Clover nodule bacteria as bioindicators of soils contaminated with heavy metals

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Abstract. The article presents the results of a study of vital activity of nodule bacteria of Trifolium pratense, Trifolium hibridum, Trifolium alexandrinum and Trifolium incarnatum as environmentally friendly bioindicators in soil toxicity assessment. The study focused on the number of nodules and their stain with leghemoglobin. The materials were taken from the plots artificially contaminated with heavy metals (lead (Pb), arsenic (As), mercury (Hg), fluorine (F), zinc (Zn) and cobalt (Co)). The results of the experiments showed that environmental distress occurs when the nitrogen-fixing nodules are stained pink or red in the range of 8-15%, which indicates that the lead content in the soil exceeds the threshold limit value (TLV) by 1.2-1.5 times. A significant decrease in nodule activity and weak or no stain were observed with an increase in mercury concentration by 1.1 times. It was found that the mass of rhizobium or the supply of organic nitrogen obtained by plants from fixation from the air is directly dependent on the grass density. At high concentrations of heavy metals in the soil, the nodule bacteria lose the amount of leghemoglobin, therefore the stain of bacteria is disrupted, so this indicator can be used as a bioindicator in soil toxicity assessment.

1. Introduction
Today, according to the latest data, the number of toxic elements polluting the environment is increasing on average by 4% annually and the increasing distribution of technogenic chemical compounds, in particular, heavy metals in the soil, creates a primary task of early identification of the most active pollutants. To solve this environmental problem, it is necessary to propose simple safe methods to assess toxicity in the natural habitat and reduce the cost of labor-intensive chemical tests [1].

At present, when the anthropogenic impact on natural processes has become one of the most significant environmental factors that determines the new conditions for the existence of biological systems, there is obviously no need to specifically substantiate and prove the fundamental nature of the researches aimed at finding criteria and methods for assessing the critical value of anthropogenic load on a person, plants and animals. The organization of a unified biological monitoring service in the Russian Federation is the main focus of this method [2].
In contrast to chemical and analytical control, the biological monitoring makes it possible to correctly assess and predict deviations in the state of biological systems from normal reaction when they are exposed to anthropogenic and technogenic factors [3, 4]. Undoubtedly, the biological monitoring does not allow us to associate the registered effect with a specific factor, but it provides an integrated assessment of the consequences of the environmental pollutant complex for the wildlife and the quality of the human environment [5, 6]. Moreover, it is required to develop new approaches and improve old ones to the assessment and prediction of the environment state based on the data provided by bioindicators [3, 4].

Plants can be used as the main bioindicators of soil contamination [7-11]. In our opinion, nodule bacteria located the root nodules of legumes can serve as such plants. It was determined that due to the fixation of atmospheric nitrogen, bacteria of Rhizobium genus in symbiosis with legumes can accumulate from 100 to 600 kg/ha of bound nitrogen per year depending on the biological characteristics of the crop [2]. This is of great importance in improving nitrogen nutrition and intensification of the production process of plants. The question of the actual size of atmospheric nitrogen fixation still remains understudied and debatable, while the study of methods for determining the size of symbiotic nitrogen in the field is one of the most urgent. Without the correct solution to this question, the problem of biological nitrogen is difficult to solve [6].

We propose using nitrogen-fixing legumes of Trifolium L. genus as bioindicators. Clover, as a representative of legumes, grows on more acidic soils and nodule bacteria are more variable under such conditions. A certain value in symbiosis with the soil and as an environmental factor affects the activity of nodule bacteria and the chemical composition of the soil. Much attention is paid to the effect of rhizospheric microflora on nodule bacteria, which has both a stimulatory and antagonistic character depending on the composition of microorganisms [3]. Researches of E. N. Bron [4], E. P. Trepachev [5] established that clover nitrogen fixation most intensively occurs during budding and beginning of flowering. During this period, generation of macronutrients in plants due to photosynthesis far exceeds their consumption, therefore a large amount of nutrients, in particular nitrogen, accumulate in the root system of clover. After cutting, due to a sharp decrease in the intake of carbohydrates in the nodules, their mass decreases, and then increases up until the next cutting. The value of nitrogen-fixing ability of clover is largely influenced by soil-climatic and agricultural conditions. The research data show that clover fixes about 150 kg/ha of nitrogen from the atmosphere per year. There are various data on the amount of nitrogen fixation by clover plants. A. A. Kutuzova [6] notes that in the central regions of Russia in a wetter year, the legume grasses contained 126 kg of biological nitrogen, in the dry year–512 kg, and in some years, under especially favorable conditions, up to 300 kg/ha. A high level of agricultural technology, providing clover plants with the necessary macro and microelements throughout the growing season is the key not only to a high increase in green mass, but also to a good development of the root system and an increase in nitrogen accumulation.

Therefore, the basis of our research was the study of the vital activity of nodule bacteria of Trifolium pratense, Trifolium hibridum, Trifolium alexandrinum and Trifolium incarnatum as bioindicators in assessing the toxicity of soils contaminated with such heavy metals as lead, arsenic, mercury, zinc fluoride and cobalt.

2. Materials and research methods
The studies were conducted at the experimental base of the North Caucasus Research Institute of the Vladikavkaz Scientific Center of the Russian Academy of Sciences in 2016-2018. In order to study the vital activity of the clover nodule bacteria, we conducted studies in natural phytocenoses and on arable land. An important property of legumes is the ability to fix air nitrogen, and such an indicator as the amount of rhizobium in the clover roots can serve to determine factors that inhibit or vice versa, contribute to the normal growth and development of the plants.

The number of nodules and their stain was determined in a 0–20 cm soil layer in the roots of legume grasses. Artificial contamination of the soil was carried out at the beginning of the
development of clover plants and observations were made until the budding-flowering phase with recording of the number of nodules and their color. The lack of color of more than 80% of the studied plants was assessed as an environmental distress. When at least 50% of the studied plants colored in pink or red, we concluded that the territory was in satisfactory condition. The lack of color indicates pollution not only of the soil, but also of the air that nodule bacteria breathe fixing molecular nitrogen.

During the biodiagnostics, we took into account the indicators of the soil chemical composition in the root layer and the maximum formation of the number of nitrogen-fixing nodules. At the same time, we compared the threshold limit value (TLV) of heavy metals at optimal soil moisture. The presence of pink or red nodules indicates a high nitrogen fixation activity. If the nodules are small and have a green or brown color, then a conclusion is drawn on the contamination of the studied plots. This color indicates the absence of leghemoglobin pigment in the nodules, causing pink or red color.

Unlike the known methods, when the chemical composition of the soil is determined, in our method it is possible to determine the toxicity of the soil by staining nodule bacteria located in the root system of legume grasses. Moreover, such a diagnosis can be carried out throughout the growing season: from the moment of spring regrowth of the legumes and until the flowering phase.

If there is an excessive amount of toxins in the soil, the pH regimen in the root soil layer is disturbed, which leads to a decrease in the leghemoglobin and nitrogen-fixing effect of the nodules and, consequently, to the lack of color (pink or red). The leghemoglobin binds nitrogen, regulating the nitrogen metabolism of the plant. With a lack of the leghemoglobin, the amount of protein and seed yield are reduced, soil fertility is also reduced.

The regrowth of clover plants, in the roots of which in the 15-20 cm soil layer there are nodule bacteria that fix atmospheric nitrogen, begins in the crop rotation system of agricultural lands and in natural phytocenoses susceptible to contamination by heavy metals and radionuclides, with humidity indicators sufficient for microorganisms (60-70%) and heat (at least 10-15°C).

The rationale for choosing the period for determining soil toxicity (2-3 weeks after the beginning of spring regrowth) is explained by the beginning of the formation of nodule bacteria at optimal humidity and temperature. Soil toxicity was determined directly on the plots by visual assessment of legume plants without laboratory and chemical tests, which greatly simplified the soil contamination assessment.

In various places of the plot with an area of 0.5 ha which was contaminated with lead, ten clover plants were sampled and 15 cm of their root system was dug out. The total number of nodules in the roots was counted with an accent to pink nodules. The plants were sampled at optimal soil moisture (65-70%) under conditions favorable for nodule bacteria since during the drought period they fix atmospheric nitrogen insufficiently and toxicity assessment may be inaccurate. The total number of nodules was determined (within 150-200 nodules per a plant). After nodules were cut with a scalpel, 118 nodules were pink at incision, which is 14.3%. As a result, it was concluded that the studied plot belongs to the 3rd category (environmental distress).

By active nodules in the studied plot, we determined three categories of soil toxicity:
1—stain of more than 50% of nodules—satisfactory condition;
2—stain of 20-50% of nodules—environmental risk;
3—stain of less than 20% of nodules—environmental distress.

This assessment of nitrogen fixation and soil toxicity determination can be carried out in the period from regrowth in early spring to the flowering phase. In the later period during the seeds formation, nodule bacteria reduce their ability of atmospheric nitrogen fixation and the leghemoglobin formation that is the basic stainer inside the nodule.

Two weeks after the legume grasses regrowth in a natural phytocenosis infected with arsenic, at a soil moisture content of 65%, the plants’ root system was dug at eight points from a depth of 20 cm and the number of nodules in the roots was counted, 13.9% of them were found stained. We concluded that the plot belongs to the 3rd category (environmental distress).

Three weeks after spring clover regrowth, in the plot contaminated with lead and mercury, at a soil moisture content of 70%, the root systems of 15 plants (10 of each species) were dug from a depth of
20 cm. The total number of nodules of various legumes amounted to 1835 ones. 156 of them (or 8.5%) were pink or red, which was classified as an environmental distress.

At the plot contaminated with fluorine, 20 clover plants were sampled, 5 plants of each species. Their root systems were dug out from a depth of 15-18 cm and the number of nodules formed in the plants' roots were counted. The total number of nodules was 2136; 862 of them (or 40.4%) were stained (the environmental risk category).

The samples taken in the plot contaminated with zinc and cobalt had 1385 nodules in 10 plants in total, 726 (or 52.4%) of them were stained. This indicator was classified as satisfactory condition.

3. Results and discussion

The research results showed that high content of iron was observed in the soil where the clover species grew. It exceeded the threshold limit values of iron by 4-5 times, of cadmium by 2 times, of copper by 5 times (table 1).

| Name   | Zn, mg/kg | Cd, mg/kg | Cu, mg/kg | Fe, mg/kg | Ni, mg/kg |
|--------|-----------|-----------|-----------|-----------|-----------|
| Clover | 18.4      | 0.91      | 5.9       | 600.0     | 3.9       |
| TLV    | 28.0      | 0.5       | 30.0      | 285       | 4.0       |

Therefore, in soil samples taken at the experimental plot of the North Caucasus Research Institute of the Vladikavkaz Scientific Center of the Russian Academy of Sciences, the average content of heavy metals amounted to: 1.07 mg/kg of cadmium and 487.78 mg/kg of iron.

The exceeding of the heavy metal threshold limits in the soil also contributed to their accumulation in clover plants.

The maximum rhizobium mass in the clover roots accumulates in the soil at the highest grass density i.e. when seeding with a row spacing of 16 cm and a sowing rate of 15 kg/ha. The maximum rhizobium mass was 13.1-14.3 c/ha. The minimum rhizobium mass (7.9-9.1 c/ha) was formed at the lowest grass density. Therefore, the rhizobium mass or the organic nitrogen amount obtained by plants due to fixation from the air depends directly on the grass density. The experiment results of the rhizobium mass in clover roots study showed that during the Trifolium alexandrinum branching phase, the average amount of rhizobium for three years of research was in the range of 22-42 pcs per plant. The spring sowing correspondingly produced 28 and 30 pieces per plant. Counting the rhizobium number in the clover roots, counting the clover plants number per 1 m² and rhizobium mass determination allowed to establish its potential stock in the soil. The maximum rhizobium mass in the clover roots accumulates in the soil at the highest grass density and when seeded with a row spacing of 16 cm and a sowing rate of 15 kg/ha.

The maximum rhizobium mass was: 6.09-14.3 c/ha; 11.10-13.3 c/ha; 6.03-13.1 c/ha; 6.04-13.8 c/ha. The minimum rhizobium mass was formed at the lowest grass density and amounted: 6.09-7.9 c/ha, 11.10-8.1 c/ha, 6.03-8 c/ha и 96.04-.5 c/ha.

Therefore, the rhizobium mass or the organic nitrogen amount obtained by plants due to fixation from the air depends directly on the grass density. At high concentrations of heavy metals in the soil, the nodule bacteria lose the amount of leghemoglobin, therefore the stain of bacteria is disrupted, so this indicator can be used as a bioindicator in soil toxicity assessment.

The results of the study of the plots toxicity showed that the lead content in the soil (48 mg/kg) exceeds the threshold limit by 16 mg/kg (32 mg/kg), the arsenic dose (25 mg/kg) exceeds the limit by 5 mg/kg (20 mg/kg). Lead with mercury combination (2.5 mg/kg) exceeds the threshold limit values (2.0 mg/kg) by 0.5 mg/kg, and fluorine (18 mg/kg) by 8 mg/kg (fluorine TLV is 10 mg/kg). The combination of zinc and cobalt (37 mg/kg) exceeds the TLV by 14 mg/kg (23 mg/kg). Therefore, the greatest environmental threat was shown by the variant of high lead doses in the soil, in comparison with all other experimental variants. The results in table 2 show that nodules staining due to
leghemoglobin is disrupted when exceeding the threshold limit values of heavy metals. This is characterized by a hazard category of the studied area.

| No. | Soil toxicants | Threshold limit values (mg/kg) | Toxicants content at plots (mg/kg) | Total number of nodules in samples (pcs.) | Number of nodules stained with leghemoglobin (pcs.) | Percentage of stained nodules | Hazard category |
|-----|----------------|-------------------------------|-----------------------------------|-----------------------------------------|-------------------------------------------------|----------------------------|----------------|
| 1.  | Lead           | 32.0                          | 48.0                              | 826                                     | 118                                             | 14.3                       | 3              |
| 2.  | Arsenic        | 20+1.0                        | 25+8                              | 612                                     | 85                                              | 13.9                       | 3              |
| 3.  | Lead + Mercury | 2.0                           | 2.5                               | 1835                                    | 156                                             | 8.5                        | 3              |
| 4.  | Fluorine       | 10.0                          | 18.0                              | 2136                                    | 862                                             | 40.4                       | 2              |
| 5.  | Zinc + Cobalt  | 23.0+5.0                      | 37+8                              | 1385                                    | 726                                             | 52.4                       | 1              |

The results of nodules color study showed 14.3% at lead, which exceeded the number at arsenic doses (13.9%) and lead with mercury (8.5%).

At the fluorine and zinc + cobalt, the numbers were 40.4% and 52.4%, respectively.

4. Conclusion
Nitrogen fixation by clover species makes it possible to increase the accuracy of assessing a contaminated area and simplify the determination of its toxicity with the bioassay of nodule bacteria directly in the field and without additional chemical test costs, which means that clover nodule bacteria can serve as bioindicators in soil toxicity assessment of a particular area.

The environmental distress occurs when 8-15% of the nitrogen-fixing nodules are stained and when the content of toxic elements in the soil exceeds the TLV by 1.2-1.5 times. A significant decrease in nodule activity was observed with an increase in mercury concentration by 1.1 times.

This study revealed that the maximum rhizobium mass in the clover roots accumulates in the soil at the highest grass density and when seeded with a row spacing of 16 cm and a sowing rate of 15 kg/ha.

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