SOIL NITROGEN FIXING BACTERIA UNDER BIOFERTILIZER APPLICATION IN DIFFERENT TILLAGE SYSTEMS IN MAIZE AND SUNFLOWER CROPS

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Abstract

It is well known that chemical inputs are the main determinant of yields, but their impact is considerable, causing significant changes in the soil. Soil microbiota is a very important component of the agricultural ecosystem and is involved in nutrient cycling. Its structure and dynamics are easily influenced by tillage, fertilizers and crops. The aim of the present study was to observe the effects of different tillage systems combined with the application of inorganic fertilizers and a phosphorus-solubilizing bacterial fertilizer in different rates and combinations on nitrogen-fixing bacteria in the soil. The study was conducted in 2023 growing season at the Ezareni Farm from Iasi. The tillage systems were represented by conventional (CT) and no-tillage (NT). Sunflower and maize crops were fertilized with: NPK (20% N, 10% P_2O_5, 5% K_2O), phosphorus solubilizing bacteria (PSB) fertilizer containing *Bacillus megaterium* var. *phosphaticum*, applied twice, and Corona N foliar fertilizer (21% N), applied to 3 of the 5 treatment formulations. For the quantification of nitrogen-fixing bacteria populations (*Azotobacter* spp. and *Clostridium pasteurianum*), soil samples were collected four times and the method used in the laboratory was that of successive dilutions. During the growing season, an increase in colony numbers was observed in CT maize when treated with the manufacturer's recommended dose (10 l/ha) of biofertilizer in combination with foliar application of nitrogen. In contrast, in sunflower, the number of bacterial populations was higher in NT under biofertilizer treatment at the prescribed dose but without foliar fertilizer application. At the beginning of the growing season, *Clostridium* sp. is more numerous, but this is reversed 4 weeks after the second application of PSB, which corresponds to the flowering period of the plants, when *Azotobacter* spp. predominates. The results indicate increased populations of nitrogen-fixing bacteria in both the recommended and higher-dose PSB treatments, and in sunflower this is more evident in the no-tillage system.

Key words: soil, no-tillage, phosphorus solubilizing bacteria, *Azotobacter* spp., *Clostridium* spp.

In the current conditions of increasing food needs, the agricultural sector is forced to take the necessary measures to increase production, the main action being the use of more chemical inputs. Their use produces a number of undesirable effects, such as reduced soil fertility, increased acidity, reduced the number of micro-organisms, leading to nutrient imbalances in the soil and as well as high levels of water pollution (Nosheen S. *et al.*, 2021; Allouzi M.M.A. *et al.*, 2022).

A complementary or even alternative solution to chemical fertilizers is the application of microorganism-based fertilizers to soil (Li X. *et al.*, 2022). Biofertilizers are preparations containing specialized living organisms that can fix, mobilize, solubilize, or decompose nutrient sources which, when applied through seed or soil, enhance nutrient uptake by plants (Mohanram S.and Kumar P., 2019). De Mandal S. *et al.* (2021) considered that biofertilizers could supplement commercial fertilizers to enhance productivity, maintain soil health and protect the microbial diversity. Microorganisms fulfil a wide range of ecosystem functions in soil such as the release of nutrients from minerals and organic matter, synthesis of proteins, and nucleic acids, N_2 fixation and soil aggregation (Hemkemeyer M. *et al.*, 2021).

Phosphorus is one of the essential nutrients for crops, but it is known to become easily inaccessible and is used in very low rates by plants. Finding ways to improve the efficiency of P-fertilizer use is very important for achieving sustainable agriculture (Liu H. *et al.*, 2022). Phosphate-solubilizing microorganisms are the most important component of the P cycle and have several mechanisms for increasing phosphate availability in the soil, being involved in soil nutrient dynamics and various interactions with microbiota (Silva L.I. *et al.*, 2023).

Nitrogen (N) is unique among the major soil nutrients in that it originates from the atmosphere, and its transformations and movement in an

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ecosystem are mediated almost entirely by the water cycle and biological processes (Ladha J. K. *et al.*, 2022). In biological fixation, gaseous N_2 is assimilated and transformed only by a select group of microorganisms, either plant symbionts or free-living diazotrophs (Sepp S-K. *et al.*, 2023). The last ones live freely in the soil and are classified into various classes based on different criteria as some are oxygendependent, e.g. *Azotobacte*r (aerobic) whereas some are anaerobic, e.g. *Clostridium* (Neeraj A. and Yadav R.H., 2021). N_2-fixation by the free-living diazotrophs is an enzymatic process, and several environmental factors have been suggested to influence the rates of this process, including soil temperature, moisture, oxygen, carbon quality and quantity, nitrogen availability and the availability of other elements, such as P, Mo and Fe (Tang Y. *et al*., 2017).

The application of biofertilizers influences the chemical and physical properties of the soil as well as the structure and functions of the soil microbiota (Javoreková S. *et al*., 2015). Changes in soil microbial communities induced by environmental changes could influence the relationships between microorganisms and plants and may negatively influence soil fertility and crop productivity. Studying the effects of fertilizers of all types on the natural microbial community is therefore crucial importance (Dincă L.C. *et al*., 2022). Hence, research shows an improvement in soil phosphorus and nitrogen content by applying phosphorus-solubilizing bacteria as biofertilizers (Khan H. *et al*., 2022).

The influence of the tillage system on soil microorganism populations is widely known from research. Thus, conservation agriculture practices, such as reduced or no-tillage system, cover crops and fertilization, are often associated with greater microbial biomass and activity that are linked to improvements in soil quality (Mbuthia L.W. *et al*. 2015). Main benefits of NT system are reduced soil erosion, moisture evaporation, and compaction, which result in more fertile and resilient soils (Binder A. *et al*., 2022).

CT causes physical disruption of the upper soil horizon, creating a homogeneous layer of soil with relatively uniform physical characteristics and nutrient distribution. This management practice promotes short-term bacterial growth by aerating the soil and by breaking apart soil aggregates to expose organic matter that had been protected from microbial decay, but affects soil chemical properties and may result in decreased quantity of organic matter, altered composition of bacterial, fungal and arthropod soil communities, reduced biodiversity of soil species, and increased levels of aerobic microorganisms (Kraut-Cohen J., 2019).

The practice of different tillage systems and the application of biofertilizers leads to changes in the structure and functions of the microbiota in response to variations in soil chemical and physical properties (Javoreková S. *et al*., 2015), therefore, this study is based on the hypothesis that the application of phosphorus-solubilizing bacteria increases the nitrogen-fixing population in the soil.

MATERIAL AND METHOD

The study was conducted in the 2023 growing season in the experimental field of the Soil Management Department at the Ezăreni Farm (47°07`N, 27°30`E) of the „Ion Ionescu de la Brad" Iași University of Life Sciences, eastern Romania (*figure 1*). The specific soil of this area is classified as Chernozem, with clay-loam texture and slightly acid reaction (Țopa D. *et al*., 2021). The climate is temperate, with annual rainfall of 435.6 mm and average temperatures of 12.05°C in 2022.

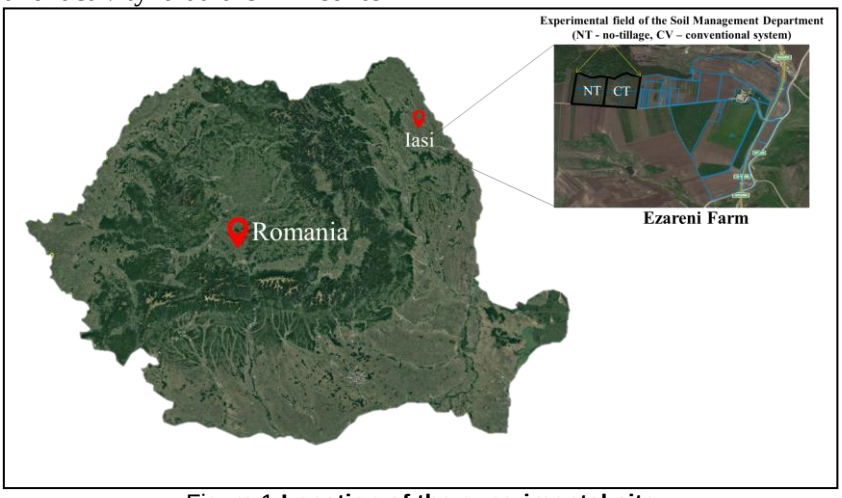

Figure 1 **Location of the experimental site**

The experiment is two-factorial design as follows: two tillage methods, including (1) conventional tillage (CT), with mouldboard ploughing to a depth of 28-30 cm; (2) no-tillage (NT), with direct seeding with Fabimag FG-01 and four fertilizer treatments (*table 1*) and control. Each

variant covered 48 m^2 resulting in experimental plots of 240 m^2 for each tillage system.

The first application of biofertilizer was performed a few days before sowing, when preparing the seedbed for CT, and in NT it was applied on the day of sowing (table 1). Both crops were sown in early May, hybrid maize P9889 was sown in the CT and NT plots at a seeding rate of 72000 pure live seeds/ha, and sunflower was sown with the late hybrid P64LE99, at a rate of 58000 pure live seeds/ ha. The second application of the tested biofertilizer was carried out 40 days after sowing (DAS), when the maize plants reached 4-6 leaves and the sunflower plants to 10-12 leaves, depending on the tillage system.

Table 1

CT and NT fertilization for maize and sunflower crops

Moments of application	Treatment		Doses
sowing	P-solubilizing Bacillus megaterium var. phosphaticum bacteria (PSB) (1 x 10^{10}cfu/ml)		T_0 – control (no-PSB) T_1 – 10.0 l/ha T_2 - 7.5 l/ha T_3 – 10.0 l/ha T_4 - 12.5 l/ha
	Inorganic fertilizer NPK (20% N, 10% P_2O_5, 5% K_2O)	maize	170 kg/ha
		sunflower	240 kg/ha
40 DAS	P-solubilizing Bacillus megaterium var. phosphaticum bacteria (PSB) (1 x 10^{10}cfu/ml)		T_0 – control (no-PSB, no-foliar fertilizer) T_1 – 5.0 l/ha, no-foliar fertilizer T_2 - 3.75 l/ha T_3 – 5.0 l/ha T_4 - 6.25 l/ha
	Foliar fertilizer (21% N + microelements)		3 kg/ha

Soil samples for microbial community assessment were collected 2 and 7 weeks after the first biofertilizer application (16.V.2023; 20.VI.2023), 4 and 7 weeks after the second application (18.VII.2023; 10.IX.2023), from 0-7 cm soil depth. For each treatment, a composite sample was obtained by sampling soil from three points with a sterile spoon and stored in sterile bags.

To quantify the colony numbers of N-fixing bacteria, *Azotobacter* spp. and *Clostridium pasteurianum*, soil samples were prepared by drying, removing plant residues and grinding in a hand mortar.

Determination of bacterial populations was carried out in the microbiology laboratory of the University of Life Sciences of Iasi, by the method of successive dilutions in solid medium using the dilution coefficient 10. To obtain the dilutions 1 g of soil was added in test tubes containing 9 ml of sterile water and agitated for 5 minutes, resulting in the dilution 10^{-1}. The suspension obtained (10^{-1}) was mixed with a sterile pipette, then 1 ml was transferred to the second test tube, this being dilution 10^{-2} (Ulea E. and Lipșa F.D. 2012). Dilutions of 10^{-3} were used for the analysis in this study, of which 1 ml of suspension was introduced in Petri plates in triplicates on Ashby culture medium (glucose 20 g, KH_2PO_4 0.2 g, K_2SO_4 0.2 g, agar 15-18 g, $MgSO_4$ 0.2 g, NaCl 0.2 g, $CaCO_3$ 5 g, in one litre distilled water). Petri plates were incubated for 5 days at 28°C.The total number of N-fixing bacteria was recorded using the HD Scan 1200 colony counter, which automatically saves images and results, and the individual identification of *Azotobacter* and *Clostridium* was performed using the plate count technique (Gafencu A.M. and Ulea E., 2023).

RESULTS AND DISCUSSIONS

Figure 2 illustrates a high abundance of *Azotobacter* spp. in all treatments in CT maize at first determination. This phenomenon is due to the fact that, in spring, climatic conditions and soil tillage for sowing are favorable to the activity of microorganisms. At this moment of the determinations, the applied biofertilizer variants are outnumbered by the control treatment (18.3×10^{-3} cfu g $soil^{-1}$), but in June, however, the situation is reversed. In July and September, of the four variants treated, those in which the product was applied at the dose recommended by the manufacturer stand out.

In the NT system, in May and June, no considerable differences were observed between the treated variants, with values ranging from 3.3 to 6.0×10^{-3} cfu g $soil^{-1}$, with the exception of the T_2, where 2.7 and 2.0×10^{-3} cfu g $soil^{-1}$, respectively. An increase in the *Azotobacter* spp. population was observed after the second biofertilizer application in the July determinations (*figure 3*).

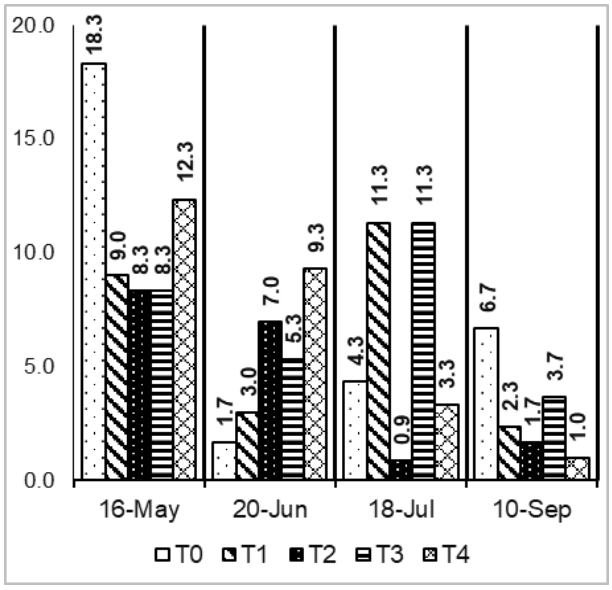

Figure 2 ***Azotobacter*** **sp. colonies in CT maize (cfu g soil^{-1})**

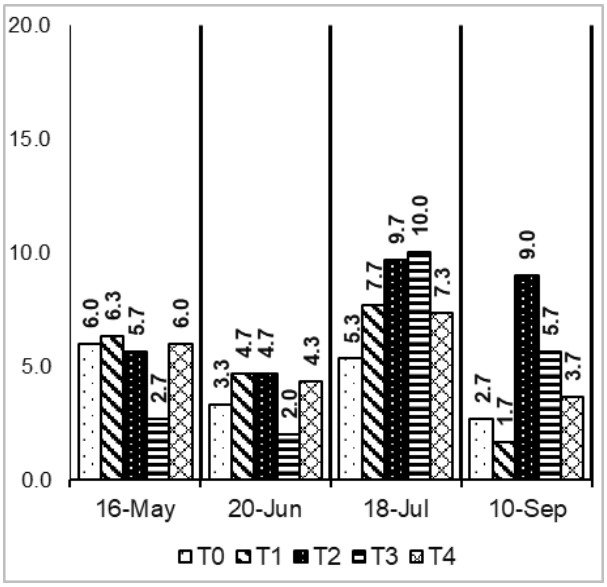

Figure 3 ***Azotobacter*** **sp. colonies in NT maize (cfu g soil^{-1})**

Also, *Clostridium* spp. in maize in both systems in the May determinations was more numerous (*figure 4; 5*), but the values are higher in CT, between 9.7-22.3×10^{-3} cfu g soil^{-1} compared to NT (5.3-13.3×10^{-3} cfu g soil $^{-1}$). In CT system after the second application of the product, the July determinations shows a lower colony count in T_2 (2.5×10^{-3} cfu g soil^{-1}), where a reduces dose of biofetilizer was applied, compared to the control and the other treatments. In September, the number of N-fixing bacteria of this species was low, with minor fluctuations between treatments.

In the NT system, in the June determinations, control exceeds the fertilized variants, but in July the T_1 treatment (10 l/ha PSB), with the highest colony count (5×10^{-3} cfu g soil^{-1}), T_2 and T_3 were higher than the control. In September, *Clostridium* spp. in maize soil showed an increased bacterial population in control and T_3 compared to the other treatments. In general, in the NT system, the activity of N-fixing bacteria is lower, which was also reported in the study of Garmashov V. M. *et al*. (2022).

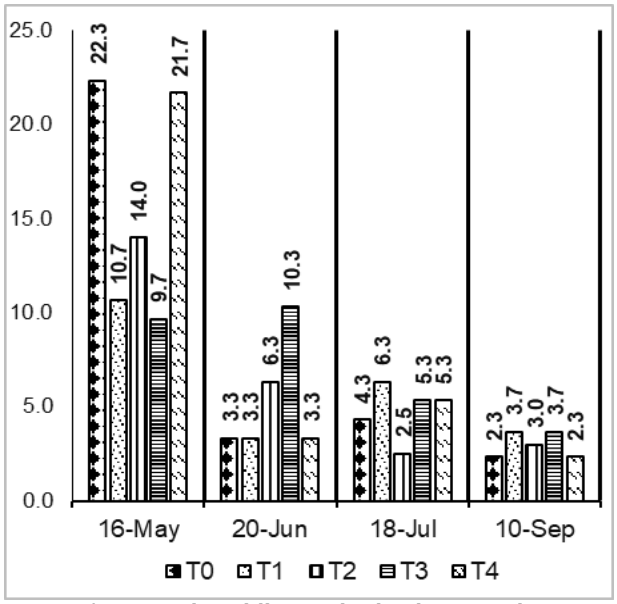

Figure 4 ***Clostridium*** **colonies in CT maize (cfu g soil^{-1})**

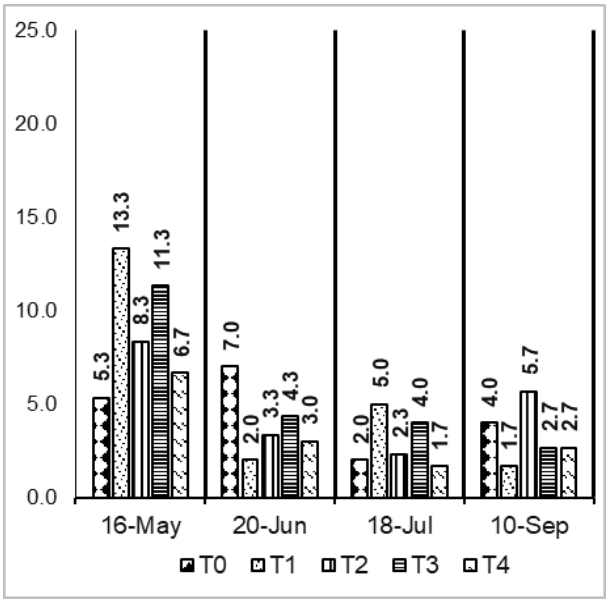

Figure 5 ***Clostridium*** **colonies in NT maize (cfu g soil^{-1})**

In sunflower, differences in the number of *Azotobacter* spp. colonies were observed with high values in CT determinations performed shortly after the two product applications (16 May and 18 July), as shown in *figure 6.* In May, all treatments show a high density, but the control is exceeded by T_2 and T_3. In July, in the variant where Ecofertil was applied at the recommended dose (T_1), a higher presence of bacterial colonies was observed (5.7×10^{-3} cfu g soil^{-1}) compared to the other treatments. At the last determination of N-fixing bacteria, in the treated variants, *Azotobacter* spp. colonies were reduced compared to the control.

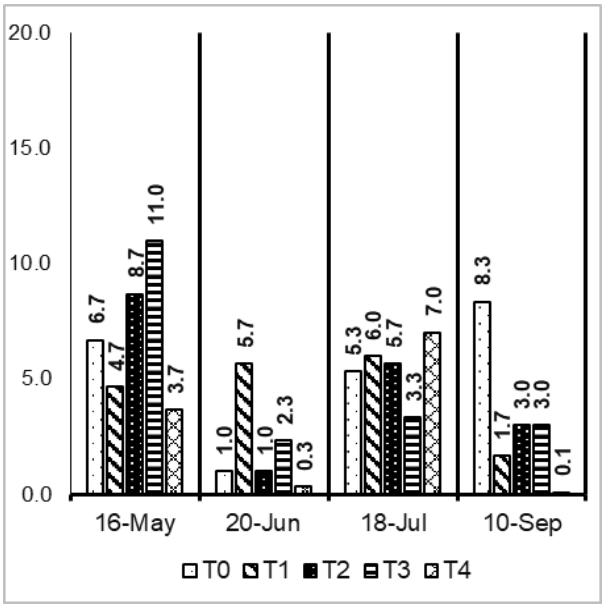

Figure 6 ***Azotobacter*** **sp. colonies in CT sunflower (cfu g soil^{-1})**

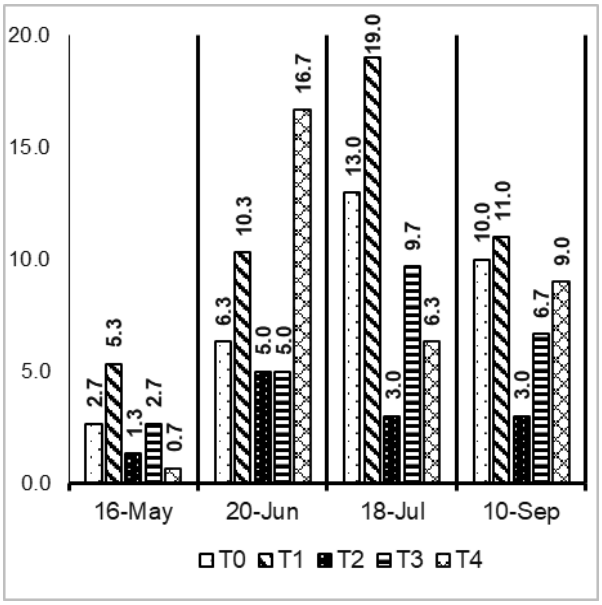

Figure 7 ***Azotobacter*** **sp. colonies in NT sunflower (cfu g soil^{-1})**

In the NT system, the 100% Ecofertil + no foliar fertilization treatment had a higher bacterial colony density, exceeding the control at all time points. In June the *Azotobacter* spp.population is more abundant in T_4 with 16.7×10^{-3} cfu g soil^{-1} compared to the other variants. After the second application of the product, the determinations showed high values in all variants except the one where a lower dose of product was applied (T_2) (*figure 7*).

In both tillage systems, *Clostridium* spp. numerical density is high in May, in CT the control is higher than all treated variants, and in NT it is exceeded by T_3 and T_4. Also, in both systems, T_1 showed a higher population in June, with no relevant differences in colony numbers between the other variants, and this was also noticed in the results of the next two determinations in the NT system (*figure 8; 9*).

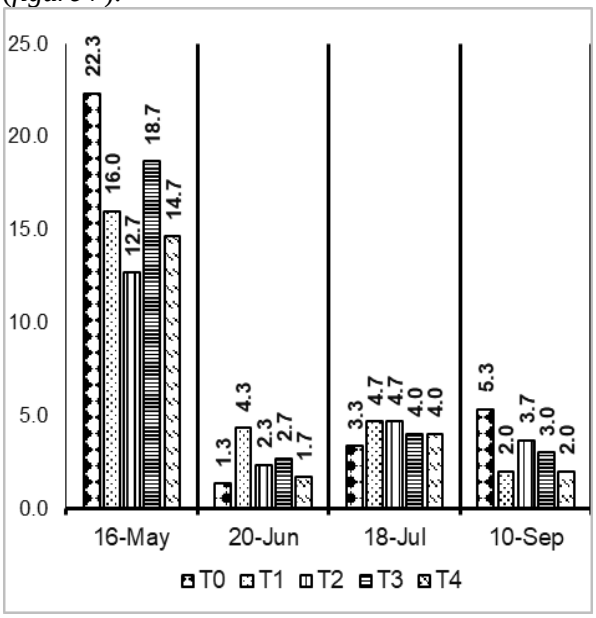

Figure 8 ***Clostridium*** **colonies in CT sunflower (cfu g soil^{-1})**

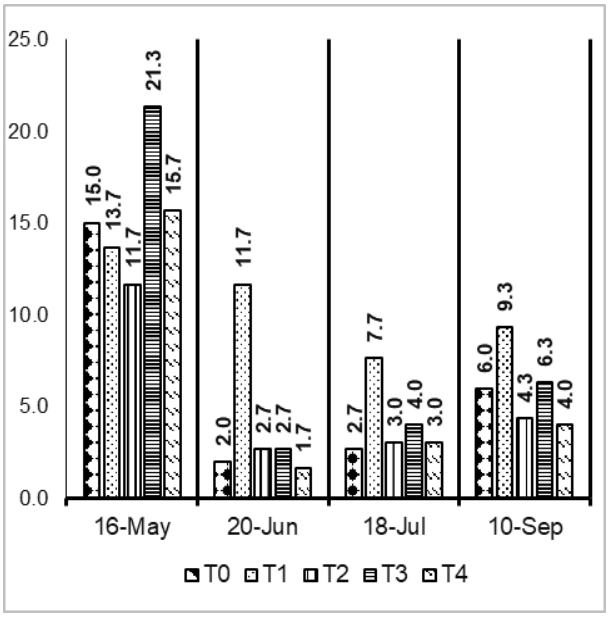

Figure 9 ***Clostridium*** **colonies in NT sunflower (cfu g soil^{-1})**

CONCLUSIONS

In the present study we investigated the evolution of *Azotobacter* spp.and *Clostridium* spp. colony numbers in different tillage systems and fertilization treatments. The results indicated that in both maize and sunflower crops, N-fixing bacteria population in the CT system were higher in May, at first determination, due to favorable environmental conditions and soil aeration by seedbed preparation tillage. There are differences between fertilization variants, but these are not consistent.

The results obtained so far do not support the hypothesis because the data are not consistent between treatments and tillage systems, and the relationship between soil N-fixing bacteria and those applied as fertilizer is based on interaction for growth and reproduction space rather than a direct influence of some on the others

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