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Research Article

Effect of Seven Major Plant Micronutrient on Growth of Bio-inoculant *Rhizobium leguminosarum* – Host – *Pisum sativum* (Pea).

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Abstract

The nitrogen assimilating bacterium *Rhizobium leguminosarum* is a symbiotic nodule forming bacterium having host specificity for *Pisum sativum*. Ammendment of compounds of plant micronutrients such as MnO, KCl, $CuSO_4$, NH_4MoO_4, $ZnSO_4$, $FeSO_4$, H_3BO_3 at various concentrations in Y.E.M.A could not completely suppress the growth of bacterium, whereas at concentrations of 0.5% (MnO), 0.5% (NH_4MoO_4) and 0.7% ($FeSO_4$) the bacterium showed same growth as compared to control and optimum bacterial growth were lesser in 1.5% (KCl), 1.3% ($CuSO_4$), 0.1% ($ZnSO_4$) and 1.3% (H_3BO_3) over the control (Y.E.M.A). However by incorporating optimum concentration of these seven compounds of plant micronutrients (multi-micronutrient), the in vitro studies synergistically showed similar growth of *Rhizobium leguminosarum* than the growth in control. The thus modified media with seven vital plant micronutrients support the minimum viability standards as per Bureau of Indian standards as well as the Fertiliser control order 1985, and this strengthens the concept integrated organic nutrient (major as well as seven minor) management for the agricultural crop i.e. *Psivum sativum*.

Keywords: *Catharanthus roseus*, Antimicrobial activity and Phytochemical analysis.

Introduction

Commercial Agricultural crop yields mainly show dependencies on soil fertility, thus the healthy flora is characterized by the thin layer of soil covering earth's surface (Doran and Zeiss2000). Soil is the living mixture of minerals and organisms that provides vital nutrient that nurtures crops growth, therefore the soil is divided into two parts as biotic and abiotic (living and non living).

Microbial Inoculants that enhances the plant growth by nitrogen fixing, solubilizing or mobilizing phosphates and potash, sulphur etc. (Gomare, *et al*., 2013) forms together with other rhizospheric micro organsms helping flora - the biotic factor, such microorganisms are now being called as agriculturally important microorganisms. The abiotic part contains elements in its free or in compound forms, which are required by microorganisms and or flora for their growth. They are divided into two types as major nutrient and minor nutrient.

The optimum availability of a-biotic factors are in fidelity with "rhizospheric" agriculturally important microorganisms (Jonas *et al*., 2011). An ideal microcosms is needed for agriculturally important microorganisms to harbor in rhizospheric soil and provide nutrient to the crop and this favor the crop commercial yield.

PGPR (Plant Growth Promoting Rhizobacteria), are grown in scientifically proven media then mixed with suitable inert carriers such as lignite to produce bio-inoculants. These bio-inoculants so produced provide nutritional support to crops like Soyabean, Cowpea, Groundnut, Black-gram, and Red-gram (Gomare, *et al*., 2013). The present study is made to find the effect

of seven major compounds of plant micronutrients on the growth of *Rhizobium leguminosarum* inhabiting *Pivum sativum* (Pea).

Materials and Methods

Sample Collection

Bacterial Strain

Rhizobium leguminosarum are the native nodule forming bacteria in *Pisum sativum*. The bacterium used in the present study is *Rhizobium legumanosarum* which is also the production strain of Biofertilizer Production Plant, of the M.P. State Agro Industries Development Corporation Limited, Bhopal, India and has high nitrogen fixing efficiency.

Studies on Effect of Micronutrients on Bacterial strain

The pure culture of *Rhizobium leguminosarum* are inoculated in yeast extract mannitol broth and incubated at room temperature for 48 hours to 72 hours till the cell concentration exceeds the optical density (OD) 1 at 620 nm and a viable cell count of 1.0×10^{10} per ml of matured (stationary phase bacteria) broth. This matured broth is then used to inoculate the experimental medium supplemented with various micronutrients under study.

a) Studies on Various Concentration of Micronutrients on Bacterial strain:

Yeast extract mannitol broth was modified by incorporating various percentage concentrations of the each compound of micronutrient (Table. 1). 5% of YEM broth with *Rhizobia* cells was inoculated in these modified broths, incubated for 48 hours then viable cells were counted by plate count method at 10^9 dilution.

b) Screening of Optimum Concentration of Micronutrient for the Growth of Bacteria

Repeating the experiment by using micronutrient in a concentration separately that were showing maximum readable growth in previous experiments for each compound using the concentration of micronutrients ranging from above and below the concentrations with maximum growth from the previous experiments (table 2).

c) Growth of bacteria in Optimized Multi-micronutrients Concentration.

5% of YEM broth with Rhizobia cells, were inoculated in a broth containing all seven compound of micronutrient with optimized concentration as found in earlier experiments and incubated for 48 hours then viable cells were again counted by plate count method at 10^9 dilution. The overall growth was compared with the growth in the control nutrient suspension (table 3).

Results and Discussion

a. Effect of Various Concentration of Micronutrient on the Growth

Maximum bacterial growth (growth/ 0.1 ml of inoculum size) is observed in each experimental media containing MnO, KCl, $CuSO_4$, NH_4MoO_4, $ZnSO_4$, $FeSO_4$ and H_3BO_3 are 0.5%, 1.5%, 1.3%, 0.5%, 0.1%, 0.7% and 1.3% respectively. (Table 1). Trace elements/metal function as co-factors in enzymatic reactions, stabilizing structure of enzyme itself. (Zhuoer Lin *et al*., 2009). Zinc is a multi-functional element found in almost 300 enzymes, and is involved in catalytic, co-catalytic, and/or structural functions; enzymes containing zinc in the reactive center are widespread in nature (Tubek*et al*., 2008). Sodium and potassium maintains osmoregulation, electrolytic balance and membrane transport system; copper and manganese are vital components of biological redox reactions (Zhuoer Lin *et al*., 2009). Boron is an indispensible micronutrition for bacteria, fungi and higher plants essential in sugar transport, cell wall synthesis and lignification, cell wall structure, carbohydrate metabolism, RNA metabolism, respiration, indole acetic acid metabolism, phenol metabolism, and membrane transport (Blevins and Lukaszewski1994; Camacho-Cristóbal*et al*., 2008); although it is toxic to microbes above a certain level. (Ahmed and Fujiwara 2010)

b. Screening of Optimum Micronutrient Concentration for Bacterial Growth

Both prokaryotic and eukaryotic organisms essentially utilize the elements including iron, cobalt, zinc, manganese, nickel, copper, phosphorus, boron, potassium, molybdenum etc in suitable concentration for various vital activities of biological processes (Zhuoer Lin *et al*., 2009).

As the experiments for screening of optimum concentration of micronutrients suitable for the growth of *Rhizobium japonicum* were designed on the basis of the observations of previous experiments in such a way that the concentration of the compound that is showing maximum readable growth of the bacteria, the percentage concentration below and above were used to find the optimum concentration. With reference to the table 2, the optimum concentration of the compounds MnO, KCl, $CuSO_4$, NH_4MoO_4, $ZnSO_4$, $FeSO_4$ and H_3BO_3 in the medium showing maximum bacterial growth are 0.5%, 1.5%, 1.2%, 0.6%, 0.09%, 0.7%, and 1.3% respectively. All the growth responses were compared with the standard yeast extract mannital media as a control. (Table 2.)

c. Effect of Optimum Concentration of Multiple Micronutrients on Bacterial Growth

The achievement of optimized fermentation conditions predominantly depends on physical and chemical factors which are highly significant in the development of any process as they affect the economics and feasibility (Duta *et al*., 2006).

The compounds used in present study when mixed collectively in the optimum concentration in the growth medium of bacteria as per the observations in the previous set of experiments in a concentrations of 0.5%, 1.5%, 1.2%, 0.6%, 0.09%, 0.7%, and 1.3% for the compounds MnO, KCl, $CuSO_4$, NH_4MoO_4, $ZnSO_4$, $FeSO_4$ and H_3BO_3 respectively (Table 3); the results showed astonishingly mat growth whereas the control (standard Y.E.M. media) showed Profuse growth.
The chief objective of the present investigation is to know the synergistic effect of concentration of compound of plant micronutrients in maximum growth of *Rhizobium leguminosarum* enabling it to be incorporated in the standard universal media by commercial industries of the strains of Biofertiliser/ Bio-inoculant. This ensures the plant for requirement of micronutrient which is in part per million (p.p.m) level and therefore can be provided through incorporating it in the universal standard media, ascertaining bacterial viability standards at par with

Bureau of Indian Standards and Fertiliser Control Order act, 1985.

Rhizobium is the Gram negative aerobic rod shaped bacteria first isolated by Beijerinck in 1888 fixing atmospheric nitrogen and exist in nature harboring in the nodule of the leguminious crops (Tilak 1998; Kumarsen 2005; Singh 2008). Further field trial research is needed to understand the crop yield, grain index, chlorophyll content, dry ash content etc to know the effect of *Rhizobium leguminosarum* thus grown in the modified, universal standard media containing various optimum concentration of compounds of plant micronutrients and then coated on seeds of *Psivum sativum* (Pea) crop and then sown during Rabi cropping season.

The bacterial viability in the form of colony forming units (CFU) per 0.1ml values at various concentrations (weight/volume) of separately used compounds of micronutrients showed no consistency and co-relativeness, however remarkable consistent and co-relative in the growth of bacteria were seen between the immediate next concentrations (weight/volume) of compounds of micronutrient and bacterial viability in the experiment to find out the optimum concentration range. In the final experiment when optimum concentrations of each compound of micronutrients were incorporated in the universal standard media where much more C.F.U/ 0.1 ml has been observed than the control i.e. universal standard yeast extract mannitol media without micronutrients. This clearly reveals that all such optimum concentration of compounds of micronutrient incorporated in the medium are the sure necessity of *Rhizobium leguminosarum* for its growth.

The microbial or bacterial viability in the form of Colony Forming Units (C.F.U) per ml values as per the Fertiliser Control Act 1985 standards for biofertilisers are 1 X 10^7 C.F.U/ ml. Though certain maximum viability values (C.F.U/ 0.1 ml at 10^9 dilution) of media devised in first set of experiments by individually incorporating compounds of plant micronutrients are less than the control (C.F.U/ 0.1 ml at 10^9 dilution), but since these value (C.F.U/ 0.1 ml at 10^9 dilution) confirm the standards of Fertiliser Control Order act 1985 as well as of Bureau of Indian standards, therefore these values (C.F.U/ 0.1 ml at 10^9 dilution) of concentration of compounds of plant micronutrients were considered optimum for further

Table 1: Effect of various concentration of each micronutrient on growth of bacteria (*Rhizobium leguminosarum).*

S. No	Concentration (wt/ vol)	C.F.U/ 0.1 ml at 10^9 dilution						
		MnO	**KCl**	**$CuSO_4$**	**NH_4MoO_4**	**$ZnSO_4$**	**$FeSO_4$**	**H_3BO_3**
1	0.1%	2432	1368	564	668	**7490**	982	688
2	0.3%	5464	4268	896	1284	1622	564	1536
3	0.5%	**Profuse**	3642	6212	**Profuse**	1358	1516	2184
4	0.7%	2658	6576	2354	1536	542	**Profuse**	3124
5	0.9%	6612	5818	1196	4518	3684	1262	1274
6	1 %	4286	4326	4672	3972	2382	4196	4968
7	1.1%	3516	6890	3596	5734	624	5412	3268
8	1.3%	1594	6574	**9256**	2648	1464	1794	**8342**
9	1.5%	1298	**8894**	3218	3624	1736	1276	2864
10	Control	Profuse	Profuse	Profuse	Profuse	Profuse	Profuse	Profuse

Table 2: Optimization of the micronutrient concentration on growth of *Rhizobium leguminosarum*.

S.no	Compound of micronutrient.	Concentration of micronutrient (%) (wt/ vol)	C.F.U/ ml at 10^9 dilution
1	**MnO**	0.4%	++++
		0.5%	**+++++**
		0.6%	++++
		Control	+++++
2	**KCl**	1.4%	++++
		1.5%	**+++++**
		1.6%	+++
		Control	+++++
3	**$CuSO_4$**	1.2%	**++++**
		1.3%	+++
		1.4%	+++
		Control	+++++
4	**NH_4MoO_4**	0.4%	+++
		0.5%	+++
		0.6%	**++++**
		Control	+++++
5	**$ZnSO_4$**	0.09%	**+++++**
		0.1%	++++
		0.2%	+++
		Control	+++++
6	**$FeSO_4$**	0.6%	+++
		0.7%	**++++**
		0.8%	+++
		Control	+++++
7	**H_3BO_3**	1.2%	++++
		1.3%	**+++++**
		1.4%	+++
		Control	+++++

Table 3: Growth of *Rhizobium leguminosarum* in optimized multi-micronutrient.

S.no	Compound of micronutrient	Optimum concentration of micronutrient (wt/ vol)	C.F.U/ ml at 10^9 dilution
1	MnO	0.5%	Mat growth
2	KCl	1.5%	
3	$CuSO_4$	1.2%	
4	NH_4MoO_4	0.6%	
5	$ZnSO_4$	0.09%	
6	$FeSO_4$	0.7%	
7	H_3BO_3	1.3%	
8	Control	Standard Y.E.M.B.	Profuse growth

research. On the other hand where the microbial viability values were more than the values (C.F.U/ 0.1 ml at 10^9dilutions) of control, it clearly reflects the necessity of such compounds of micronutrients for the bacterial growth and therefore were taken into consideration for further research. (Table 1, 2 & 3)

Conclusion

The compounds of micronutrients are well known to impart healthy effect on the plant growth as they are the prime requirement (trace elements) for various vital activities of plants. The experiment hereby confirms that the universal standard media for growth of *Rhizobium* i.e. Yeast extract mannitol media scientifically managed by incorporating in it optimum concentration of all seven plant micronutrients showed increased growth of *Rhizobium leguminosarum* than the growth in control (Yeast extract mannital media). In such circumstances where the trace element compounds that are well know for proper plant health, and simultaneously are also proved to support the microbial growth in modified production medium, and that this makes sense to utilize this concept in biotechnological industries for commercial Bio-inoculant production upon further studies on scale up.

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