https://doi.org/10.59893/abud.24(2).002

Acta Biol. Univ. Daugavp. 24(2) 2024
ISSN 1407 – 8953

THE PROCESS OF NITRIFICATION IN THE ROOT ZONE OF WINTER RYE PLANTS UNDER THE ACTION OF MINERAL NITROGEN AND INOCULATION

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Korotka I., Volkogon V. 2024. The process of nitrification in the root zone of winter rye plants under the action of mineral nitrogen and inoculation. *Acta Biol. Univ. Daugav., 24(2): 165-176.*

Abstract

Among the most important components of the nitrogen cycle in nature, the nitrification process is the least studied. Furthermore, nitrification features in soils depend on many factors, the role of some of which is almost unknown. The inhibitory approach to determining the nitrification activity allows for a differential assessment of the participation of auto- and heterotrophic nitrifiers in the oxidative link of the nitrogen cycle. Our study was conducted in a field experiment on cultivated sod-podzolic dusty-sandy soil. Winter rye was grown without inoculation and with *Diazobacteryn* treatment on different backgrounds. The experiment determined the content of water-soluble protein with Folin reactive and the activity of nitrate reductase in the flag leaf, protein content of grain, carried out yield accounting. The intensity of nitrification increases with the application of mineral nitrogen fertilisers in the experiment. When fertilizers were applied at a dose of N_{120}, the activity of the process in the phase of tube emergence increased by 38%, flowering − by 54%, and milk ripeness − by 93% compared to the control (without fertilizers). We found that pre-sowing inoculation of winter rye seeds with a biological product based on *Azospirillum brasilense,* stimulates the activity of autotrophic and heterotrophic nitrification, which may be one of the factors of positive influence of bacteria introduced into the agrocenosis on the root nutrition of plants. Autotrophic nitrification significantly exceeds the activity of the heterotrophic process. At the same time, the activity of autotrophic nitrification relative contribution to the formation of the nitrate pool in the rhizosphere soil of plants increases within the growing season. The use of *Diazobacteryn* helps to intensify the activity of heterotrophic nitrifiers, especially in the early stages of plant vegetation.

Keywords: Autotrophic nitrifiers, heterotrophic nitrifiers, inoculation, winter rye, nitrification inhibitor.

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INTRODUCTION

The biological nature of nitrate formation in the soil was established in the second half of the nineteenth century by Schlesing and Münz (1877a, 1877b). For the first time, Pasteur (1862) suggested the participation of microorganisms in the process of nitrification. However, it was not possible to identify the microorganisms involved in the formation of nitrates for a long time. Winogradsky (1888) made a significant contribution to the study of this issue. To isolate microorganisms, he used an elective medium, which is a solution of pure mineral salts, including ammonium sulfate, with which he impregnated silica gel plates. In 1891, he was able to isolate the microorganisms that cause the nitrification process. It was shown that there are two groups of nitrifiers: the first one actively oxidizes ammonium to nitrite, and the second one oxidizes nitrite to nitrate. The scientist first showed that the main source of carbon for nitrifying bacteria is air CO_2, which is assimilated in the Calvin cycle, and the energy required for this comes from the oxidation of ammonia and nitrite. Autotrophic nitrification occurs in two phases. The first phase of ammonium oxidation to nitrite has such a high reaction potential that it cannot be carried out by any physiological electron carriers. The first phase of nitrification is carried out by representatives of the genera *Nitrosomonas* (*N. europaea* Winogradsky), *Nitrosococcus* (*N. oceanus* Watson*, N. mobilis* Koops*, N. halophilus* Koops), *Nitrosospira* (*N. briensis* Winogradsky), *Nitrosolobu*s (*N. multiformis* Watson), *Nitrosovibrio* (*N. tenuis* Harms). The second phase of nitrification consists in the oxidation of nitrite to nitrate and is carried out by bacteria belonging to the genera *Nitrobacter* (*N. winogradskyi* Winslow*, N. hamburgensis* Bock), *Nitrospina* (*N. gracilis* Watson), *Nitrospira* (*N. marina* Watson), *Nitrococcus* (*N. mobilis* Watson).

Autotrophic nitrifiers make a significant contribution to the formation of nitrous oxides in soils. As early as 1971, T. Yoshida and M. Alexander first showed that *Nitrosomonas europea* Winogradsky releases N_2O during the oxidation of ammonium or hydroxylamine to nitrite. Later, using ^{15}N compounds, it was found that when cells were incubated in a medium with ammonium and nitrite in an atmosphere of 0.1 % O_2, most of the N_2O was formed from nitrite. That is, phase I nitrifiers (chemolithotrophic ammonium-oxidizing bacteria) contribute to the emission of NO, N_2O, and N_2 (Yoshida & Alexander 1970). The formation of gaseous nitrogen compounds by autotrophic nitrifiers is confirmed by the data of a number of authors (Chen et al. 2023, Davidson et al. 1986, Davidson & Swank 1986, Goreau et al. 1980, Peixoto & Petersen 2023). It is now known that representatives of heterotrophic microorganisms, including micromycetes, are also capable of producing nitrite and nitrate during the oxidation of ammonium and other reduced organic and inorganic nitrogen compounds (Arp & Stein 2003, Antošovský et al. 2024, Zhang et al. 2020).

Even in the days of Winogradsky, the development of nitrifiers on organic media was repeatedly reported, indicating the possibility of the heterotrophic component of nitrification. Warington (1884) and Munro (1886) argued that organic substances are not a direct substrate for nitrification; only substances that have been pretreated to simplify their molecular structure (ammonification) are nitrified.

Heterotrophic nitrification consists in the oxidation of ammonium and amine nitrogen of organic compounds with the formation of intermediate products − hydroxylamine, oximes, hydroxamic acids, nitroso compounds, nitrites; the final product is nitrates (Verstraete & Alexander 1972a, Verstraete & Alexander 1972b). Heterotrophic nitrification does not serve as a source of energy for microorganisms. In nature, heterotrophic nitrification occurs wherever ammonia is formed in conditions of excess organic matter, for example, in compost heaps, water bodies, etc (Castignetti & Hollocher 1984, Stroo et al. 1986). It is interesting that during heterotrophic nitrification, microorganisms can also oxidize nitro compounds that are part of pesticides.

The optimal course of the crop production process often limits the level of nitrogen supply to plants, and agricultural technologies therefore involve the use of nitrogen fertilizers. However, even when mineral nitrogen is applied, conditions may arise in the soil where the absorption of the active ingredient from fertilizers by plants is low and environmental pollution is intense. For example, when ammonia fertilizers are used, ammonia becomes available to nitrifying microorganisms and is oxidized during the nitrification process. The nitrates produced in this process are used by plants, which is a positive aspect of the process. At the same time, their excess is washed out into water bodies and is also the basis for the development of another process of nitrogen cycling - biological denitrification (De Boer et al. 1996, Li et al. 2018, Shukla et al. 2020, van Niel et al. 1993, Yuan et al. 2019). Therefore, nitrogen fertilizers should be used rationally both from the standpoint of economic effect and environmental considerations. In this regard, it is important to study the peculiarities of the processes of biological transformation of nitrogen compounds in the soil.

It should be noted that among the most important components of the nitrogen cycle in nature, the process of nitrification is the least studied, and the peculiarities of its course in soils depend on many factors, while the role of some of them is practically unknown. For example, it is believed that autotrophic nitrification prevails over heterotrophic nitrification in agrocenosis soils, since the presence of ammonia and the lack of organic matter optimizes the living conditions of autotrophic nitrifiers (Brierley & Wood 2001, Barneze et al. 2015). Autotrophic nitrifiers account for almost 84−99% of ammonium nitrogen oxidation in cultivated soils of agrocenoses (Mukhtar & Lin 2019). At the same time, the main vector of biochemical activity in soils is plants, which release a large amount of organic compounds, including those containing nitrogen, into the root zone (Nardi et al. 2020). Theoretically, conditions for active heterotrophic nitrification should be created in the plant root zone. Our research aimed to find out the peculiarities of autotrophic and heterotrophic nitrification processes in the root zone of winter rye plants at different levels of nitrogen fertilization.

MATERIAL AND METHODS

Study site

To study the peculiarities of nitrogen assimilation by winter rye (*Secale cereale* L.) plants of Synthetic-38 variety under the influence of *Diazobacteryn* (biological agent of the preparation − *Azospirillum brasilense* 18-2) inoculation and dependence on backgrounds, field experiments were conducted on sod-podzolic dusty-sandy cultivated soil of the experimental field of the Institute of Agricultural Microbiology and Agroindustrial Production of NAAS. This biopreparation is organic and safe, does not harm the human body, animals and insects, including bees, is approved for use in organic production and has the Organic Standard certificate.

The experimental field is located in the Chernihiv district of the Chernihiv region, in the Polissya zone of the Prydniprovska lowland. The average height above sea level is 120 meters. The climate of the Chernihiv region is temperate continental, mild, and quite humid. The winters are snowy, stable and relatively warm in most years, while summers are warm and moderately humid. The average annual temperature is 6-8° C. Over the past 10 years of observations, there has been a clear upward trend in the average annual air temperature, mainly due to the winter months. The average temperature of the coldest month of the year (January) is 6-7° C, and the warmest month (July) reaches 19-20° C, but in some years the air temperature deviates significantly from these values.

The soil cover of the Chernihiv region is formed mainly by low-humus soils of light particle size distribution, which determines their low absorption capacity, low buffering capacity, low saturation with soil colloids, and thus increased vulnerability to technogenic and anthropogenic impact. The humus content in sod-podzolic soils is 1.02%. They have a neutral reaction of the soil solution, with a weighted average pH of 7.2. The availability of mobile phosphorus forms is significantly increased (330 mg kg^{-1} of soil), exchangeable potassium is average (148 mg kg^{-1} of soil) and easily hydrolyzed nitrogen is low (54.9 mg kg^{-1} of soil). They are characterized by a low content of exchangeable forms of calcium and magnesium, respectively, 3.2–4.1 and 0.6-0.7 mg-eq 100 g^{-1} of soil. Sod-podzolic soils are the least fertile soils, with their bonitas ranging mainly 31–38 points.

The crop predecessor – pea and oat mixture

Scheme of experiment:
I Without bacterization
1. without fertilizers;
2. $N_{30}K_{20}$ (N_{20} in fall + N_{10} in early spring);
3. $N_{60}K_{40}$ (N_{30} in fall + N_{30} in early spring);
4. $N_{90}K_{60}$ (N_{30} in fall + N_{30} in early spring + N_{30} in the tube emergence phase);
5. $N_{120}K_{80}$ (N_{30} in fall + N_{45} in early spring + N_{45} in the tube emergence phase).

II Inoculation with *Diazobacteryn*
6. without fertilizers;
7. $N_{30}K_{20}$ (N_{20} in fall + N_{10} in early spring);
8. $N_{60}K_{40}$ (N_{30} in fall + N_{30} in early spring);
9. $N_{90}K_{60}$ (N_{30} in fall + N_{30} in early spring + N_{30} in the tube emergence phase);
10. $N_{120}K_{80}$ (N_{30} in fall + N_{45} in early spring + N_{45} in the phase of tube emergence).

The scheme of the experiment did not provide for phosphorus fertilizers due to high content of water-soluble phosphates in the soil. The dose of nitrogen fertilizers 120 kg ha^{-1} and potassium fertilizers 80 kg ha^{-1} was calculated on the basis of the maximum planned yield of 35 kg ha^{-1}.

To study the process of nitrification and assess the contribution of autotrophic and heterotrophic nitrifying microorganisms to the formation of nitrates, the composting method by D. Zvyagintsev was used. An average sample of rhizosphere soil was prepared, sieved through a sieve with a mesh diameter of 2 mm, 10 g weights were placed in 40 ml vials and moistened to 60-70% of the full field moisture capacity. The samples were incubated for 21 days at 26–28°C, and the humidity was maintained by adding water after control weighing of the vials. The activity of autotrophic nitrification was inhibited by the nitrification inhibitor 4-amino-1,2,4-triazole (100 µg per g soil). It is known from the literature (Wu et al. 2019, Li et al. 2020) that aminotriazole (ATG) in this concentration, which exceeds its specific activity by almost 2 orders of magnitude, does not reduce the growth rate, biomass accumulation, and nitrate production in heterotrophic microorganisms. The nitrate content was determined after 21 days using ion-selective electrodes. The experiments were repeated 3 times.

Nitrification activity in the rhizosphere soil was analyzed using the two-way ANOVA algorithm with a probability level (<5%) using Statistica 6.0 software (StatSoft Inc., USA). The least significant difference was calculated for (a) the whole experiment, (b) only for different doses of fertilizers, (c) only for inoculation with *A. brasilense* and their interaction.

RESULTS AND DISCUSSION

According to the data obtained, the intensity of nitrification increases with the application of mineral nitrogen fertilizers in the experiment. Thus, when fertilizers were applied at a dose of N_{120}, the activity of the process in the phase of tube emergence increased by 38%, flowering − by 54%, and milk ripeness − by 93% compared to the control (without fertilizers). The use of aminotriazole to inhibit the activity of autotrophic nitrifiers indicates a significant decrease in the intensity of the nitrification process when it is added (Hao et al. 2020, Li et al. 2020, Wu et al. 2019).

Table 1. Nitrification activity in the rhizosphere soil of winter rye plants, phase of tube growth (36 days after early spring mineral nitrogen application), µg $N{-}NO_3$ per g soil.

Variants	Soil (total activity of processes)	Soil + inhibitor of autotrophic nitrification
	Without inoculation	
Control, without fertilizers	40.87	6.60
$N_{30}K_{20}$	43.47	7.13
$N_{60}K_{40}$	49.20	7.60
$N_{90}K_{60}$	49.40	19.80
$N_{120}K_{80}$	56.93	22.87
	Inoculation by Diazobacteryn	
Without fertilizers	50.67	12.00
$N_{30}K_{20}$	51.87	14.00
$N_{60}K_{40}$	50.67	14.33
$N_{90}K_{60}$	58.13	25.73
N120K80	59.47	26.80
LSD_{05} for the experiment	11.15	4.17
for fertilizers	4.99	1.87
for inoculation and interaction	6.44	2.41

Thus, the inhibitory approach allows for a differential assessment of the participation of autotrophic and heterotrophic nitrifiers in the oxidative link of the nitrogen cycle (Belser & Mays 1980, Okey et al. 1996, McGeough et al. 2016).

When studying the activity of the process in the first period of sampling (36 days after early spring mineral nitrogen application), it was found that the activity of autotrophic nitrification in the rhizosphere soil of winter rye plants grown on low agricultural backgrounds significantly exceeds the corresponding indicators of heterotrophic nitrification (Tab. 1). However, with increasing doses of mineral fertilizers in the rhizosphere soil of rye plants, not only the activity of autotrophs but also heterotrophic nitrifiers increases, and the latter to a much greater extent. Thus, if the share of nitrates from the activity of heterotrophic nitrifiers was 16% of the total scale of the process in the control (without nitrogen application to the soil), then in the variant with the highest dose of fertilizer in the experiment, this figure reached 40%. We explain this by the possibility of increasing both the size of root exudates and the amount of nitrogen-containing organic compounds in them under these conditions. Tate III R.L. (1977) also notes a significant increase in the activity of heterotrophic nitrification under conditions unfavourable for the development of autotrophic nitrifiers. These data suggest that the heterotrophic population may be responsible for some of the nitrate produced in soil.

The use of the microbial preparation *Diazobacteryn* in rye cultivation technology increases the scale of nitrification. A comparison of autotrophic and heterotrophic nitrification rates indicates that the observed increase is due to the activation of heterotrophic microorganisms. Thus, the contribution of heterotrophic nitrifiers to the total scale of the process ranged from 24% in the control to 45% in the variant with a high dose of fertilizer (as already noted, the corresponding indicators in the block of variants without bacterization were 16 and 40%). Since the amount of mineral nitrogen (as well as all other factors) in the soil was the same as in the non-inoculated variants, this effect can be explained by an increase in the amount of root exudates in bacterized rye plants, which can be an additional source of organic matter containing nitrogen compounds in addition to carbon. This assumption is quite acceptable, given the numerous literature data on the effect of pre-sowing inoculation on the activity of photosynthesis in bacterized plants (Wang et al. 2024).

In the next period of research − the flowering phase (62 days after early spring application of mineral nitrogen and 25 days after fertilizing plants in the respective variants), the above-described features of nitrate formation depending on the agrophage as a result of nitrification are generally observed. However, there are also some differences. For example, the share of heterotrophic nitrifiers in the formation of the nitrate pool decreases during this period. And although the effect of bacterization can be traced − the indicators differ by more than two times − it should be concluded that autotrophic nitrification dominates even under these conditions. Its share reaches 83−86% (Tab. 2).

The decrease in the activity of heterotrophic nitrification in the flowering phase in the block of variants with the use of *Diazobacteryn* is associated with some regularities of interaction between the inoculant and the plant. Thus, it is known that in the initial phases of organogenesis of bacterized plants, introduced bacterial strains actively colonize the root spheres of plants, dominate among microorganisms, and over time, their relative number in the microbial community begins to decrease due to the desire of the young ecosystem to stabilize. Therefore, the number of introduced bacteria gradually decreases to the level occupied by the microorganism in the natural environment (Wang et al. 2024, Ying et al. 2017). It is natural that the impact of bacterization on both the production process of crops and soil biochemical processes in the root zone of plants decreases over time.

At the end of the growing season, the nitrification activity decreases, which may be due to the deficiency of ammonium (substrate for autotrophs) and organic nitrogen compounds (substrate for heterotrophic nitrifiers) in the rhizosphere soil of winter rye plants (Tab. 3). It is interesting to note that in the block of experimental variants with bacterization, the activity of nitrification (both autotrophic and heterotrophic) is lower than the corresponding indicators of variants without inoculation.

Table 2. Nitrification activity in the rhizosphere soil of winter rye plants, flowering phase (62 days after early spring mineral nitrogen application and 25 days after plant nutrition), µg $N-NO_3$ per g soil.

Variants	Soil (total activity of processes)	Soil + inhibitor of autotrophic nitrification
	Without inoculation	
Control, without fertilizers	36.00	3.68
$N_{30}K_{20}$	40.00	3.69
$N_{60}K_{40}$	43.13	3.40
$N_{90}K_{60}$	46.00	3.72
$N_{120}K_{80}$	55.53	4.65
	Inoculation by Diazobacteryn	
Without fertilizers	50.33	8.60
$N_{30}K_{20}$	55.60	8.87
$N_{60}K_{40}$	64.33	9.20
$N_{90}K_{60}$	64.80	9.40
$N_{120}K_{80}$	67.33	10.00
LSD_{05} for the experiment	9.88	1.37
for fertilizers	4.42	0.61
for inoculation and interaction	5.70	0.79

This is because: firstly, plants initiated by bacterization more actively absorb nutrients, which contributes to their faster depletion from the soil, and secondly, bacterized rye plants, as evidenced by long-term observations, go through the phases of organogenesis faster and mature earlier. Accordingly, the biochemical activity in the root zone of such plants will also be reduced.

Table 3. Nitrification activity in the rhizosphere soil of winter rye plants, phase of milk ripeness (91 days after early spring mineral nitrogen application and 54 days after plant nutrition), µg $N{-}NO_3$ per g soil.

Variants	Soil (total activity of processes)	Soil + autotrophic nitrification inhibitor
Without inoculation		
Control, without fertilizers	13.00	2.63
$N_{30}K_{20}$	12.93	2.67
$N_{60}K_{40}$	13.53	3.52
$N_{90}K_{60}$	16.73	4.73
$N_{120}K_{80}$	25.13	7.53
Inoculation by Diazobacteryn		
Without fertilizers	11.47	3.80
$N_{30}K_{20}$	13.07	2.74
$N_{60}K_{40}$	13.20	2.89
$N_{90}K_{60}$	13.80	4.19
$N_{120}K_{80}$	13.33	4.32
LSD_{05} for the experiment	3.01	1.33
for fertilizers	1.35	0.60
for inoculation and interaction	1.74	0.77

Barraclough and Puri (1995) obtained similar results in their research. They showed that a maximum of 8% of the observed nitrification could be the result of heterotrophic nitrifiers oxidizing organic nitrogen to nitrate without passing through the exchangeable soil ammonium pool.

CONCLUSIONS

Thus, the activity of nitrification in the root zone of winter rye plants increases with increasing dose of mineral nitrogen. Autotrophic nitrification significantly exceeds the activity of the heterotrophic process. At the same time, its relative contribution to the formation of the nitrate pool in the rhizosphere soil of plants increases with the growing season. The use of Diazobacteryn helps to intensify the activity of heterotrophic nitrifiers, especially in the early stages of plant vegetation. This research is important for preserving soil fertility and the environment. Further study of the nitrification process will, on the one part, allow us to influence the metabolism of excess nitrogen runoff from the soil (and its reduction to gaseous nitrogen), and, on the other part, reduce greenhouse gas emissions from agricultural activities.

ACKNOWLEDGEMENTS

We are grateful to the Laboratory of Soil Microbiology of the Institute of Agricultural Microbiology and Agro-industrial Manufacture of the NAAS for their assistance in organizing and conducting field and laboratory experiments. We are also sincerely grateful to the reviewers who provided their suggestions and recommendations.

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Received: 30.07.2024
Accepted: 01.11.2024