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Research Article

Effects of cobalt and manganese on biomass and nitrogen fixation yields of a free-living nitrogen fixer - *Azotobacter chroococcum*

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ABSTRACT

The effects of different concentrations of cobalt and manganese on the biomass and the ability of *Azotobacter chroococcum* to fix nitrogen were investigated. *In vitro* trials were conducted in Jensen's (nitrogen free) broth (half strength) under continuous air flow, incubated at ambient room temperatures for seven days. Results obtained showed that 12.5 mg/l, 25 mg/l, 50 mg/l, 100 mg/l, and 200 mg/l concentrations of cobalt and manganese respectively enhanced microbial growth of *Azotobacter chroococcum* concomitantly. However, nitrogen fixation was enhanced only at 12.5 mg/l and 25 mg/l concentrations for cobalt, and only at 12.5 mg/l concentration for manganese. Statistical analysis revealed no significant difference in the specific growth rates and nitrogen fixations respectively, between the cobalt and manganese trials. Kinetic modeling revealed that nitrogen fixation was associated with biomass concentration, and not with cell mass growth.

Keywords: Diazotroph; Micronutrients; Biostimulation; Toxicity; Luedeking-Piret model.

1. INTRODUCTION

Nitrogen is the most abundant element in the earth's atmosphere. However, due to the fact that atmospheric nitrogen is extremely un-reactive, it is also the most limiting nutrient to most plants [1]. Nitrogen is a constituent of proteins, enzymes, chlorophyll, and plant growth regulators; and its deficiency causes reduced growth and increased functional stress [2]. Biological nitrogen fixation is the process of converting elemental nitrogen into the forms, ammonium (NH^+_4) and nitrates (NO^-_3), available to plants [3], which are subsequently incorporated into amino acids [4]. Nitrogen fixation is the second most important biological process after photosynthesis, and it is mediated exclusively by prokaryotes [2]. Some bacteria fix nitrogen in a free living state (non-symbiotic fixation). Others are closely associated with plant roots (associative nitrogen fixation), and still others form a mutualistic symbiosis [4].

Although free living nitrogen fixing organisms are widely distributed in soils, the quantity of nitrogen they fix seldom approaches that of the symbiotic systems. It is not that they are inherently incapable of vigorous nitrogen fixation, but abundant substrates to support their growth and fixation are commonly lacking in the soil;

whereas, plants can directly supply the high energy demands of this process in associative and symbiotic systems [5]. The nitrogen fixing activity of non-symbiotic, non-photosynthetic aerobic bacteria is strongly dependent on favourable moisture conditions, oxygen concentrations, and a supply of organic carbon substrates [6]. Nitrogen fixers are generally more active in rhizosphere soil; with plants that are capable of releasing exudates promoting higher nitrogen fixation in soil [7].

Azotobacter species are free living, obligate aerobic nitrogen fixers dominantly found in soils. They are also capable of growth under low oxygen tension [8]. *Azotobacter species* are non-symbiotic heterotrophic bacteria capable of fixing an average of 20 kg N/ha/year. They also produce plant growth promoting substances and are known to be antagonistic to plant pathogens [2]. *Azotobacter chroococcum* is the most prevalent species found [9], but other species include *A. vinelandii, A. beijerinckii, A. armeniacus, A. nigricans,* and *A. paspali* [10].

Micronutrients are important components of biological systems; they are required at low concentrations for growth and metabolic functions. These essential elements serve several functions: they represent prosthetic groups in many proteins and dictate the configuration of the active sites of enzymes; they serve as co-factors for some enzymatic reactions; they serve as multidentate centres for porphyrin molecules; and they serve as redox centres, transferring electrons in important redox reactions in cells - these functions are not mutually exclusive [11]. Some essential micronutrients are Cu, Zn, Fe, Ni, Mn, and Co. Microbes have evolved mechanisms that vary in specificity to accumulate these micronutrients from the surrounding environment [12].

The objectives of this study were to: determine the nitrogen fixing capacity of *Azotobacter chroococcum* in pure culture; measure the effects of different concentrations of cobalt and manganese (as $CoCl_2$ and $MnCl_2$) respectively on the nitrogen fixing capacity and cell mass growth of *A. chroococcum*; to explore the relationship between the nitrogen fixing capacity of *A. chroococcum* and its cell mass growth at different concentrations of cobalt and manganese respectively.

2. MATERIALS AND METHODS

2.1. Collection of soil samples

Rhizosphere soil samples were collected from *Musa paradisiaca* (plantain). Soil samples were collected at a depth of about 10-20 cm using sterile corers (sterilized with 95% ethanol). Soil samples were then passed through a coarse sieve (< 2 mm) to pulverize, remove any foreign material, and thoroughly mix them. Soil samples were transported to the laboratory in sterile containers at 4^{o}C, where they were kept refrigerated at 4^{o}C until required for analysis.

2.2. Isolation of test isolate (*Azotobacter chroococcum*)

10 g soil sample was homogenized in 90 ml sterile distilled water (with agitation for 15 minutes), and serially diluted in sterile distilled water in a proportion of 1:10 up to 10^{9}. 0.1 ml aliquot from each dilution was inoculated onto sterile solid Jensen's (nitrogen free) agar plates, and plated out using the spread plate method. Plates were incubated at prevailing room temperatures for 3-7 days. Discrete colonies were enumerated, and sub-cultured severally on Jensen's agar (SRL, India) to get axenic cultures. Pure isolates were stored on Jensen's agar slants at 4^{o}C.

2.3. Microbial analysis

The colonial morphology of pure isolates on agar plates were observed and recorded. Isolates were Gram-stained, spore-stained, and subjected to different biochemical tests: motility test; oxygen utilization test; catalase test; utilization of rhamnose, caproate, caprylate, meso-inositol, mannitol, malonate. Bacteria isolates were identified by comparing their morphological, microscopic, and biochemical characteristics with those of known taxa using the schemes of Bergey's Manual of Determinative Bacteriology [10].

2.4. Determination of effects of cobalt and manganese concentrations on the cell growth and nitrogen fixing capacity of *Azotobacter chroococcum*

A stock broth culture of *A. chroococum* was prepared. To prepare stock broth culture of *A. chroococum*, a sterile wire loop was used to introduce *A. chroococum* from Jensen's agar slant to sterile Jensen's (nitrogen free) broth (Jensen's medium manufactured by Sisco Research Laboratory PVT. Ltd, Mumbai, India), and incubated at prevailing room temperatures for 7 days under continuous airflow. From this stock broth, 0.5 McFarland standards were prepared [2]. Then 200 mg/l, 100 mg/l, 50 mg/l, 25 mg/l, 12.5 mg/l, and 0 mg/l sterile $CoCl_2$ and $MnCl_2$ solutions were prepared in half strength Jensen's broth [11]. Then 0.1 ml aliquot of 0.5 McFarland standard of *A. chroococcum* was added to 9.9 ml of each of the above sterile $CoCl_2$ and $MnCl_2$ solutions (concentration). This set up was incubated for 7 days at prevailing room temperatures under continuous airflow in a sterile chamber plugged to a Airfree T800 Filterless Air Purifier, (USA). The optical density at 600nm (OD_{600}) (using model 722 visible spectrophotometer, manufactured by Shanghai Third instrument Factory, China), nitrate-N concentration, and amino-N concentration of each test was measured at days 0 and 7 respectively [13].

2.5. Determination of nitrate nitrogen and amino nitrogen

Broth culture experiments were analyzed for nitrate nitrogen (NO_3-N) and amino nitrogen (Amino-N) at inception (Day 0) and at the end (Day 7) of the experiment. Nitrate-N concentration was determined by Cataldo's method [14]: 2.5 µl of sample solution was taken into a 1.5 ml Eppendorf tube, and 10 µl of salicylic acid-sulfate solution (500 mg of salicylic acid was dissolved in 10 ml of concentrated sulfuric acid) was mixed and kept for 20 minutes. Then, 250 µl of 2M NaOH solution (8.00 g of NaOH was dissolved in 100 ml of water) was mixed and kept for 20 minutes. 200 µl of the reaction solution was put in a 722 visible spectrophotometer and the absorption at 410 nm was measured. Standard solution was made by dissolving 42.5 mg of $NaNO_3$ in 100 ml of water, which contains 5 mM nitrate (70 mg N l^{-1}). Diluted solutions (0, 1, 2, 3, 4, 5 mM) were used for the calibration and plotting of standard curve.

Amino-N concentration was determined by ninhydrin method [15]: 2.5 µl of sample solution was taken into a 1.5 ml Eppendorf tube, and 75 µl of citrate buffer (5.6 g of citrate and 2.13 g of NaOH was dissolved in 100 ml of water) was mixed. Afterwards, 60 µl of ninhydrin solution (958 mg of ninhydrin and 33.4 mg of ascorbate was dissolved in 3.2 ml of water and filled up to 100 ml with methoxyethanol in a flask. The flask was secured with its cork and heated at 100^{o}C for 20 minutes in a hot air oven (Quincy Hydraulic Gravity Convection Oven, USA.). Then, 300µl ethanol was added and cooled to room temperature for 10 minutes. 200 µl of the reaction solution was put in a 722 visible spectrophotometer and the absorption at 570 nm was measured. Standard solution was made by dissolving 66.1 mg of asparagine (or 70.1 mg of asparagine monohydrate) plus 73.1 mg glutamine in 100 ml of water, which contains 5 mM asparagine + 5 mM glutamine (280 mg N L^{-1}). Diluted solutions (0, 28, 56, 84, 112, 140 mg N L^{-1}) were used for the calibration and plotting of standard curve.

2.6. Determination of specific growth rate

The specific growth rate of *Azotobacter chroococum* was determined using the formula proposed by Stanier et al. [16].

$$\text{Specific growth rate} = \frac{\text{Log } OD_1 - \text{Log } OD_0}{T_1 - T_0} \times 2.303$$

Where,

Log OD_1 = Log value of optical density (OD) of culture at time T_1 days

Log OD_0 = Log value of optical density (OD) of culture at time T_0 days.

2.7. Estimation of nitrogen fixed

The amount of nitrogen fixed was estimated with the formula:

$$\text{Nitrogen fixed} = N - N_0$$

Where,

N = the total concentrations of nitrate nitrogen and amino nitrogen in culture medium after incubation

N_0 = the total concentrations of nitrate nitrogen and

amino nitrogen in culture medium before incubation (at inception).

2.8. Estimation of nitrogen fixation rate

Nitrogen fixation rate was estimated using the formula:

$$\text{Nitrogen fixation rate} = \frac{N - N_0}{T - T_0}$$

Where,

$N - N_0$ = Nitrogen fixed

$T - T_0$ = incubation period

2.9. Statistical analysis

All measurements were made in triplicate, and values reported as mean of triplicate values. Student t tests and Pearson's correlation analysis for all possible variable pairs were estimated using Minitab 17 software. Significant difference was taken at 5% level of significance ($p<0.05$).

2.10. Kinetic modeling for product synthesis (nitrogen fixation)

The Luedeking-Piret model was applied to analyze product synthesis (nitrogen fixation) kinetics, using Curve Expert Professional 2.4.

3. RESULTS AND DISCUSSION

Experimental data revealed that the trials with no micronutrient (Co and Mn) added (control) had a cell mass yield of X_0 = 0.042 OD_{600} units, and a nitrogen fixation yield of P_0 = 0.847 ppm respectively, after seven days incubation. Similarly, experimental data revealed maximum cell mass concentrations, X_{max}, (which occurred at 200 mg/l for both cobalt and manganese) of 0.100 OD_{600} units and 0.092 OD_{600} units for cobalt and manganese respectively (plots not shown).

Cobalt enhanced nitrogen fixation of *Azotobacter chroococcum* at 12.5 mg/l and 25 mg/l $CoCl_2$ concentrations, while 50 mg/l, 100 mg/l, and 200 mg/l $CoCl_2$ concentrations abated the nitrogen fixation of *A. chroococcum*. On the other hand, manganese enhanced nitrogen fixation of *Azotobacter chroococcum* at 12.5 mg/l $MnCl_2$ concentration, while 25 mg/l, 50 mg/l, 100 mg/l, and 200 mg/l $MnCl_2$ concentrations abated the nitrogen fixation of *A. chroococcum* (Figure 1). Figure 1 also showed that maximum nitrogen fixations, P_{max}, occurred at 12.5 mg/l concentration for both cobalt (1.936 ppm) and manganese (1.368 ppm) respectively.

Contrary to the results of nitrogen fixation, the specific growth rate of *A. chroococcum* was enhanced by all the micronutrient concentrations tested for both cobalt and manganese; with specific growth rate increasing concurrently with increasing cobalt and manganese concentrations respectively. Thus, in these experiments, increase in cell mass of *A. chroococcum* did not always translate to increase in nitrogen fixation. Maximum growth rates, μ_{max}, recorded for Co and Mn were 0.131 OD units/day and 0.119 OD units/day respectively.

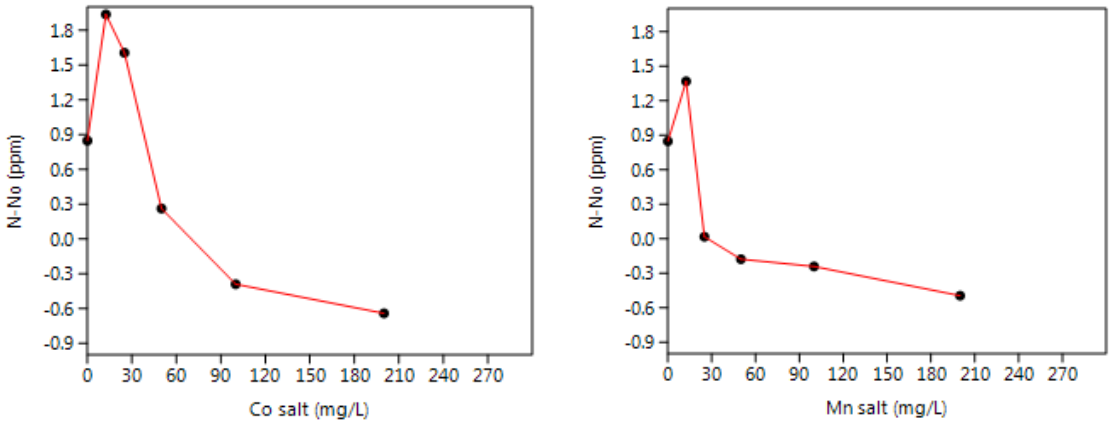

Figure 1. Nitrogen fixation yields of *Azotobacter chroococcum* at different cobalt and manganese concentrations.

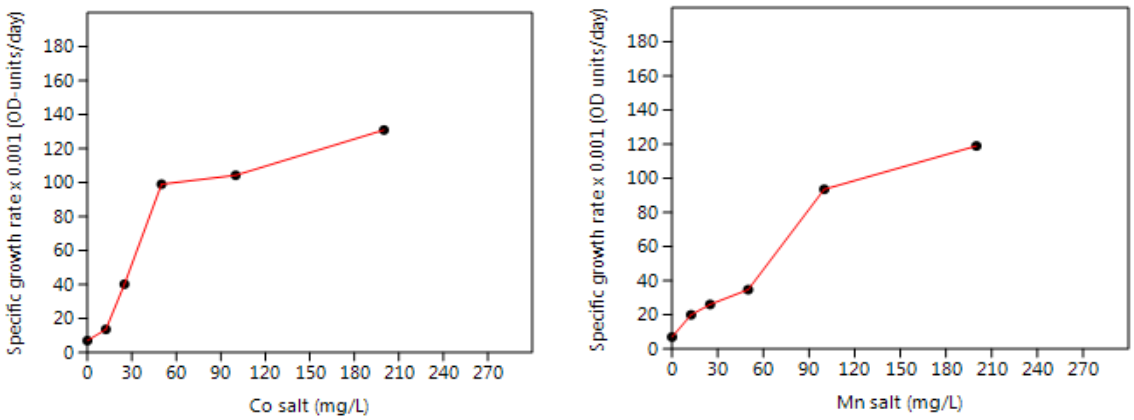

Figure 2. Specific growth rates of *Azotobacter chroococcum* at different cobalt and manganese concentrations.

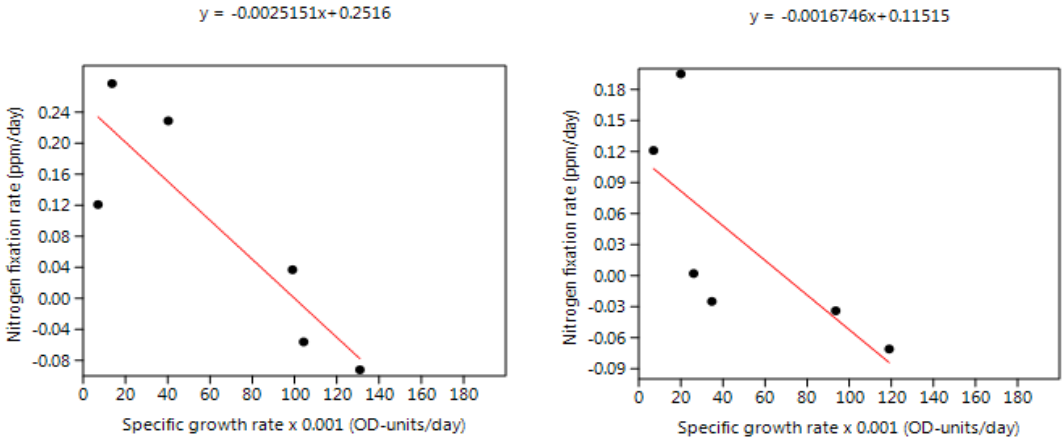

Figure 3. Luedeking-Piret model of *Azotobacter chroococcum* for different of cobalt and manganese concentrations.

Though experiments revealed comparatively higher nitrogen fixation and specific growth rate values in cobalt trials, Student t tests showed no significant difference ($p>0.05$) in the specific growth rates and nitrogen fixation rates respectively between the cobalt and manganese broth trials of *A. chroococcum*. Pearson's correlation analysis showed very strong and direct correlations ($p<0.05$) between micro-element (Co and Mn) concentrations and cell mass growth, with coefficient of correlations (r) of 0.913 and 0.975 for Co and Mn respectively. However, correlations between micro-element concentrations and amount of nitrogen fixed were indirect and relatively weaker, with coefficient of correlations (r) of -0.822 ($p<0.05$) and -0.732 ($p>0.05$) for Co and Mn respectively. This indirect relationship between nitrogen fixation and population size was also reported by Chang and Knowles [17].

The Leudeking-Piret model [18] was applied to determine the type of relationship existing between cell mass and nitrogen fixation by *A. chroococcum in vitro.*

$$r_{fp} = \alpha r_{fx} + \beta x \quad (1)$$

Where,

r_{fp} = rate of product formation

r_{fx} = rate of biomass formation

α = coefficient of proportionality between the rate of product formation and growth rate (ppm/OD-units)

β = coefficient of proportionality between the rate of product formation and biomass concentration (ppm/OD-units/day).

According to this model, the product formation rate (nitrogen fixation rate) depends linearly upon the growth rate and the cell mass concentration.

$$\frac{\delta P}{\delta t} = \alpha \frac{\delta x}{\delta t} + \beta x \quad (2)$$

$$\beta = \frac{(dP/dt)\ \text{stationary phase}}{X_s} \quad (3)$$

X_s = cell concentration at stationary phase

The other kinetic constant, α, can be calculated using the yield coefficient, $Y_{p/x}$, which is given as:

$$Y_{p/x} = \frac{\text{Mass of product formed}}{\text{Mass of cell formed}} = \frac{\Delta P}{\Delta X} = \frac{P - P_0}{X - X_0} \quad (4)$$

Integrating equation 3 gives:

$$P(t) - P(0) - \beta(X_s/k)\,[1 - X_0/X_s\,(1 - e^{kt})] = \alpha(X_t - X_0) \quad (5)$$

A linear plot of nitrogen fixation rate against specific growth rate generated α and β coefficients for Co and Mn respectively (Figure 3). For Co, α = -0.0025151 and β = 0.2516; while for Mn, α = -0.0016746 and β = 0.11515. If $\alpha \leq 0$, then nitrogen fixation is associated with cell mass concentration; on the other hand, if $\beta \leq 0$, then nitrogen fixation is associated with cell mass growth; however, if $\alpha > 0$ and $\beta > 0$, then nitrogen fixation is associated with both cell mass growth and cell mass concentration [19]. From the results obtained in this work, nitrogen fixation (product synthesis rate) of *A. chroococcum* was associated with cell mass concentration, X_c, and not with cell (bacterial) mass growth (for both Co and Mn trials). According to Wright and Weaver [20], a sizeable population may be present without providing the enzyme activity needed for significant rates of nitrogen fixation; nevertheless at the attainment of a critical population size the needed biomass and nitrogenase for significant rates of nitrogen fixation is provided. Since nitrogen fixation in both experiments depend solely on biomass concentration, it implies that the substrates consumed (Co and Mn) were required for both the fixation of nitrogen and also for the growth of *A. chroococcum* [21]. Also since nitrogen fixation was solely dependent on biomass concentration for Co and Mn, it means that the nitrogen fixed by *A. chroococcum* in these trials is a secondary metabolite [22]. When non-growth associated product formation is modeled as a phenomenon associated with the secondary metabolite formation, the rate of the product formation is linked to the endogenous rate of the cellular degradation (endogenous metabolism). The product formation is thus a process that is secondary to the biomass growth. In addition, Zerajic and Savkovic-Stevanovic [23] stated that in such a situation, product formation but not growth is subsequently inhibited by the concentration of the substrate. This phenomenon expressed by Stevanovic [23] agrees with the data obtained in this work, where after a certain concentration, increased Co and Mn concentrations concurrently abated nitrogen fixation, but still enhanced cell mass growth.

4. CONCLUSIONS

The results obtained in this study suggest that the micronutrients, cobalt and manganese, impacted both on the cell mass growth and nitrogen fixation of *Azotobacter chroococcum*, but in different ways. All the concentrations of cobalt and manganese tested enhanced cell mass growth, while at given concentrations nitrogen fixation started to wane for both cobalt and manganese. Nitrogen fixation was found to be associated with biomass density, rather than with cell mass growth.

Further studies are recommended were similar investigations may be done *in situ* in soil, and compared with *in vitro* results.

AUTHOR'S CONTRIBUTION

JO: Project supervisor, research design; CN: Research design/development, experimental design, mathematical/ statistical analysis; HA: Sample collection and laboratory assistance; AA: Sample collection and laboratory assistance. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors have no conflict of interest to declare.

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