BO_3 (0.1%). Notably, NH_4MoO_4 showed an equal growth response at 0.09% and 0.2% before optimizing at 0.9%.

Effect of Optimum Concentration of Multiple Micronutrients

The successful optimization of fermentation conditions relies heavily on fine-tuning physical and chemical factors to ensure economic feasibility and process efficiency (Duta *et al.,* 2006). When all seven compounds were combined in the growth medium at their identified optimum concentrations (MnO 0.4%, KCl 1.0%, $CuSO_4$ 1.0%, NH_4MoO_4 0.9%, $ZnSO_4$ 1.0%, $FeSO_4$ 1.0%, and H_3BO_3 0.1%), the results demonstrated an astonishingly dense, mat-like growth (Table 3).

The primary objective of this investigation was to evaluate the synergistic effect of these trace compounds on maximizing the yield of *A. chroococcum*, aiming to incorporate them into the standard universal media for commercial bio-inoculant production. Because the plant requirement for micronutrients is at the parts-per-million level, this can be efficiently accomplished by embedding them directly within the biofertilizer matrix.

According to the Fertilizer Control Order (1985) and Bureau of Indian Standards, the minimum bacterial viability for commercial biofertilizers is 1.0×10^7 CFU/ml. In our final experiment combining all optimized micronutrients, the Colony Forming Units (CFU) greatly exceeded those of the plain Jensen's media control. This firmly establishes that incorporating these optimal concentrations is not only safe but highly beneficial, constituting a crucial necessity for maximizing the commercial viability and biological efficacy of *A. chroococcum*.

In conclusion, Micronutrient compounds are well-documented for their positive impact on plant health, acting as essential trace elements for vital physiological activities. This study confirms that modifying the universal standard growth medium (Jensen's media) by incorporating optimal concentrations of seven specific plant micronutrients significantly increases the growth and viability of *Azotobacter chroococcum* compared to the standard control.

Given that current Indian agricultural soils are increasingly deficient in essential micronutrients—driven by an over-reliance on chemical macronutrient fertilizers, the burning of crop residues, and the lack of farm-compost conversion—this integrated production approach is highly relevant.

Table.1 Effect of various concentrations of each micronutrient on the growth of *Azotobacter chroococcum*. (Cell counts recorded as CFU / 0.1 ml at 10^9 dilution.)

S. No.	Concentration (wt/vol)	MnO (CFU)	KCl (CFU)	$CuSO_4$ (CFU)	NH_4MoO_4 (CFU)	$ZnSO_4$ (CFU)	$FeSO_4$ (CFU)	H_3BO_3 (CFU)
1	0.1%	1146	248	62	544	126	308	2116
2	0.3%	3744	Mat	80	Mat	Profuse	362	376
3	0.5%	Mat	1464	780	1200	556	162	44
4	0.7%	142	2840	112	40	74	204	32
5	0.9%	264	Mat	1892	3652	Profuse	64	46
6	1.0%	120	Mat	840	Mat	2218	3428	74
7	1.1%	26	200	36	150	22	Mat	20
8	1.3%	32	312	740	26	496	24	16
9	1.5%	54	88	28	40	06	252	52
10	Control	1786	1786	1786	1786	1786	1786	1786

Table.2 Optimization screening of specific micronutrient concentrations for *Azotobacter chroococcum*.

S. No.	Compound	Concentration (wt/vol)	Bacterial Growth (CFU / ml at 10^9 dilution)
1	**MnO**	0.4%	+++++
		0.5%	+++
		0.6%	++
		Control	+++
2	**KCl**	0.8%	++
		0.9%	+++
		1.0%	+++++
		Control	++++
3	**$CuSO_4$**	0.8%	+++
		0.9%	++
		1.0%	++++
		Control	++++
4	**NH_4MoO_4**	0.9%	++++
		1.0%	++
		1.1%	+++
		Control	+++++
5	**$ZnSO_4$**	0.8%	++
		0.9%	+++
		1.0%	++++
		Control	+++++
6	**$FeSO_4$**	1.0%	++++
		1.1%	+++
		1.2%	++
		Control	+++++
7	**H_3BO_3**	0.09%	+++
		0.1%	+++++
		0.2%	+++
		Control	++++

Table.3 Final growth analysis of *Azotobacter chroococcum* utilizing the combined, optimized multi-micronutrient formulation.

S. No.	Compound	Optimum Concentration (wt/vol)	Observations (CFU / ml at 10^9 dilution)
1	MnO	0.4%	
2	KCl	1.0%	
3	$CuSO_4$	1.0%	
4	NH_4MoO_4	0.9%	Astonishing Mat Growth
5	$ZnSO_4$	1.0%	
6	$FeSO_4$	1.0%	
7	H_3BO_3	0.1%	
8	Control	Standard Y.E.M.B.	Profuse Growth (Standard Baseline)

Adopting this optimized, micronutrient-enriched bioprocess in biotechnological industries will allow for the superior production of commercial bio-inoculants.

This paves the way for targeted *Azotobacter* applications customized for specific, micronutrient-deficient agricultural soils as identified by regional soil health cards.

Author Contributions

Rahul Bansod: Investigation, formal analysis, writing—original draft. Ravi Upadhyay: 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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