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PHYLOGENETIC STRUCTURE OF COMMUNITY OF PROCARIOTS OF SODDY-PODZOLIC SOIL UNDER THE COVER OF WINTER RYE IS NOT INFLUENCED BY AGROTECHNICS

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Abstract

Soil microbial communities are complex multicomponent systems that form under the influence of a wide range of factors, among them — soil type, plant species, climate, agricultural technology — in general, determining the physical and chemical characteristics of the environment. The plant, according to many researchers, is the main factor determining the structure of the soil microbial community, due to the extensive number of compounds released into the soil. There is still a discussion about the specific nature of the action of various plants on soil microbiome, which is very important both for understanding the mechanism of interaction of microorganisms and plants, and for building optimal crop rotations, as well as organizing measures to protect agricultural crops from phytopathogenic microorganisms and pests. Winter rye is one of the few crops that can grow continuously for decades. It has a powerful root system, comparable to the biomass of the above-ground part of plants. The root excretions of winter rye reach 21 % of the synthesized plant mass. This paper presents the results of research aimed at studying the phylotypical structure and diversity of prokaryotic microorganisms in rye crops grown in permanent culture and six-field crop rotation for almost 100 years, in the long-term multifactorial field experiment of the Moscow Timiryazev Agricultural Academy. The aim of our work was to study the influence of various agricultural technicians such as crop rotation and liming, under the conditions of a long field experiment on the phylogenetic structure of prokaryotic micro-organisms in rye crops. The results of the high-throughput DNA sequencing of the soil microbiome and the subsequent analysis of the phylogenetic structure and diversity of the prokaryotic microorganisms of the sod-podzolic soil under the conditions of perennial rye culture showed that the plant is one of the key factors in the formation of the prokaryotic community. Regardless of the agrotechnical methods under the cover of winter rye, the same core structure of prokaryotes, including a small number of types of proteobacteria and actinobacteria, develops in the earing phase. The dominant position among them is occupied by the bacteria of the *Rhizobiaceae* family, which in this case is to some extent related to the history of the experimental field. Apparently, the bacteria of this family and, above all, the nodule bacteria, find favorable conditions for their development in the rye rhizosphere. It is possible that a kind of associative symbiosis is formed between them, which was observed by some authors with other cereal crops. In this connection, studies of the viability of *Rhizobiaceae* in winter rye crops, and their evolution to associative endosymbiotic relationships with rye in the course of a long coexistence are of undoubted interest. The effect of liming on the genetic structure of the prokaryote community of acidic soils may be different. At the same time, apparently, the specific type of plants, as well as the history of the field (crop rotation, permanent culture, fertilizer system, etc.) are of significant importance.

Keywords: phylogenetic structure, biodiversity of prokaryotes, *Rhizobium* sp., *Proteobacteria*, sod-podzolic soil, winter rye.

The soil is a complex ecological system, where a critical role is played by

microbial communities, which ensure the normal functioning of the biosphere [1, 2]. For agriculture, the taxonomic and functional structure of the microbial community, which is formed under a variety of physical and chemical environmental conditions under the cover of different plant species, is of special interest. Most researchers are of the opinion that the main factor in the formation of the rhizosphere microbiota, and especially the rhizoplane, are plants, but, given the complexity of the interaction in the soil—microorganism —plant system, many of them recognize the significant influence of the soil type, agrotechnical measures, and climate on the structure and diversity of rhizosphere microorganisms [3-5].

Until now, there is a discussion about the specific nature of the effect on various plants on the soil microbiome, which is very important for understanding the mechanism of interaction between microorganisms and plants, building optimal crop rotations, as well as organizing measures to protect agricultural crops from phytopathogenic microorganisms and pests. In this regard, a special role is given to the study of soil microbial flora under the conditions of long field experiments in which the same crop is under cultivation in the field for many years. Such experiments are conducted in Russia, Germany, Great Britain, the USA, Canada, and France [6]. Total studies of the phylogenetic structure of the soil microbiome in the multifactorial long-term (more than 100 years) field experiment (Moscow Timiryazev Agricultural Academy) showed [7] that the key factor in the microbiome phylogenetic diversity is the type of cultivated plant, liming is in the second place. The systematic use of mineral fertilizers had no noticeable effect on the phylogenetic structure of the soil microbiome. At the same time, microbiomes of the soil sown with various plants respond differently to liming [7].

The structure of the soil microbiome under winter rye is of special interest. It is one of the few crops that can grow continuously for decades. It has a powerful root system, comparable to the biomass of the above-ground part of plants and reaching 6 t/ha [8]. The total area of the roots is about 6 thousand m^2 and their surface exceeds the surface of the above-ground part by 130 times [9]. According to some authors, the root excretions reach 21% of the synthesized plant mass. The root excretions of winter rye and plant tissues include organic acids, sugars, cyclic hydroxamic acid glucosides, as well as the products of their secondary transformation, α-glucans and benzoxyzolinone derivatives [11-13]. Hydroxamic acid derivatives and their transformation products have herbicidal, fungicidal and insecticidal properties, providing protection of the crop from phytopathogenic fungi and its high competitiveness with weeds [14-16]. This suggests a significant effect of winter rye on the soil microbiome.

E. Kurek et al. [17] showed that the number of prokaryotes in the rhizosphere of winter rye is higher than in the soil. According to their data, gram-positive bacteria prevail in the soil and in the rhizosphere of plants. Other researchers note that gram-negative bacteria prevail in the rhizosphere of plants of almost all field crops, especially at an early age [2, 9]. According to A.O. Zverev et al. [18], the phylogenetic structure of prokaryotes and their diversity in the rhizosphere of winter rye at the age of 42 days and in the fallow soil almost do not differ. I.G. Shirokikh et al. [19] found in the rye rhizosphere a significant number of actinomycetes; their species composition and number changed during the ontogeny of plants. The dominant position was occupied by streptomycetes. Data on the long-term effect of rye cultivation on soil microbial flora are not available.

This paper for the first time presents data on the phylotype structure and diversity of prokaryotic microorganisms in the soil when growing rye as a permanent crop in six-field crop rotation for almost 100 years (long-term multifactorial field experiment of the Moscow Timiryazev Agricultural Academy). The

results indicate that the plant is a major factor in the formation of a soil prokaryotic community.

The purpose of this study was to assess the effect of different agricultural technologies (crop rotation, liming) under the conditions of a long-term field experiment on the phylogenetic structure of microorganisms in rye crops.

Techniques. Soil samples were collected in 2010 at the site of the long-term experiment of the Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, which was located on an area of about 1.5 hectares with a slope of 1° to the north-west on the morainic plain in the southern part of the Klin-Dmitrov Upland. The altitude above the sea level was 162 m, the average precipitation was about 600 mm per year, about half of which occurred in May-August; the average annual temperature was 4.1 °C. The soil was sod-podzolic, sandy large-silt loam, old-arable (over 200 years in tillage) [20]. Plots planted with winter rye (*Secale cereale* L.), which has been cultivated as a permanent crop in six-field crop rotation since 1912, were used for phylogenetic analysis of the prokaryotic microorganisms system in the soil. Crop rotation included bare fallow, winter rye, potatoes, barley with clover undersowing, first-year clover, and flax. Mineral fertilizers were applied to experimental plots annually. The total volume of these fertilizers over the years of study (1912-2009) was 5820 kg of nitrogen, 7990 kg of phosphorus, 6716 kg/ha of potassium [20].

Soil samples were collected during the winter rye paniculation phase to a depth of arable horizon A1 (0-20 cm) in 5 replications, of which an average sample was made, which was thoroughly mixed.

When isolating DNA from a soil sample, a weighed portion (0.2 g) was placed in an Eppendorf tube with a volume of 2 ml, then an equal volume of glass beads with a diameter of 0.1 mm (Innomed, Hungary), 350 μl of A solution (20 mM of sodium phosphate buffer, 240 mM of guanidine isothiocyanate, pH 7.0), 350 μl of B solution (500 mM of Tris-HCl, 1% SDS, pH 7.0) and 400 μl of a phenol and chloroform mixture were added. The tube was placed in a FastPrep-24 homogenizer (MP Biomedicals, USA) and the sample was destroyed within 10-15 min. Then it was centrifuged at 10,000-15,000 g for 5 min, the aqueous phase was collected. After homogenization, 400 μl of chloroform was added into the sample, then it was vigorously shaken using a vortex for 1 min, centrifuged under the same conditions as in the previous stage, the aqueous phase was collected. An equal volume of isopropyl alcohol was added to the extracted DNA; then it was vortexed, centrifuged, washed twice with 70% ethanol, and dried in air. The precipitate was dissolved in 100 μl of water at 65 °C for 15 min.

DNA was purified from impurities using electrophoresis in 1% agarose gel. A cut agarose block containing DNA was placed in an Eppendorf tube (1.5 ml), 2 volumes of C solution (3 M of guanidine isothiocyanate, 20 mM of Tris-HCl, 20 mg/ml of Triton X-100, pH 7.0) were added and incubated at a temperature of 65 °C until complete dissolution of the block. 20 μl of D solution (C solution with the addition of silica, 40 mg/ml) was added to the solution, then it was stirred and incubated for 5 min at room temperature and shaken occasionally. Then it was centrifuged at 10000-15000 g for 1 min, the supernatant was completely removed, the precipitate was suspended in 200 μl of D solution (25% of ethanol, 25% of isopropanol, 100 mM of NaCl, 10 mM of Tris-HCl, pH 7.0), centrifuged at 10,000-15,000 g for 1 min, then the supernatant was removed, the precipitate was resuspended in ethanol, centrifuged again for 1 min, and then the supernatant was removed. The precipitate was dried in air for 15 minutes, 50 μl of elution buffer (10 mM of Tris-HCl, 1 mM of EDTA, pH 8.0) was added and vortexed for 30 minutes. After that, the samples were centrifuged and the supernatant was collected, avoiding the ingress of silicon oxide into the

purified DNA sample.

The isolated total soil DNA was used as a template for sequencing nucleotide sequences. Universal primers for the V4 variable region of the 16S rRNA gene (F515 – 5′-GTGCCAGCMGCCGCGGTAA-3′, R806 – 5′-GGATACVSGGGTATCTAAT-3′) were used with the addition of oligonucleotide identifiers for each sample and service sequences necessary for high-throughput DNA sequencing using the Roche protocol (Switzerland). Samples preparation and sequencing were performed using GS Junior (Roche, Switzerland) in accordance with the manufacturer's recommendations. Taxonomic identification of DNA sequences and comparative analysis of microbial communities were performed using VAMPS (Visualization and Analysis of Microbial Population Structure) (http://vamps.mbl.edu/). In addition, the RDP database (Ribosomal Database Project, http://rdp.cme.msu.edu/) was used for the extended phylogenetic characteristics of sequences.

Results. Agrochemical characteristics of the soil as of the date of soil sampling are presented in the table.*Результаты.* Агрохимическая характеристика почвы на дату отбора почвенных образцов представлена в таблице.

Agrochemical characteristics of sod-podzolic soil in the plots of the long-term experiment under the crops of winter rye (experimental field of the Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Moscow, 2010)

Experiment variant	N_{total}, %	P_2O_5, mg/100g	K_2O, mg/100g	C_{total}, %	$pH_{sal.}$	The amount of exchange bases, mEq/100 g
Rye crop rotation	0.090	31.45	4.25	0.79	4.2	8.00
Rye crop rotation + lime	0.098	31.85	4.63	0.93	5.7	7.75
Permanent rye	0.112	53.80	24.70	1.28	4.6	8.63
Permanent rye + lime	0.095	53.70	21.51	0.98	6.1	8.25

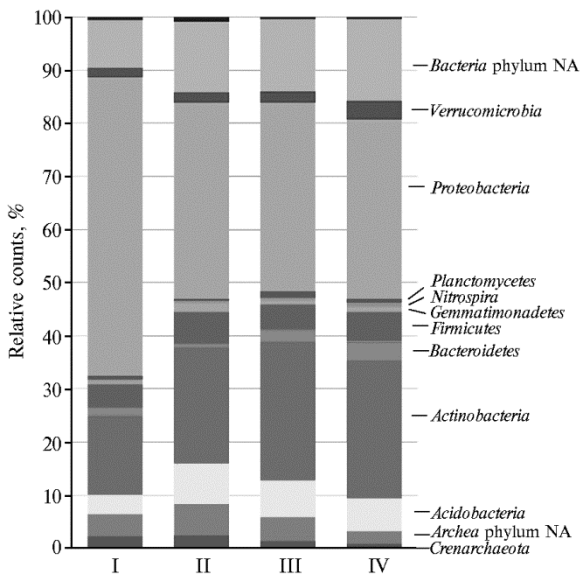

Fig. 1. Taxonomic diversity of prokaryotic microorganisms of sod-podzolic soil (at the phyla level) in the plots of the long-term experiment under the crops of winter rye depending on the cultivation technology: I – in crop rotation with soil liming, II – in crop rotation without soil liming, III – permanent crop with soil liming, IV – permanent crop without soil liming (experimental field of the Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Moscow, 2010)

High-throughput pyrosequencing of amplified DNA from soil samples as part of the microbial community of the sod-podzolic soil under winter rye crops revealed 16 phyla of bacteria and 2 phyla of archaea. The dominant position was occupied by two bacterial phyla — *Proteobacteria* (from 34 to 56%) and *Actinobacteria* (from 15 to 26%). The *Acidobacteria* and *Firmicutes* phyla were from 3.5 to 7.5%, and archaeans were from 3.0 to 8.5% of the total number of prokaryotic microorganisms (Fig. 1).

About 300 genera of microorganisms were found in of the soil prokaryotic community. Among them, only 41 (which did not exceed 13% of the total number of taxa) had a frequency of more than 1% (Fig. 2). 12 genera were encountered in all variants of the

experiment, regardless of the winter rye growing technology (rotation, permanent crop, liming). Apparently, they were the core system of prokaryotes, characteristic of the studied soil type under winter rye crops. The system was dominated by bacteria belonging to the *Proteobacteria* and *Actinobacteria* phyla, as well as unidentifiable bacteria (see Fig. 2). The dominant development of these taxa in the rhizosphere of winter rye was observed by other authors [2, 18], which is consistent with the results of this study.

Microorganism	I	II	III	IV
Acidobacteria Gp6	0.99	1.85	0.37	1.42
Acidobacteria Gp16	0.58	1.50	0.87	2.24
Acidobacteria Gp1	0.58	0.45	3.29	0.67
Acidobacteria Gp4	0.58	1.00	0.31	0.89
Acidobacteria Gp3	0.25	0.65	1.49	0.37
Actinobacteria genus NA18	4.47	7.44	5.21	6.18
Actinobacteria genus NA17	1.66	2.70	2.73	2.76
Solirubrobacter sp.	1.41	1.45	1.43	1.86
Actinobacteria genus NA16	1.08	2.15	2.42	1.86
Arthrobacter sp.	0.91	1.50	0.93	1.12
Actinobacteria genus NA11	0.58	0.70	0.74	0.97
Streptomyces sp.	0.50	1.05	0.37	1.04
Conexibacter sp.	0.50	0.35	1.05	0.60
Nocardioides sp.	0.25	0.95	0.50	1.19
Actinobacteria genus NA5	0.17	1.40	0.37	0.60
Bacteroidetes genus NA2	0.50	1.55	0.37	1.04
Crenarchaeota genus NA	2.24	0.85	2.42	1.42
Firmicutes genus NA1	1.08	1.15	1.12	1.19
Firmicutes genus NA5	0.83	1.10	1.24	1.56
Paenibacillus sp.	0.75	0.70	1.05	0.07
Bacillus sp.	0.58	0.85	0.93	0.67
Gemmatimonas sp.	0.66	1.10	1.74	0.89
Bacteria genus NA	9.02	15.43	13.08	13.56
Archaea genus NA	4.22	2.50	5.95	4.47
Rhizobium sp.	25.83	6.04	13.14	6.33
Proteobacteria genus NA7	14.90	3.55	3.60	3.80
Proteobacteria genus NA24	2.15	4.20	2.36	3.13
Proteobacteria genus NA30	1.57	1.55	3.35	2.76
Proteobacteria genus NA6	1.41	0.05	0.12	0.15
Proteobacteria genus NA28	0.75	1.35	0.43	1.04
Pseudomonas sp.	0.66	0.45	0.74	1.34
Bradyrhizobium sp.	0.58	0.85	0.93	0.60
Proteobacteria genus NA15	0.50	1.00	1.43	1.27
Sphingomonas sp.	0.41	0.95	0.50	1.27
Proteobacteria genus NA36	0.41	1.45	0.68	1.27
Proteobacteria genus NA34	0.33	1.00	0.93	0.97
Hyphomicrobium sp.	0.17	1.05	0.12	1.42
Verrucomicrobia genus NA2	1.08	2.20	1.12	1.19

Fig. 2. Heat map of the dominant prokaryotic microorganisms of sod-podzolic soil (at the genus level) in the plots of the long-term experiment under the crops of winter rye depending on the cultivation technology: I — in crop rotation with soil liming, II — permanent crop with soil liming, III — in crop rotation without soil liming, IV — permanent crop without soil liming; white to dark gray color gradations — abundance of microorganisms (in percent), respectively, ≤1; 1.01-5; 5.01-10 and > 10 (experimental field of the Russian State Agrarian University − Moscow Timiryazev Agricultural Academy, Moscow, 2010).

According to the dominance (diversity) curves of the prokaryotic system in the soil, the greatest species wealth of prokaryotic microorganisms was found under the permanent rye crop (Fig. 3). It was significantly lower in the crops of rye grown under the conditions of six-field crop rotation. The low species wealth in the latter case, apparently, is due to the fact that rye precursors were flax and fallow field, where the delivery into the soil of fresh organic matter as a source of nutrition and energy-yielding material for microorganisms is extremely limited. These observations lead to the conclusion about a certain effect of the precursor on the species wealth and diversity of the prokaryotic system of soil microorganisms in winter rye crops..

In all variants of the experiment, the *Rhizobium* genus occupied the dominant position among proteobacteria. In terms of phylogenetics, the *Rhizobium* genus is very close to the *Agrobacterium* and *Allorhizobium* genera, which are the members of the *Rhizobiaceae* family, and which are currently united in the *Rhizobium — Agrobacterium* phylogenetic group. Moreover, J.M. Young et al. [21], on the basis of phylogenetic similarity, proposed to combine these bacteria into one genus — *Rhizobium*. The results obtained on the dominant position of the *Rhizobium* genus bacteria in the soil under the cover of winter rye should be considered, at least, as the dominant position of the *Rhizobium − Agrobacterium* group bacteria, taking into account the degree of their relationship and the resolution of the method used. A significant number of microorganisms from this group in the soil and rhizosphere of plants was noted by many authors. According to M. Sadowski et al. [22], nodule bacteria (*Rhizobium* sp. and *Bradyrhizobium* sp.) are wide-

spread and are up to 8.0% of the total number of bacteria in the soil. The presence of *Rhizobiaceae* representatives, including nodule bacteria, in the rhizosphere and the roots of cereal crops has been reported in a number of papers [18, 23, 24]. Some researchers call nodule bacteria endophytes of cereal crops [25-27]. Zverev *et al.* [18], on the basis of phylogenetic analysis, found nodule bacteria of the *Mezorhizobium* genus belonging to this family in the rhizosphere of a 42-day-old winter rye crop. The pathogenesis of winter rye caused by *Agrobacterium tumefaciens* was identified in no case [28].

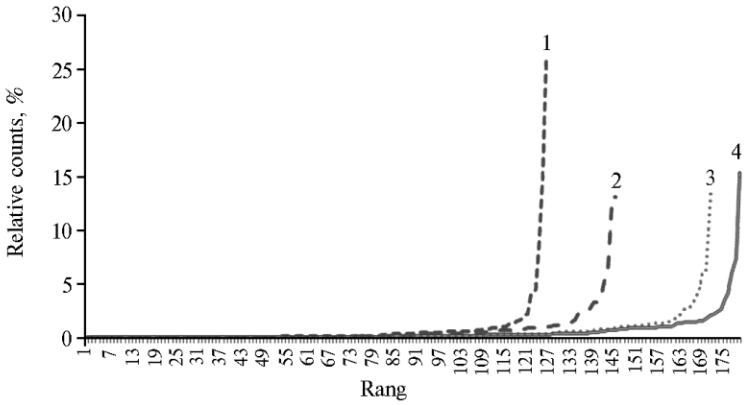

Fig. 3. Dominance (diversity) curves of the prokaryotic system in sod-podzolic soil in the plots of the long-term experiment under the crops of winter rye depending on the cultivation technology: 1 — in crop rotation with soil liming, 2 — in crop rotation without soil liming, 3 — permanent crop with soil liming, 4 — permanent crop without soil liming (experimental field of the Russian State Agrarian University —Moscow Timiryazev Agricultural Academy, Moscow, 2010).

The presence of the *Rhizobiaceae* family in the soil under the winter rye crops in long-term field experiments, in the authors' opinion, is due to the fact that in the conditions of six-field crop rotation two fields were occupied by clover. The nodule bacteria present in the clover crops survived the period when the soil was occupied by the precursors of winter rye (flax, clean fallow) and found favorable conditions for their development under the cover of this crop. The latter, in terms of biology, is of interest for assessing the adaptation and survival of nodule bacteria in agrocenoses.

As for the permanent rye crop, it should be noted that before establishing the experiment, 100 years ago this field was occupied by clover for several years [20]. In addition, bacteria of the *Rhizobium* genus can exist in the soil without fixing atmospheric nitrogen. It is possible that nodule bacteria adapted and strike roots in the rhizosphere of plants as associative endosymbionts. The presence of such association of spiked cereals of the *Bradyrhizobium* sp., *Agrobacterium* sp., *Rhizobium* sp. nodule bacteria is noted in other papers [25, 27]. It should be noted that the permanent rye crop has formed its own microbial flora, an important component of which was bacteria of the *Rhizobiaceae* family. This raises the interest for a deep analysis of the evolution of *Rhizobiaceae* and cereals relationship, in particular, winter rye, and the practical significance of these studies. Thus, biopreparations on the basis of non-pathogenic *Agrobacterium radiobacter* have already been created and are successfully used in cereal crops planting [29].

Liming almost did not affect the species wealth in the permanent rye crops, but sharply reduced it in crop rotation (see Fig. 3). A direct dependence of prokaryotes biodiversity on the pH value was not found. Large taxa at the phyla level were present in all variants of the experiment. Changes in the phylogenetic structure of prokaryotes were observed at the level of the genus, species,

and strain of microorganisms. Thus, while liming, the abundance of some *Acidobacteria* species decreased, but the abundance of others increased, which indicates a rearrangement of the taxonomic composition of prokaryotes. This contradicts the point of view about the unconditional positive effect of liming acidic soils on the microbial flora [30, 31]. However, it should be noted that material, which gives a controversial assessment of the effect of liming on the soil microorganisms community, has been accumulated in recent years. If according to some authors [32, 33], liming increased the biomass of microorganisms and the intensity of soil respiration, other researchers [34] showed that changes in the pH of the red soils, both towards acidic and alkaline, led to a decrease in the biomass of microorganisms. Kennedy *et al.* [35], along with an increase in microbiological activity during liming, show a change in the phylogenetic structure and a decrease in the diversity of the soil bacterial community. Different effects of liming on the phylogenetic diversity of prokaryotes under the cover of various plants were noted by Korvigo *et al.* [7]. In particular, liming led to a decrease in the diversity of prokaryotes in the link of crop rotation of potatoes and flax (precursors of winter rye), which corresponds to the results of this research.

Thus, the analysis of the phylogenetic structure and diversity of prokaryotic microorganisms in sod-podzolic soil under the conditions of a perennial rye crop showed that the plant is the main factor in the formation of the prokaryotic community. Regardless of the agrotechnical methods, the core system of prokaryotes with the same structure, including a small number of *Proteobacteria* and *Actinobacteria* species, is formed under the cover of winter rye during the paniculation phase. A dominant position among them is occupied by bacteria of the *Rhizobiacea*e family, in particular, the *Rhizobium* genus, which is a member of the *Rhizobium* − *Agrobacterium* group, which to a certain extent is related to the history of the experimental field. Apparently, the bacteria of this family, first of all, nodule bacteria, find favorable conditions for development in the rye rhizosphere. The effect of liming on the structure of the prokaryotic community of acid soils may be different. Apparently, the specific type of plants, as well as the history of the field (crop rotation, permanent crop, fertilizing system, etc.), are essential. Further studies of the *Rhizobiaceae* viability in winter rye crops and their evolution towards associative endosymbiotic relationships with rye plants in the process of long-term coexistence are of undoubted scientific interest.

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