doi: 10.26114/pja.iung.447.2021.44.01

**Polish Journal of Agronomy**
**2021, 44, 3–8**

# Occurrence of *Azotobacter* spp. in cultivated soils in Poland

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**Abstract.** *Azotobacter* spp. are soil bacteria capable of fixing atmospheric nitrogen and making it available to plants. This makes them microorganisms of hight importance to the agriculture. The distribution of these bacteria is a complicated subject and is correlated with diverse factors, witch determine the presence or absence of the bacteria in a specific soil. The soil properties as soil and fertility and climate conditions affect the abundance of diazotrophs. Research material consisted of 182 soil samples taken in 2015 from agricultural land in various voivodeships. The number of *Azotobacter* spp. was determined based on plate method. The presented study evaluated the abundance of *Azotobacter* spp. in various Polish soils and confirmed the relationship between soil properties and the presence of these bacteria. The studied bacteria were present in 37% of soil samples and numbers of these bacteria varied widely, from 3 to 10801 cfu $g^{-1}$ of the soil. In the case of *Azotobacter* spp., soil acidity was also an important factor restricting the occurrence of these bacteria in soils.

**Keywords:** abundance of *Azotobacter* spp., soil, soil properties

## INTRODUCTION

Aerobic bacteria belonging to the genus *Azotobacter* represent a diversified group of free-living diazotrophs which are commonly found in soils. *Azotobacter* spp. are agriculturally beneficial bacteria because of their capacity for fixing the atmospheric nitrogen and making it available to higher plants in a bioavailable form, as well as producing numerous compounds which stimulate the growth and development of plants (Aquilanti et al., 2004; Kukreja et al., Lenart, Chmiel, 2008; Patil, 2011; Vikhe, 2014; Arora et al., 2017). Moreover, they are microorganisms being strongly responsive to chemical and physical factors in the soil, so fluctuations in the abundance of these bacteria are good indicators of changes occurring in the environment (Lenart, Chmiel, 2008; Kozieł et al., 2018).

There are currently 9 species and 4 subspecies known worldwide within the genus *Azotobacter*.

- *Azotobacter armeniacus* (Thompson, Skerman, 1979),
- *Azotobacter beijerinckii* (Lipman, 1904),
- *Azotobacter bryophylli* (Liu et al., 2019),
- *Azotobacter chroococcum* (Beijerinck, 1901),
- *Azotobacter chroococcum* subsp. *chroococcum* (Jin et al., 2020),
- *Azotobacter chroococcum* subsp. *isscasi* (Jin et al., 2020),
- *Azotobacter macrocytogenes* (Jensen, 1955),
- *Azotobacter nigricans* (Krasil'nikov, 1949),
- *Azotobacter nigricans* subsp. *achromogenes* (Thompson and Skerman, 1979),
- *Azotobacter nigricans* subsp. *nigricans* (Howey et al., 1990),
- *Azotobacter paspali* (Döbereiner, 1966),
- *Azotobacter salinestris* (Page, Shivprasad, 1991),
- *Azotobacter vinelandii* (Lipman, 1903).

The species *A. chroococcum* is most widely spread throughout the soils all over the world (Döbereiner, 1983; Kole et al., 1988; Neito et al., 1989; Kumar et al., 2001; Verma et al., 2004; Tejera et al., 2005), whereas the occurrence of other species of this genus is considerably more limited, e.g., *A. paspali* inhabits only the rhizosphere of the *Paspalum notatum* grasses (Tchan, New, 1984; Dobereiner, 1995). The bacteria of the genus *Azotobacter* are characterised by being highly sensitive to the acid reaction of the soil environment, and consequently such bacteria are rarely observed in soils with a pH below 6 (Ziemięcka, 1923; Martyniuk, Martyniuk, 2003). The abundance of *Azotobacter* spp. in neutral or alkaline soils fluctuates between several and several thousand cells per 1 g of soil, whereas in acidic soils (pH < 6.0) these bacteria are typically not present or present in extremely low quantities (Marty-

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niuk, Martyniuk, 2003; Aquilanti et al., 2004; Lenart, 2012; Mahato, Kafle, 2018; Mukhtar et al., 2018). What is more, the presence and population size of the bacteria from this group is strongly tied to various environmental conditions, such as: soil properties (organic matter content, humidity, fertility, C/N ratio, pH) and climate (Tejera et al., 2005). In 1923 Ziemięcka published the results of her pioneering research on the occurrence of the bacteria belonging to the genus *Azotobacter* in selected Polish soils. During her research, these types of bacteria were found in 50% of soil samples collected from 28 regions of Poland. The characteristic feature of the soils in which *Azotobacter* spp. was not found was acidic pH, while the most abundant populations inhabited fertile soils with a high content of floatable fraction and with pH close to neutral. Similar results were presented by Martyniuk, Martyniuk (2003), who found these bacteria in 52% of soils samples taken from various regions of Poland. Another research which confirmed the thesis, whereby these microorganisms are vulnerable to acidic pH and are mostly found in neutral or slightly alkaline soils, was the study conducted by Lenart (2012) on the occurrence of *Azotobacter* in 100 soil samples collected from Małopolskie and Śląskie voivodeships in the south of Poland.

The aim of the research was to estimate the current level of the colonisation of Polish agricultural soils by bacteria of the genus *Azotobacter*. The discussion about the impact of some soil properties on the occurrence and the population size of this group of bacteria has also been elaborated in this work.

## RESEARCH MATERIALS AND METHODS

The research material consisted of soil samples collected in 2015 by the employees of the Department of Soil Science, Erosion and Land Protection belonging to the Institute of Soil Science and Plant Cultivation – State Research Institute during the national monitoring of soil chemistry. This program covered selected agricultural lands in various voivodeships. Soil samples for laboratory analyses were collected from 216 permanent measurement and control points located on arable lands characteristic for the soil cover of the country, from a depth of 0–20 cm, and then mixed to obtain an average sample. In order to determinate the total number of bacteria of the genus *Azotobacter*, 182 average soil samples from sixteen voivodeshipswere used.

The total number of bacteria from the genus *Azotobacter* in soil samples was determined using the method of dilution plating (Fenglerowa, 1965), by sowing appropriately diluted suspensions of the tested soils onto Petri plates. After 10 g of each soil was weighed and shaken for 30 minutes, a series of $10^1$–$10^2$ dilutions were made under sterile conditions. Then 1 $cm^3$ of the appropriate dilution of each sample was poured onto sterile Petri plates with a pipette and immersed in 5 $cm^3$ of Jensen medium. After 5 days of incubation at 28 °C, the abundance of bacterial cells (CFU) was quantified. The number of bacteria cells was converted to 1 gram of dry soil mass. The analyses were performed three times.

At the Department of Soil Science, Erosion and Land Protection, the pH of the tested soils in the water suspension was determined using the potentiometry at the soil/solution ratio of 10 g : 40 $cm^3$. The granulometric composition of soil was indicated with usage of the Casagrande method in Prószyński's modification (Lityński et al., 1976), and the granulometric groups of the topsoil were determined according to classification created in 2008 by the Soil Science Society of Poland.

## RESULTS

The soils under study belonged to different classes and types and were characterized by different pH values, and the number of the studied group of bacteria was varied. The presence of these bacteria was noted in 67 (37%) of 182 soil samples that had been analysed. The total number of the studied group of bacteria ranged from 3 to 10801 CFU $g^{-1}$ dm of soil.

The most abundant populations of bacteria of the genus *Azotobacter* were observed in clayey silt, loamy silt, silt, sandy loam (Table 1). The large differences in the numbers of these bacteria, both within the same soil class and between individual soil class, ought to draw attention. Their highest abundance was found in clayey silt (10801 CFU $g^{-1}$ dm of soil) at pH 7.7, while the lowest one was found in sandy loam (3 CFU $g^{-1}$ dm of soil) with pH 5.9.

Bacteria of the genus *Azotobacter* were most abundant in Fulvic cambisols, Gleyic chernozems, Degraded gleyic chernozems, Haplic luvisols (Table 2). These bacteria were not found in the Gleyic fulvisols, Entic podzol. As in the case of soil species, a large variation in the number of *Azotobacter* spp. was found between and within particular soil types.

In order to verify the relationship between the presence of *Azotobacter* and the pH of the soil environment, the pH in $H_2O$ was determined for 182 tested soils. The conducted research demonstrated that over half (63%) of the analysed soils did not contain *Azotobacter* spp. The soils in which no bacteria of this genus were found are mainly acidic. When the tested soils were divided into 5 groups based on their pH (Table 3) and the percentage of soils with *Azotobacter* spp. was calculated for each group, the results confirmed that in very acidic soils (pH up to 4.5) these bacteria were not present in general, while the vast majority of neutral (85%) and alkaline (94%) soils are inhabited by free-living bacteria of the genus *Azotobacter*. The high correlation coefficient (R = 0.963) between the average measured pH values for the soil groups listed in Table 3 and the percentage of soils with *Azotobacter* spp. in these groups confirms the close relation between these parameters (Fig. 1).

Table 1. Occurrence and number of *Azotobacter* spp. (cfu $g^{-1}$ soil d.m.) in different granulometric groups of arable soils in Poland (n = 182).

| Granulometric group | Number of soils | Number of soils with (+) or without (-) *Azotobacter* spp. | pH in $H_2O$ | Range of *Azotobacter* spp. [cfu $g^{-1}$ d.m. soil] |
|---|---|---|---|---|
| Clay loam | 2 | 2 + | 6.7–7.1 | 726.7–1996.1 |
| Light loam | 5 | 4 +<br>1 - | 5.8–7.7<br>7.3 | 7.9–1062.6 |
| Sandy loam | 47 | 18 +<br>29 - | 5.8–7.6<br>3.8–6.7 | 3.2–3117.5 |
| Silt clay loam | 1 | 1 + | 7.4 | 490.9 |
| Loam | 7 | 5 +<br>2 - | 6.1–7.2<br>5.3–5.5 | 19.6–147 |
| Loamy sand | 44 | 5 +<br>39 - | 5.8–7.4<br>4.1–7.0 | 3.4–220.4 |
| Loose sand | 10 | 1 +<br>9 - | 5.9<br>3.7–7.1 | 7.5 |
| Weakly loamy sand | 12 | 2 +<br>10 - | 6.6–6.7<br>4.2–5.8 | 10.7–86 |
| Loamy silt | 38 | 17 +<br>21 - | 5.4–7.7<br>4.3–6.6 | 8.2–5159 |
| Clayey silt | 10 | 8 +<br>2 - | 6.2–7.8<br>5.3–5.9 | 11.9–10801 |
| Silt | 6 | 4 +<br>2 - | 5.8–7.0<br>5.0–6.5 | 22.5–4058.3 |

Source: authors' own study

Table 2. Occurrence and number of *Azotobacter* spp. (cfu $g^{-1}$ soil d.m.) in different types of arable soils in Poland (n = 182).

| Soil type | Number of soils | Number of soils with (+) or without (-) *Azotobacter* spp. | pH in $H_2O$ | Range of *Azotobacter* spp. [cfu $g^{-1}$ d.m. soil] |
|---|---|---|---|---|
| Fulvic Cambisol | 13 | 7 +<br>6 - | 6.2–7.7<br>4.6–5.9 | 147–10801 |
| Fulvic Phaeozem | 4 | 3 +<br>1 - | 6.9–7.5<br>5.5 | 96.8–2003.6 |
| Gleyic Fulvisol | 2 | 2 - | 5.2–5.7 | |
| Gleyic Chernozem | 6 | 4 +<br>2 - | 6.7–7.7<br>5.9–6.0 | 726.7–4552.9 |
| Degraded Gleyic Chernozem | 6 | 2 +<br>4 - | 6.9–7.7<br>4.9–5.9 | 895–5159 |
| Haplic Chernozem | 1 | 1 + | 6.7 | 220.8 |
| Degraded Haplic Chernozem | 2 | 1 +<br>1 - | 7.5<br>5.0 | 1662.1 |
| Entic Podzol | 1 | 1 - | 5.6 | |
| Acidic Cambisol | 17 | 3 +<br>14 - | 5.8–7.1<br>4.3–7.1 | 7.9–2102.5 |
| Cambisol | 13 | 10 +<br>3 - | 5.8–7.4<br>5.8–7.3 | 14.4–1378.6 |
| Eutric Cambisol | 32 | 13 +<br>19 - | 5.4–7.3<br>4.8–6.6 | 3.5–2199.6 |
| Haplic Luvisol | 55 | 14 +<br>41 - | 5.8–7.6<br>3.8–6.7 | 3.2–4640.9 |
| Brunic Arenosol | 26 | 5 +<br>21 - | 5.8–7.4<br>3.7–7.0 | 3.4–86 |
| Cambic Leptosol | 3 | 3 + | 7.1–7.3 | 55.9–3117.5 |
| Dolomitic Cambisol | 1 | 1 + | 7.8 | 294.6 |

Source: authors' own study

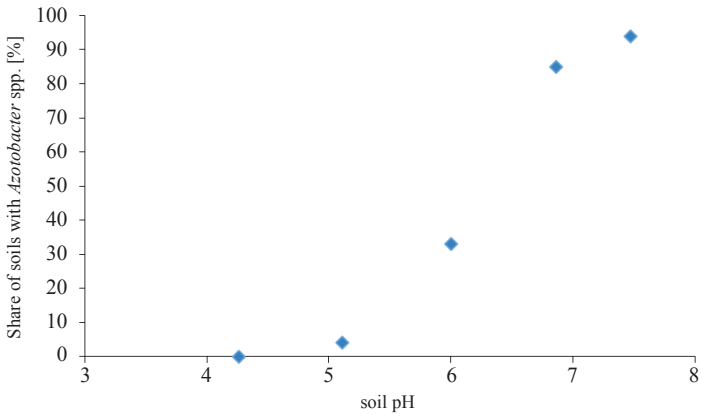


Figure 1. Correlation between the mean pH values of five soil groups and the % share of soils with *Azotobacter* spp. in these groups

Table 3. Occurrence of *Azotobacter* spp. in soils grouped with respect to their pH

| pH range (mean measured) | % of soils with *Azotobacter* spp. | Mean numbers of *Azotobacter* spp. |
|---|---|---|
| do 4.5 – very acidic soils (4.26) | 0 | 0 |
| 4.5-5.5 – acidic soils (5.11) | 4 | 0.3 |
| 5.6-6.5 – slightly acidic soils (6.00) | 33 | 146 |
| 6.6-7.2 – neutral soils (6.86) | 85 | 754 |
| >7.2 – alcalic soils (7.49) | 94 | 1649 |

Source: authors' own study

Analysing the relationship between the average number of *Azotobacter* in five soil groups listed in Table 3 and the average soil pH in these groups, a statistically significant correlation (R = 0.887) between the parameters mentioned was found. The data presented in Table 3 clearly indicates that the soils of group 5, i.e., alkaline soils (pH>7.2), were characterized by the highest number of *Azotobacter* spp. Conversely, in the group of soils with a very acidic (pH up to 4.5) and acidic pH (pH 4.5–5.5), i.e., adverse to the development of the discussed bacteria, many of these soils were not inhabited by *Azotobacter*.

The conducted research confirmed the results of previous studies whereby the bacteria belonging to the genus *Azotobacter* are vulnerable to the acidity of the soil and rarely occur in soils with a pH below 6. The obtained results clearly indicate that the pH of the soil is a significant factor determining the colonization of soils by the bacteria of the genus *Azotobacter*.

## DISCUSSION

The interest taken in the bacteria belonging to the genus *Azotobacter* dates to the beginning of the 20th century. Then, in 1901, Beijerinck isolated and marked the first species belonging to this genus – *Azotobacter chroococcum* (Tchan, New, 1984). It was at this point that the research on the occurrence, physiology and genetics of these important for agriculture microorganisms began.

Based on the results of the research, the frequency of *Azotobacter* bacteria was estimated at 37%, and their numbers ranged from 3 to 10801 cfu $g^{-1}$ d.m. in soil. A higher frequency of the occurrence of these bacteria in Polish soils, at the level of about 50%, was indicated by Ziemięcka (1923) and Martyniuk and Martyniuk (2003). In the studies conducted by Ziemięcka over the period of 1917–1918, 50% of the analysed soils in Poland were inhabited by *Azotobacter* spp. In 2000, 31 soils were sampled from the same area and subjected to similar analyses. The conducted research was aimed at verifying whether the intensification of agriculture, which took place in the 20th century, had caused changes in the colonization of soils by bacteria of the genus *Azotobacter*. The results of the research turned out to be similar to the results obtained by Ziemięcka (1923), as about 52% of the studied soils were inhabited by the discussed group of bacteria (Martyniuk, Martyniuk, 2003). Also, the quantity of *Azotobacter* spp. in the soils of the compared periods was similar and ranged from a few cells to almost 10,000 cells per gram. In the research conducted by Zawiślak (1973), the quantity of this group of bacteria in agricultural soils was at a similar level and ranged from several hundred to a thousand cells in 1 g of soil. Similar studies described in the work by Lenart (2012) referred to 100 soil samples collected from Małopolskie and Śląskie voivodeships, from areas with different methods of land use. However, the frequency of the occurrence of *Azotobacter* bacteria in soils collected from agricultural lands was only 22.2%. This result differs greatly from the results obtained by Ziemięcka (1923), Martyniuk (2008), and from this research. Such discrepancies may result from different sites of soil sampling, differences in the practices of agricultural engineering, applied plant protection products and fertilizers in researched locations as well as differences in the properties of the researched soils. Also, the abundance of *Azotobacter* spp. was much lower and ranged from 7 to 47 cfu $g^{-1}$ d.m. of soil.

The dependence of the occurrence and the quantity of the bacteria of the genus *Azotobacter* on the type, species and soil pH was also analysed. Taking into account the influence of the soil type on the *Azotobacter* spp. quantity, it can be noticed that the greatest quantities of these bacteria were found in Fulvic cambisols, Gleyic chernozems, Degraded

Gleyic chernozems and Haplic luvisols. Their lowest abundance was noted in Brunic arenosols, while in Gleyic fulvisols and Entic podzols no presence of these microorganisms was found (Table 2). Lenart (2012) noted the presence of the discussed bacteria group in alluvial soils and cambisols. Otherwise, the author did not demonstrate any significant correlation between the soil type and the occurrence of *Azotobacter* spp. In 2015 Siebielec et al. (2015) quantified the quantity of nitrogen-fixing bacteria of the genus *Azotobacter* in different types of soil subject to long-term cultivation of grain crops. Their greatest amount was found in eutrophic cambisols and Cambic leptosol, but they were also found in chernozem and fulvic cambisol. While examining the effect of soil type on the quantity of bacteria belonging to the genus *Azotobacter*, it can be concluded that clayey silt, loamy silt, silt and sandy loamfavour the growth of these bacteria, because these species were characterized by the highest abundance of the studied group of bacteria. The populations of these free-living nitrogen assimilators with the lowest abundance were recorded in the case of loose sand (Table 1). The obtained results of the dependence of the presence of *Azotobacter* spp. on the soil pH in the analysed samples, confirmed the thesis whereby these bacteria prefer soils with a neutral and slightly alkaline pH, whereas they rarely occur in acidic soils with a pH below 6 (Martyniuk, 2008; Sartaj et al., 2013; Mazinani, Asgharzadeh, 2014; Gothandapani, 2017). The optimal pH for the bacteria belonging to the genus *Azotobacter* ranged from 6.6 to 7.8, which is consistent with the results of similar studies obtained by various scientists (Limmer, Drake, 1996; Aquilanti et al., 2004; Lenart, 2012). Over a dozen percent of soil samples with a lower pH contained bacteria of the genus *Azotobacter*, while in the soils with a pH below 5.0 the presence of these microorganisms was not found (Figure 1). As presented in the literature data (Ziemięcka, 1923; Martyniuk, 2008) the most numerous populations of *Azotobacter* spp. inhabit fertile soils with a high amount of silt fraction and pH closer to neutral. Zawiślak (1973) noticed in her research that the bacteria occurred more frequently and numerously in agricultural soils and it is associated with a higher quality of arable soils due to more intensive ventilation, more careful fertilization and alternate cropping. This suggests that agrotechnical treatments may create more favourable conditions for the development and survival of these bacteria.

## CONCLUSIONS

1. The presence of the bacteria of the genus *Azotobacter* was found in 67 (37%) out of 182 analysed soil samples. The general abundance of these bacteria ranged from 3 to 10801 cfu $g^{-1}$ d.m. of soil.

2. The highest abundance of *Azotobacter* spp. was recorded in clayey silt, loamy silt, silt and sandy loam, while the lowest one was recorded in loose sand.

3. The most numerous populations of the bacteria belonging to the genus *Azotobacter* were found in Fulvic cambisols, Gleyic chernozems, Degraded gleyic chernozems and haplic luvisols. Their presence was not found in Gleyic fulvisols and Entic podzol.

4. The analysis of the dependence of the presence of *Azotobacter* spp. on soil pH confirmed the thesis whereby these bacteria prefer neutral and slightly alkaline soils, while rarely occurring in acidic soils with a pH below 6. The pH of the soil is a very important factor determining the colonization of soils by the bacteria of the genus *Azotobacter*.

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received – 26 October 2020
revised – 3 December 2020
accepted – 2 February 2021