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Dear Dr. Bahadur,

Your revised manuscript: "Effect of osmium on microbial nitrogen fixation in the presence and absence of molybdenum" has been received and reviewed once more by the editorial committee of this journal. Unfortunately, no recommendation has been given to accept the revised manuscript. This is mainly due to the following reasons:

- The paper has not been reduced in size, as recommended.
- The answers to the questions of the editorial committee have not been incorporated in the revised manuscript satisfactorily.
- No more data have been given to support the conclusions as had been suggested.
- In your tables columns "Difference against beginning" have been included. However, it appears the numbers really give the differences to zero treatment. No information on the time course of the nitrogen fixation is given. Hence, these new columns do not make sense.

Therefore, I am sorry, I cannot accept your manuscript in this journal for publication, even though the results are very interesting.

Please, find enclosed your manuscript.

Sincerely yours,

A. Jungk

Prof. Dr. A. Jungk
Editor Plant Nutrition

Enclosure

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# EFFECT OF OSMIUM ON MICROBIAL NITROGEN FIXATION IN THE PRESENCE AND ABSENCE OF MOLYBDENUM.

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Die Wirkung des Osmiums auf mikrobielle Nitrifizierung durch Bakterien in Anwesenheit und Abwesenheit von Molybdän.

## Key words

Osmium, molybdenum, nitrogen fixers, mg nitrogen fixed per g carbon consumed.

Osmium, Molybdän, Stickstoff-Fixierer

## ABSTRACT

The effect of various concentrations of osmium, in the culture media on the nitrogen fixing activity of the three nitrogen fixing microorganisms isolated from Allahabad soil, is studied in the presence and absence of molybdenum. Our studies show that osmium in the culture media acts as a very good activator for nitrogen fixing activity of all the three organisms. Also, in absence of molybdenum the organisms fix almost negligible amounts of nitrogen. The joint effect of molybdenum and osmium is quite favourable for nitrogen fixation, under the conditions studied.

## Übersicht

Die Wirkung von verschiedenen Konzentrations Mengen des Osmiums in Anwesenheit und Abwesenheit von Molybdenum in den Nährboden auf die Stickstoff-Fixierungs tätigkeit der drei nitrifizierenden Mikroorganismus die aus Allahabad-Erdteil abgesendet wurden, wird untersucht. ES wird beobachtet, daß in Anwesenheit von Osmium im Nährboden die Nitrifizierung durch den Organismus erheblich aussteigt. In Abwesenheit von Molybdenum, die Nitrifizierung durch den Organismus ist unwesentlich. Die Unter Suchungen ergeben daß die Anwesenheit von beide Osmium and Molybdenum im Nährboden günstig beeinflußt.

INTRODUCTION

Considering electron activation two site hypothesis for the mechanism of biological nitrogen fixation (HARDY et al 1971), it is said that two different sites exist for nitrogen fixation. The first one being the electron activation site named as X site and the second, the substrate complexing site named as Y site. Substrates of nitrogenase are complexed to Y, activated and held in juxta position for reduction by electrons from X reduced. A transition metal is suggested for Y. This leads us to study the effect of Osmium salt on the nitrogen fixing activity of the organisms.

According to BOROD'KO et al (1967), osmium complexes of nitrogen form a new class of compounds,with nitrogen in the metal coordination sphere. These complexes are of interest as possible intermediates in catalytic fixation of molecular nitrogen (BOROD'KO et al 1967; KHAN and MARTELL 1975). Compounds of Os, Rh, Co, Ru and Ir with emphasis on the studies of mechanisms by which atmospheric nitrogen is fixed have also been reported (CHATT, J, 1969; CHATT, J and FOGG 1969). They reported that transition metals can form stable complexes with molecular nitrogen.

It is well known now that the key enzyme of nitrogen fixation pathway, "nitrogenase" consists of two proteins. One of these proteins consists of two molybdenum atoms (WINSTON J. BRILL 1978 and HARDY et al 1971). Other reports are also there by which it is apparent that molybdenum is indispensable for the utilization of atmospheric nitrogen by organisms(GRADOVA 1967, MORTENSON et al 1967 and ILINA 1966).

Considering above observations we have studied effect of various concentrations of osmium and also joint effect of various concentrations of molybdenum with a particular osmium concentration on the nitrogen fixing activities of the three organisms. Our studies show that osmium tetraoxide in the culture media acts as a very good activator for nitrogen fixing activity of all the three organisms. Also, in absence of molybdenum the organisms fix almost negligible amounts of nitrogen. The joint effect of molybdenum and osmium is quite favourable for nitrogen fixation, under the conditions studied.

MATERIALS AND METHODS

The culture media comprised of three solutions - A, B and C.

Solution A consisted of 0.1 g sodium chloride, 0.1 g potassium sulphate, 0.01 g sodium molybdate, 0.1 g disodium hydrogen phosphate and 0.001 g ferrous sulphate dissolved in 100 ml glass distilled water. The pH of this solution was adjusted to 7.5 by adding phosphate buffer of pH 7.5. This buffered solution was made upto 500 ml with glass distilled water. In experiments with molybdenum, sodium molybdate was not added.

Solution B - 0.2 g magnesium chloride and 0.1 g calcium chloride were dissolved in 250 ml glass distilled water.

Solution C - 15 g of mannitol was dissolved in varying amounts of double distilled water along with requisite amount of 1200 µM stock solution of osmium tetraoxide to get 250 ml solution C

containing 300, 600 or 900 µM of osmium tetra oxide. The stock solution of osmium tetra oxide was prepared by dissolving it in water and determining the metal content according to SCOTT (1956). (The osmium salt was obtained from Johnson, Mathey and Co. London).

For the experiments of joint effect of osmium and molybdenum, 15 g of mannitol was dissolved in varying amounts of double distilled water along with requisite amount of 1200 µM stock solution of osmium tetra oxide and 1200 µM or 400 µM stock solutions of molybdate solution to get 250 ml solution C containing 300, 400, 500 or 600 µM of molybdenum. The three solutions were sterilized in an auto clave at 15 lb. p.s.i.for 30 minutes. These three solutions were then mixed aseptically in the ratio A:B:C:: 2:1:1. The culture media thus prepared had 75, 150 and 225 µM of osmium, and for the second set of experiments 75, 100, 125 or 150 µM of molybdenum. Inoculation was done by transferring 0.2 ml of 5 days old culture into 15 ml aliquots of the prepared culture medium taken in several cotton plugged, sterilized flasks. All the flasks were kept in an incubator at 32°C for 15 days. After this period, 5 ml of the culture was analysed for carbon consumption by Kjeldahl method as described by ROBINSON et al (1929). Another aliquot of 5 ml culture was estimated for nitrogen by Kjeldahl method according to the procedure adopted by KOLTHOFF and STENGER (1947) and MOORE (1920).

In the procedure of nitrogen estimation the ammonia liberated was absorbed in 4% boric acid solution having Tashiro's indicator (TASHIRO, 1922). The liberated ammonia was titrated against standard sulphuric acid and the amount of nitrogen fixed was calculated.

The three nitrogen fixers studied here are designated as $D_3$, $B_3$ and B. Morphological as well as physiological features of these organisms are different from each other. All the experiments throughout the investigation are evaluated statistically.

## RESULTS AND DISCUSSION

The results of amount of nitrogen fixed and carbon consumed by the three nitrogen fixers $D_3$, $B_3$ and B at different concentrations of osmium in the culture media, and the joint effect of various concentrations of molybdenum in presence of the optimum concentration of osmium for the particular nitrogen fixer, are given in tables I and II respectively.

In general, our results show that the nitrogen fixing activity of all the three nitrogen fixers is highly increased if both osmium and molybdenum are present in the culture medium at a time, but they are unable to fix nitrogen in absence of molybdenum, even if considerable amount of osmium is present in nutrient media.

Table I shows that ~~except the organism $D_3$,~~ the nitrogen fixing activity of organisms $B_3$ and B is greatly increased in the presence of 75, 150 and 225 μM of osmium in the culture medium. Nitrogen fixation by the organism $D_3$ is inhibited in presence of 225 μM of osmium as compared to the amount of nitrogen fixed initially by the organism in a media having no osmium. This organism fixes almost double amount of nitrogen in a media having 150 μM of osmium. It is noticeable that maximum amount of mg nitrogen per g carbon consumed is fixed by this organism at this concentration only. With this optimum concentration in the nutrient media, $D_3$ fixes surprisingly high amounts of nitrogen when 75, 100 or 125 μM molybdenum is also present. Only in media having 150 μM molybdenum in addition to 150 μM osmium, nitrogen fixation is less; but this nitrogen fixation is still quite satisfactory. From the table I, it is evident that the optimum osmium concentration requirements of the three nitrogen fixers for fixing maximum nitrogen is different. Except the organism $D_3$, the nitrogen fixing activity of $B_3$ and B is linear with respect to osmium concentration from 0 to 225 μM. Presence of molybdenum in addition to optimum concentration of osmium in the nutrient media is too beneficial for these two nitrogen fixers also. The organism $B_3$ and B fix 71.71 and 83.34 mg nitrogen respectively (per g carbon consumed) in presence of 100 μM molybdenum and 225 μM osmium in the culture media. Other concentrations of molybdenum are also effective for these organisms. The most striking feature of these studies is that though the organism B fixes least amount of nitrogen initially, in a media having both osmium and molybdenum

(in media without Mo)

its nitrogen fixing activity increases remarkably. One of the remarkable features of these studies is that in presence of osmium, though the three organisms fix high amounts of nitrogen at different concentrations but sugar consumption remains more or less steady; whereas this is not true while observing the joint effect of osmium and molybdenum. Here, the sugar consumption by the organisms is also increased with increase in nitrogen fixing activity. From the studies it is clear that molybdenum is indispensable for nitrogen fixation and this when present in nutrient media along with osmium favours the nitrogen fixation tremendously. Also, osmium alone, when present in culture media at different concentrations enhance the nitrogen fixing activity of all the three nitrogen fixers. This transition metal may exert its effect by forming stable nitrogen complexes (CHATT. J, 1969 and CHATT. J and FOGG 1969) and catalyzing the fixation of nitrogen (VOL'PIN et al 1968) by stabilization of nitrogenases (BENEMANN et al 1972).

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TABLE I

Amount of nitrogen fixed and carbon consumed by the organisms in 5 ml culture media having varying concentrations of Osmium Oxide at pH 7.5.

| Organism | Osmium concentration (μM) | Total nitrogen fixed (μg) | Statistical error | Difference against beginning | Carbon consumed (mg) | Statistical error | Difference against beginning | mg nitrogen fixed/g carbon consumed |
|---|---|---|---|---|---|---|---|---|
| $D_3$ | 0 | 339.36 | ± 1.57 | - | 7.89 | ± 0.03 | - | 42.98 |
| | 75 | 509.04 | ± 2.44 | +169.68 | 8.25 | ± 0.04 | 0.36 | 61.63 |
| | 150 | 640.08 | ± 2.52 | +300.72 | 8.52 | ± 0.06 | 0.63 | 75.07 |
| | 225 | 328.44 | ± 1.57 | − 10.92 | 8.77 | ± 0.05 | 0.88 | 37.42 |
| $B_3$ | 0 | 304.08 | ± 2.52 | - | 9.30 | ± 0.05 | - | 32.69 |
| | 75 | 405.72 | ± 2.14 | +101.64 | 9.32 | ± 0.06 | 0.02 | 43.50 |
| | 150 | 448.56 | ± 1.57 | +144.48 | 9.61 | ± 0.05 | 0.31 | 46.64 |
| | 225 | 465.36 | ± 1.57 | +161.28 | 9.83 | ± 0.04 | 0.53 | 47.30 |
| B | 0 | 173.88 | ± 1.02 | - | 7.39 | ± 0.05 | - | 23.50 |
| | 75 | 318.36 | ± 1.57 | +144.48 | 7.63 | ± 0.05 | 0.24 | 41.69 |
| | 150 | 367.92 | ± 2.14 | +194.04 | 7.81 | ± 0.05 | 0.42 | 47.08 |
| | 225 | 413.28 | ± 2.52 | +239.40 | 8.32 | ± 0.05 | 0.93 | 49.64 |

TABLE II.

Amount of nitrogen fixed and carbon consumed by the organisms in 5.0 ml culture media having optimum osmium and varying concentration of molybdenum at pH 7.5.

| Organism | Osmium concentration(μM) | Molybdenum concentration (μM) | Total N fixed (μg) | Statistical error | Difference against beginning | Carbon consumed (mg) | Statistical error | Difference against beginning | mg nitrogen fixed/g carbon consumed. |
|---|---|---|---|---|---|---|---|---|---|
| $D_3$ | 150 | 0 | 231. 4 | ± 2.86 | - | 6.78 | ± 0.08 | - | 34.09 |
| | | 75 | 640.08 | ± 2.52 | 408.68 | 8.52 | ± 0.04 | 1.74 | 75.07 |
| | | 100 | 972.16 | ± 1.37 | 740.76 | 12.43 | ± 0.03 | 5.65 | 78.21 |
| | | 125 | 767. 2 | ± 0.74 | 535. 8 | 9.24 | ± 0.06 | 2.46 | 83.01 |
| | | 150 | 687.68 | ± 2.08 | 456.28 | 11.60 | ± 0.04 | 4.82 | 59.27 |
| $B_3$ | 225 | 10 | 235. 2 | ± 3.06 | - | 8.52 | ± 0.23 | - | 27.58 |
| | | 75 | 465.36 | ± 1.57 | 230.16 | 9.83 | ± 0.04 | 1.31 | 47.30 |
| | | 100 | 608.16 | ± 1.37 | 372.96 | 8.48 | ± 0.04 | — 0.04 | 71.71 |
| | | 125 | 829.36 | ± 1.37 | 594.16 | 11.60 | ± 0.04 | 3.08 | 70.09 |
| | | 150 | 260.96 | ± 3.36 | 25.76 | 8.56 | ± 0.05 | 0.04 | 30.45 |
| B | 225 | 0 | 70.56 | ± 3.35 | - | 6.77 | ± 0.07 | - | 10.41 |
| | | 75 | 413.28 | ± 2.52 | 342.72 | 8.32 | ± 0.05 | 1.55 | 49.64 |
| | | 100 | 657.44 | ± 2.24 | 586.88 | 7.88 | ± 0.08 | 1.11 | 83.34 |
| | | 125 | 564.48 | ± 2.09 | 493.93 | 9.84 | ± 0.04 | 3.07 | 57.31 |
| | | 150 | 592.48 | ± 2.74 | 521.92 | 9.23 | ± 0.05 | 2.46 | 64.18 |