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.

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 7 months (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...
1. INTRODUCTION

Some of the useful soil microflora are those with strong capacity to transform gaseous nitrogen of the air to nitrogen utilisable 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 Phosphobacteria 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 Storzyk [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 (A6) 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, gibberellins and cytokinins [13]. One ml of a mixture of commercial hormones at the concentration mentioned above was added to another two flasks (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 (A6), Azotobacter chroococcum (A6) + Phosphobacteria (PB) and A6 + Plant hormones (PH); and were cultivated as described by [9].

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

### 2.2 Assessment of Soil Bacteriostasis

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 *Azotobacters* pecies (A. *chroocucum* A6, A. *beijerinckii* A4 and A. *vinelandii* A5). 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 rhizosphere are 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* (x—x) no. g⁻¹ dry rhizosphere soil, and bacteriostatic activity towards *Azotobacter* (% of colonies) in control (o—o) and inoculated (e—e) rhizosphere, affected by NPK fertilizer (----).](image-url)
Table 1. Effect of soil bacteriostatic factors on Azotobacter in control and Azotobacter-inoculated rhizosphere

| Weeks after inoculation | Inoculation treatment | % of colonies* |  |  |  |
|-------------------------|-----------------------|----------------|---|---|---|
|                         |                       | Azotobacter(A) | A4*** | A5 | A6 |
| 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

***A4 = A. beijerinckii, A5 = A. vinelandii and A6 = 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) |  |
|-----------------------|-------------------------|------------------------|---|
| 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 |  |
|-----------------------|---------------------------------------------------------------|---|
| 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}) |  |
|-----------------------|---------------------------------|---|
| 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 10^5 g dry rhizosphere soil 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.

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