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Investigation of Effects of Ecological Factors on the Establishment of *Azotobacter* in the Rhizosphere of Thyme (*Thymus vulgaris*)

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Authors' contributions

This work was carried out in collaboration among all authors. Author NNN designed the study, wrote the protocol and the first draft of the manuscript. Authors NNN and ALO managed the analyses of the study and literature searches, read and approved the final manuscript.

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ABSTRACT

Aim: The present study investigates the effect of phosphate-solubilizing bacteria (phosphobacteria (PB) and activity of soil bacteriostasis on the development of *Azotobacter* in *Thymus vulgaris* rhizosphere.
Place and Duration of Study: The study was conducted at Kenule Beeson Polytechnic botanical garden and at the Science Laboratory Department of the institution for a period of 7months (from March 2018- September 2018).
Methods: The impact of phytohormones produced by phosphate-solubilizing bacteria *in vitro* and in the rhizosphere of *T. vulgaris* was used to assay for *Azotobacter* colonization. Bacteriostasis activity of the soil was determined by comparing the number of *Azotobacter* microcolonies on discs incubated over soil with respect to those on the controls.
Results: Decisive stimulation of *Azotobacter* population and establishment was observed in *Thymus vulgaris* rhizosphere when inoculated with phosphobacteria than when inoculated alone as reflected in 5% (0.05) least significant difference. *Azotobacter* was susceptible to the bacteriostatic

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factors in potted soils inoculated with it and without (*Azotobacter)*. The increase in susceptibility of this rhizospheric bacteria was time dependent and reached a maximum and thereafter remained almost constant. However, this was overcome by the addition of NPK fertilizer to the plant at some critical stage of the assay.

Conclusion: The Presence of nitrogen fixing bacteria (NFB) in vegetation could play significant role in the sustainability and improvement of plant growth and yield. Soil bacteriostasis can also be an important factor that limits the survival and development of NFB.

Keywords: Thymus vulgaris; sensitivity; bacteriostasis; phosphobacteria; Azotobacter.

1. INTRODUCTION

Some of the useful soil microflora are those with strong capacity to transform gaseous nitrogen of the air to nitrogen utilizable by a variety of microorganisms and flora [1]. Without these nitrogen fixers, most life-forms on earth would become extinct [2]. The most important ones are *Azotobacter*, a genus of free-living soil organisms, and *Rhizobium*, a root nodule symbiotic type.

Azotobacter has been classified as being in the group of plant growth-promoting rhizobacteria (PGPR) with phytohormone synthesizing ability [3,4,5] an effect which is especially marked in fertile soils with a high organic matter content and a near neutral pH [6]. The beneficial action of the organisms is actually associated with its production of bioactive materials [1,7,8].

Apart from the significance of NFB in stimulating plant growth, recent research interests have focused on different environmental factors influencing the progressive development of *Azotobacter* following their inoculation in the rhizospheres of florae. Some factors such as soil fertility, manuring and mutual interaction between *Azotobacter* and Phosphobcateria which impacted on *Azotobacter* colonization has been previously reported in literature [9]. At harvest, it was shown that there were abundance of *Azotobacter* in the rhizosphere of lavender when both bacteria were inoculated together. Addition of 2% farmyard manure to the organic carbon content richer soil enhanced this effect. Such that plant growth also was greatest when seedlings were inoculated with both groups of bacteria.

Amensalism, according to Storzky [10] was undoubtedly a significant factor to the survival and ecology of *Azotobacter* in soil [9]. Microorganisms antagonistic towards growing *Azotobacter* cells, were abundant in rhizosphere of lavender. These organisms were stimulated when inoculated with *Azotobacter* but decreased in number at the end of the experiment. Microorganisms that were capable of lysing *Azotobacter* resting cells predominated also irrespective of the *Azotobacter* inoculation treatment. This activity fluctuated throughout the study but was highest at the time of harvest.

Soil bacteriostasis has been described as an important factor in limiting the growth of soil bacteria [1,11]. Moreover, it was suggested as the cause of the inhibition, under certain conditions of the germination of *Azotobacter* cysts in soil [12]. Ocampo et al. [9] and Barea et al.[13] pointed out that bacteria which produced plant hormones (PHs) stimulated natural and introduced *Azotobacter* populations as well as growth of other microorganisms in the rhizosphere [14-17].

2. MATERIALS AND METHODS

Six conical flasks containing N-deficient liquid medium, prepared as described by [1] ~~which~~ were each inoculated with 1ml of a suspension of *Azotobacter* (A_6) cysts in sterile distilled water. Two of these flasks were also inoculated with 1ml of a phosphobacteria culture (PB treatment) prepared as described by [2]. This culture contained 0.1µg of each of the phytohormones; auxins, giberellins and cytokinins [13]. One ml of a mixture of commercial hormones at the concentration mentioned above was added to another two flasks (~~p~~ PH treatment). Numbers of *Azotobacter* in the three treatments were counted after 1, 3 and 5 days of incubation at 25º C on a rotary shaker.

2.1 Treatment with Bacterial ~~Bacaterial~~ Consortium

Thyme seedlings were inoculated by treating their roots with *Azotobacter chroococcum* (A_6), *Azotobacter chroococcum* (A_6) + Phosphobacteria (PB) and A_6 + Plant hormones (PH); and were cultivated as described by [9].

During the experiment, rhizosphere soil was sampled at 15-day intervals and *Azotobacter* counted as described by [2].

2.2 Assessment of Soil Bacteriostatsis

For assessing soil bacteriostasis, rhizosphere soil from each sample of *Azotobacter*-inoculated and uninoculated control was placed in Petri dishes and moistened to 70% of filled capacity. Disks of Whatman No. 1 Filter paper were either placed on the soil, or in sterile dishes as controls. Agar discs 7mm in diameter (9 replicates per sample) cut from 1.5% sterile distilled water agar, were placed on the filter paper. Dishes were kept at 25°C for 15h and were then inoculated with 0.01ml suspensions of each of these *Azotobacter*s pecies (*A. chroocucum* A_6, *A. beijerinckii*, A_4 and *A. vinelandii*, A_5). Three replicates per *Azotobacter* spp were prepared. After 48h incubation at 25°C the discs were removed, stained with 10% dilute carbol fuchsin and examined under the microscope. Bacteriostasis was assessed by comparing the number of micro-colonies which grew on discs incubated over soil with those on the controls.

3. RESULTS AND DISCUSSION

The results of effect of soil bacteriostatic factors on *Azotobacter* in control and *Azotobacter*-inoculated rhizosphereare presented in Table 1. The effect of phosphobacteria (PB) and plant hormones (PHs) on numbers of *Azotobacter* (A) in culture are presented in Table 2. The effect of phosphobacteria (PB) and plant hormones (PH) on numbers of *Azotobacter*(A) inoculated in thyme rhizosphere are presented in Table 3. Table 4 shows the effect of bacterial fertilizers on dry weights of thyme plants as affected by NPK fertilizer. The course of development of *Azotobacter*number per gram dry rhizosphere, soil and bacteriostatic activity towards *Azotobacter* in control and inoculated rhizosphere is presented in Fig. 1.

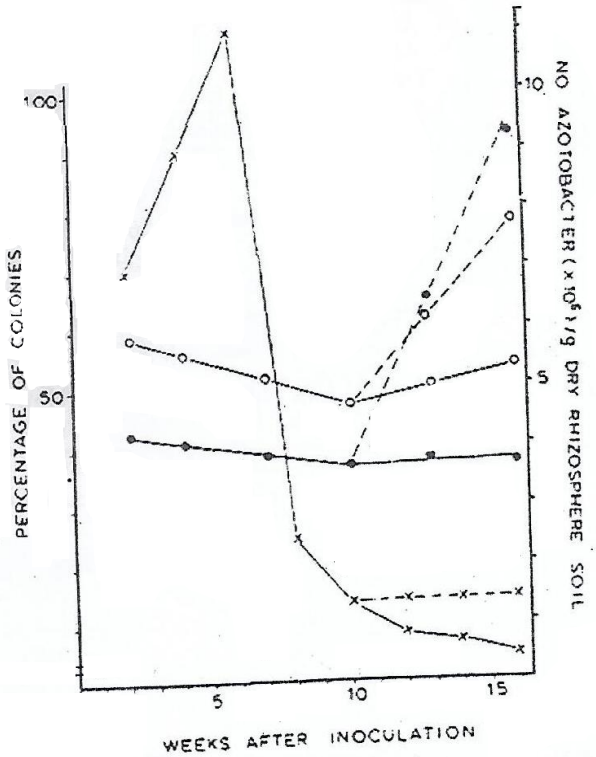

Figure 1. Course of development of *Azotobacter* (×——×) no. · g^{-1} dry rhizosphere soil, and bacteriostatic activity towards *Azotobacter* (% of colonies) in control (o——o) and inoculated (●——●) rhizosphere, affected by NPK fertilizer (– – – – –)

Table 1. Effect of soil bacteriostatic factors on *Azotobacter* in control and *Azotobacter*-inoculated rhizosphere

Weeks after inoculation	Inoculation treatment	% of colonies*		
		A_{4}***	A_5	A_6
2	Control (C)	60	63	56
	Azotobacter(A)	44	44	46
4	C	58	61	51
	A	41	40	44
	C	48	49	53
7	A	40	36	44
	C	52	46	46
10	A	38	34	42
	C	53(64)**	51(62)	49(61)
13	A	40(68)	34(64)	43(66)
	C	54(83)	53(81)	55(73)
16	A	43(97)	38(95)	33(89)

**Percent colonies in relation to control with no soil added*
***The parentheses contain % of the numbers of colonies in NPK treated rhizosphere*
****A_4 = A. beijerinckii, A_5 = A. vinelandii and A_6 = A. chroococum.*

Table 2. Effect of phosphobacteria (PB) and plant hormones (PHs) on numbers of *Azotobacter* (A) in culture

Inoculation treatment	No ($x10^7$) ml culture (Age of culture, days)		
	1	3	6
A	17.4	4.4	3.3
A+PB	32.4	16.4	15.4
A+PH	34.4	18.4	17.2
L.S.D (5%)	6.4	3.4	2.5

L.S.D (5%) = Least Significant Difference at 5%

Table 3. Effect of phosphobacteria (PB) and plant hormones (PHs) on numbers of *Azotobacter* (A) inoculated in thyme rhizosphere

Inoculation treatment	No.($X10^6$) g dry rhizosphere $soil^{-1}$ weeks after inoculation							
	2	4	6	8	10	12	14	16
A	43	100	140	25	13.2	4.9	3.6	3
A+PB	126	140	200	64	50	23	24	19
A+PH	111	120	189	47	33	10	11	7
L.S.D (5%)	16	17	3.0	9.8	8.2	7.3	6.4	2.9

L.S.D (5%) = Least Significant Different at 5%

Table 4. Effect of bacterial fertilizer on dry weights of thymus plants as affected by NPK fertilizer

Inoculation* treatment	NPK treatment** (mg $plant^{-1}$)			
	1	2	3	4
C	600	886	882	1022
A	730	1026	996	1196
PB	650	910	1046	115
A+PB	790	105	1090	1176
L.S.D (5%)	55	60	60	70

**C = Uninoculated control, A = Azotobacter-inoculated pots;*
PB = Phosphobacteria – inoculated pots.
***1 = No NPK added; 2 = NPK added at inoculation time;*
3 = NPK added at the middle of assay; 4 = 2 + 3 treatment.
L.S.D. (5%) = Least Significant Difference at 5%.

Table 1 shows that the three *Azotobacter* spp. were sensitive to bacteriostatic factors in thymus rhizosphere soil from both *Azotobacter* inoculated and uninoculated pots. Fig. 1, clearly demonstrated that the sensitivity of *Azotobacter* increased with time, and reached a maximum at a certain stage of the experiment, then remained almost constant. The living roots supplied substances which helped to promote bacteriostasis towards *Azotobacter* which was overcome by the addition of NPK fertilizer at some stage during the experiment. This corroborates the reports of other investigators that nitrogen plays an important role in plant growth and development [1,12,18]. However, the mechanism and nature of the bacteriostatic factors are still not well understood.

Tables 2 and 3 show that plant hormones play a certain role in *Azotobacter* development both *in vitro* and in the rhizosphere. The presence of Phosphobacteria, however, may have acted in synergy with *Azotobacter* to further stimulate and enhance the functionality of available phytohormones such phenomenon had been previously reported in Ramie plants [3,17] by another mechanism in addition to that based on the supply of hormones. Fig. 1 also shows that the course of development of the activity towards *Azotobacter* of soil bacteriostasis coincides with the numerical decline of *Azotobacter* in the rhizosphere. The period at which that antagonistic factor is expressed is similar to that previously found for other antagonism agents [1,9]. The lack of conclusive evidence for most of the mechanisms, *in situ*, is a general trend [10].

Regardless of the mechanisms involved the *Azotobacter* population introduced into the thyme rhizosphere was influenced by the activity of several ecological factors which govern the biological equilibrium in the root region. However, a number of *Azotobacter* becomes established. Initially, between 1 and 6 weeks after inoculation *Azotobacter* was stimulated but after 6 to 12 weeks cell numbers dropped. Finally, cell numbers became equilibrated. In the present experiments each seedling received about 10^7*Azotobacter* cells which were stimulated to 10^9gdryrhizosphere soil^{-1} and 10^{-5}-10^6 cells g^{-1} remained at harvest. Table 4 summarizes the dry weights of plants grown in the different experimental conditions and given different inoculation treatments.

In NPK treatments 1, 2 and 3, there was more *Azotobacter* at harvest when plants were inoculated with *Azotobacter* together with phosphobacteria than inoculated singly. Although this is apparently reflected in Table 4, in which differences between plant dry weights in A + PB *vs* A treatment are significant in 1, 2 and 3 treatments, but not in 4; this may be a direct effect of phosphobacteria on plant growth.

4. CONCLUSION

The *Azotobacter* and phosphobacteria used as test organisms produced *in vitro* phytohormones which could play vital role in improving and sustaining plant growth. Furthermore, soil bacteriostasis can be a significant factor that limits the growth of soil microbiota and hence agricultural productivity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ngerebara NN, Ogan AC. Effects of soil bacteriostasis and phosphobacteriaon the establishment of *Azotobacter* in the rhizosphere. J Science and Technol Research Centre for Development of Strategies. 2015;9(1):1-7.
2. Ngerebara NN, Ogan AC. Selected colonization of the rhizosphere of wheat by nitrogen-fixing bacteria. J. Gen. Microbiol. 2013;42:125-144.
3. Suryatmana P, Anggraini DA, Kamaluddin NN, Setiawati MR. Nitrogen-fixing Bacteria inoculation (NFB) and Compost Application in Phytoremediation of Petroleum-Contaminated Soils using Ramie plant (*Boehmeria nivea* L). Int'l J Agric., Environ, Bioresearch. 2020;5(04):8-18.
4. Gałązka A, Król M, dan Perzyński A, The efficiency of rhizosphere bioremediation with *Azospirillum* sp. And *Pseudomonas stutzeri*Nisoils freshly contaminated with PAHs and diesel fuel. "Pol J. Environ Study. 2012;21(2):345-353.
5. Murcia RB, Rodelas V, Salmertn MV, Martlnez-Toledo dan J, Gonzalez-Lopez. Effect of the herbicide simazine on vitamin production by *Azotobacter chroococcum* and *Azotobacter vinelandii*. Applied Soil Ecology.1997;6(2):187-193.
6. Dommergues YJ, Mangenot FT. (2001). Ecologie Microbienne du sul. Paris. Masson et C. 2001;796.

7. Mishustin AN, Shilnikova VK. Biological fixation of atmospheric nitrogen. Nature. 2000;171:189-191.
8. Brown MA. Soil bacteriostatic limitation in growth of soil and rhizosphere *bacteria.* Can. J. Microbial. 2002;18:182-190.
9. Ocampo JC, Brown JV, Minonye EC. Interactions between *Azotobacter* and phosphobacteria and their establishment in the rhiozsphere as affected by soil fertility. Can J. Microbial. 2005;20:1150-1160.
10. Stotzky FG. Activity, ecology and population dynamics of microorganisms in soil. Critical Rev. Microbial. 2003;2:52-120.
11. Brown EM. Seed and root bacterization. Ann. Rev. Phytopathol. 2001;12:180-190.
12. Jackson JJ, Brown EM. Behavior of *Azotobacter chroococcum* introduced into the plants rhizosphere. Ann. Inst. Pasteur. 2003;110:105-112.
13. Barea JV, Nelson EJ, Palomares AJ, Esther ME. A rapid microbiological assay method for auxins, gibberellic acid and kinetin using *yeast.* J. Appl. Bacteriol. 2004;37:560-580.
14. LU KC, Gilbert CN, Zigallo AB, Boyle OK. Effects of gibberellic acid on the growth and multiplication of some soil microorganisms and unicellular green algae. Nature. 2011;204:1228-1230.
15. Saono SZ. Effect of gibberellic acid on the growth and multiplication of some soil microorganisms and unicellular green algae. Nature. 2001;205:1220-1229.
16. Sullia MB. Effect of foliar spray of hormones on the rhizosphere microflora of leguminous seed. Plant and Soil.2005;27:290-295.
17. Suryatmana P, Fauzi M, Setiawati MR, Bety NF. The biofertilzer's effect on phytoremediation performance by using *Boehmeria nivea* L. Gaud on petroleum hydrocarbon contaminated soil. Academic Journal of Science. 2018;8(1):31–42.
18. Patti PS, Kaya E, danSilahooy Ch. Analisisstasiun nitrogen tanahdalam Kaitannya dengan serapan N oleh tanaman padi sawah di Desa Waimital, Kecamatan Kairatu, Kabupaten Seram bagian barat. Jurnal Agrologia. 203;2(1):51-58.

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