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# The Effect of Combined and Separate Inoculation of Alfalfa Plants with *Azospirillum lipoferum* and *Sinorhizobium meliloti* on Denitrification and Nitrogen-Fixing Activities

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**Abstract**—The effects of associative nitrogen fixer *Azospirillum lipoferum* strain 137 and root nodule bacteria *Sinorhizobium meliloti* after combined and separate inoculation of alfalfa seedlings on the background of mineral nitrogen applied at various times were studied. It was demonstrated that exudates of the alfalfa seedlings with the first pair of cotyledonary leaves already provide a high activity of these bacteria in the rhizosphere. To 74.6% of the introduced nitrate was transformed into $N_2O$ when the binary preparation of these bacteria was used. In an extended experiment (30 days), an active reduction of nitrates to $N_2O$ with inhibition of nitrogen fixation was observed in all of the experimental variants during the formation of legume–rhizobial and associative symbioses and simultaneous introduction of nitrates and bacteria. The most active enzyme fixation was observed in the case of a late (after 14 days) application of nitrates in the variants with both separate inoculations and inoculation with the binary preparation of *A. lipoferum* and *S. meliloti*. Separation in time of the application of bacterial preparations and mineral nitrogen assisted its preservation in all of the experimental variants. The variant of alfalfa inoculation with the binary preparation of *A. lipoferum* and *S. meliloti* and application of nitrates 2 weeks after inoculation was optimal for active nitrogen fixation (224.7 $C_2H_4$ nmol/flask ·24 h) and low denitrification activity (1.8 μmol $N_2O$/flask · 24 h). These results are useful in applied developments aimed at the use of bacterial and mineral fertilizers for leguminous plants.

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An intensive use of mineral nitrogen at biological cost leads to negative consequences of soil nitrating. However, up to 90 t/ha of molecular nitrogen can be fixed annually only due to leguminous plants [1].

It is known that inoculation of legumes with active strains of root nodule bacteria increases the yield of alfalfa hay by 22–113% under favorable conditions and provided that an efficient symbiosis has developed [2]; moreover, plant morbidity rate decreases, they mature quicker, and the crop quality elevates [3]. Also of importance is the fact that root nodule bacteria stimulate accumulation of vitamins in plants. As the positive effect of inoculation also covers the roots, approximately 120–150 kg/ha nitrogen remains after harvesting alfalfa and tilling its remaining parts into the soil [4]. The roots mineralize quicker and efficiently influence the next culture.

Associative nitrogen-fixing bacteria colonize plant roots and frequently invade root tissues without developing any nodules [5]. The efficiency of nitrogen fixation of associative microorganisms is lower than that of the symbiotic; however, associative nitrogen fixers stimulate plant growth, protect plants from pathogens, synthesize phytohormones, and degrade toxic substances. A combined inoculation with *Azospirillum* and *Rhizobium* assists root nodule bacteria in invading legume root hairs [6–8]. It is known [9] that rhizobial surface proteins interact with the polysaccharides secreted by *Azospirillum.* Presumably, the manifestation of such interaction explains the development of various biologically active multicomponent bacterial nitrogen-fixing associations located in the rhizosphere.

Nitrogen fertilizers significantly influence the development of symbiotic association. The character of this influence depends both on the form, dose, and time of applying mineral nitrogen and on other environmental factors (excess humidity, high acidity, and deficiency of trace elements in soil). When the concentration of mineral nitrogen exceeds a certain level, the virulence of bacteria and the susceptibility of legumes to them decrease; as a result, nodules fail to develop on roots [10]. Soil microbial toxicity, which arises due to a long-term application of nitrogen fertilizers, also has its effect [11]. This is connected with an increase in the population and diversity of species composition of phytotoxic microorganisms, in particular, micromycetes [12]. When maximal doses of mineral nitrogen is introduced with fertilizer ($P_{70}K_{140}N_{210}$), the losses of nitrogen from soil increase due to denitrification (up to 80% of the nitrogen applied); these losses may reach 1.3 μ N– $N_2O$ per 1 g soil per day [13].

A moderate application of nitrogen fertilizers elevates the activity and increases the population of microorganisms from various physiological groups: aerobic

and anaerobic nitrogen fixers, nitrifiers, denitifiers, ammonifiers, etc.

The data are also available that application of inoculation at the sites with optimal conditions (no excess moisture, neutral pH, and sufficient amount of trace elements) even without nitrogen fertilizers provided an equal or even higher yield compared to the variants with nitrogen fertilizers [14–17].

Application of a minimal dose of mineral fertilizers on the background of bacterial inoculation gives a more pronounced positive effect than application of either mineral or bacterial fertilizers alone [18].

In the majority of cases, inoculation gives a significant increase in the yield, and its application is cost effective. Nonetheless, inoculation of legumes is sometimes inefficient on the background of nitrate application; presumably, this is connected with the particular time of application of mineral fertilizers.

The goal of this work was to study the effect of combined and separate inoculations of alfalfa seedlings with the preparations of *A. lipoferum* and *S. meliloti* on the activity of nitrogen fixation and denitrification on the background of nitrates applied at a different time.

## MATERIALS AND METHODS

**Bacteria.** The associative nitrogen fixer *A. lipoferum* strain 137 and the root nodule bacteria *S. meliloti* strain 425a were taken as the objects of this study. The strains were obtained from the All-Russia Institute of Agricultural Microbiology, Russian Academy of Agricultural Sciences (St. Petersburg, Russia).

**Media.** The culture of *A. lipoferum* was passaged on Doebereiner medium containing 0.4 g/l $KH_2PO_4$, 0.1 g/l $K_2HPO_4$, 0.2 g/l $MgSO_4$, 0.1 g/l NaCl, 0.02 g/l $CaCl_2$, 0.01 g/l $FeCl_3 \cdot 6H_2O$, 0.02 g/l $Na_2MoO_4 \cdot 2H_2O$, 2.5 g/l malic acid, 1 g/l sucrose, 1 g/l yeast extract, and 14 g/l agar; pH 6.5.

*S. meliloti*, a rapidly growing culture, was passaged in pea medium (50 g peas boiled for 20 min, 0.5 g/l $K_2HPO_4$, 5 g/l sucrose, and 14 g/l agar; pH 6.8–7.0) before inoculation.

**Plants.** Alfalfa cultivar *Zheltaya shvedskaya* plants were used as the object. The plants were raised from seedlings obtained from sterilized alfalfa seeds. The seeds were sterilized from the surface for 3 min with concentrated sulfuric acid followed by treatment with 0.1% solution of mercury chloride for 20 min. Then the seeds were repeatedly washed with sterile water.

Seeds were germinated in sterile petri dishes for 2 days.

Two sets of experiments were conducted. Set 1 (short experiment) was performed for 4 days; the goal here was to test the ability of *S. meliloti* and *A. lipoferum* cultures to reduce nitrates due to the exudates of alfalfa seedlings; therefore, nitrates were applied simultaneously with the inoculation in all of the variants of experiments. Set 2 (long-term multivariant experiment) was performed for 30 days; the goal in this set was to find the optimal conditions for active nitrogen fixation and low denitrification by varying the time of nitrate application on the background of separate or binary inoculation of alfalfa plants.

**Short experiment (4 days).** The experiment was conducted under sterile microvegetation conditions. Three 2-day-old alfalfa seedlings were placed in each vial and inoculated with water suspension of *S. meliloti* and/or *A. lipoferum* either separately or as a binary preparation.

The plants were placed in small 100-$cm^3$ vials containing 20 ml of agar Thornton nitrogen-free medium, containing 0.74 g/l KCl, 0.3 g/l $K_2HPO_4$, 0.3 g/l $KH_2PO_4$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l $CaCl_2$, 0.04 g/l $FeCl_3 \cdot 6H_2O$, 6.5 g/l agar, and trace elements according to Johnson, namely, 250 μg/l boric acid, 50 μg/l $MnSO_4$, 20 μg/l $ZnSO_4 \cdot 7H_2O$, 20 μg/l $CuSO_4 \cdot 5H_2O$, 10 μg/l $Na_2MoO_4 \cdot 2H_2O$, and 50 μg/l $CoCl_2 \cdot 6H_2O$ [19].

Nitrates were immediately added to the vials as $KNO_3$ at a dose of 10 mg/vial (500 mg/l). Sterile air was the gas phase. The vials were sealed with sterile rubber stoppers. The measurements were conducted after 2 and 4 days.

The experiment was performed at 23°C, air humidity of 70%, and 16 light hours (illumination with xenon lamps).

**Long-term (30 days) multivariant experiment.** The long-term experiment comprised 12 variants; the volume of experiment flasks was 250 $cm^3$. Both time of inoculation and time of nitrate addition were varied.

Five 2-day-old alfalfa seedlings produced from sterilized seeds were planted into one flask with mineral Thornton medium. Plants were grown at 23°C, air humidity of 70%, and 16 light hours (illumination with xenon lamps).

In both experiments (short and long-term), acetylene in an amount of 5% of the flask volume was introduced under sterile conditions as a substrate for detection of nitrogenase activity and an inhibitor of $N_2O$ reductase.

All the experiments were performed in five replicates; the tables list mean values.

The number of nodules formed was recorded (the average number of nodules per flask in the long-term experiment).

Denitrification activity ($N_2O$ μmol/flask · 24 h) and the amount of nitrites ($NO_2^-$ – μmol/flask) were measured. Nitrogenase (NG) activity ($C_2H_2$ nmol/flask · 24 h) was determined by the acetylene method in inoculated plants under sterile microvegetation conditions [19, 20]. One day before the determination, cotton wool stoppers were substituted under sterile conditions with rubber stoppers with a tightly inserted glass tube sealed at the end with a rubber stopper. All stoppers were

**Table 1.** Short 4-day experiment. The activities of denitrification and nitrogen fixation after inoculation of alfalfa plants with *Azospirillum lipoferum* and *Sinorhizobium meliloti* on the mineral medium with nitrates

| Variant, no. | Denitrification, $N_2O$, μmol/vial | | | | Nitrites, $NO_2^-$, μmol/vial | | Nitrogen fixation, $C_2H_4$, nmol/vial | |
|---|---|---|---|---|---|---|---|---|
| | day 2 | | day 4* | | day 4* | | day 2 | day 4* |
| | $N_2O$, μmol/vial | % N transformed from $KNO_3$ | $N_2O$, μmol/vial | % N transformed from $KNO_3$ | $NO_2^-$, μmol/vial | % N transformed from $KNO_3$ into $NO_2^-$ | $C_2H_4$, nmol/vial | $C_2H_4$, nmol/vial |
| 1. Plants + $KNO_3$ | 0 | 0 | 0 | 0 | | 0 | 0 | 0 |
| 2. Plants + $KNO_3$ + *S. meliloti* | 29.1 | 19.2 | 0 | 0 | | 0 | 0 | 0 |
| 3. Plants + $KNO_3$ + *S. meliloti* + *A. lipoferum* | 112.2 | 74.6 | 12.9 | 8.5 | 40.5 | 29.2 | 38.9 | 43.1 |
| 4. Plants + $KNO_3$ + *A. lipoferum* | 29.2 | 19.3 | 11.4 | 7.5 | 56.5 | 40.5 | 19.5 | 13.8 |

* After addition of $KNO_3$.

boiled and washed with alcohol. After 14 days, when alfalfa plants developed their first nodules, nitrates were added depending on the variant of experiment (500 mg/l or 35 mg in 70 ml of the Thornton medium).

## RESULTS AND DISCUSSION

**Short experiment (4 days). Denitrification.** Two sets of experiments were conducted. Set 1 (short experiment) was performed for 4 days; combined and separate inoculations were tested. The goal here was to test the ability of *S. meliloti* and *A. lipoferum* cultures to reduce nitrates due to the exudates of alfalfa seedlings; therefore, nitrates were applied simultaneously with the inoculation in all the variants of experiment.

It is accepted that appearance of the first true leaves on plants, which provide the flux of carbohydrates to the roots, and secretion of exudates, stimulating the activity of bacteria in the rhizosphere, are tightly connected [10]. However, the alfalfa seedlings studied provided a high bacterial activity in the rhizosphere already with the appearance of the first pair of cotyledonary leaves. The measurements performed on day 2 (Table 1) recorded an active reduction of nitrates to nitrous oxide, which corresponded percentagewise to the reduction of 74.6% nitrogen to $N_2O$; this was observed in variant no. 3, where *S. meliloti* and *A. lipoferum* were coinoculated. In variant no. 2 (inoculation with *S. meliloti* only), 19.2% nitrogen was reduced; in the variant with *A. lipoferum* (no. 4), 19.3% nitrogen was reduced. This suggests that a synergistic effect was observed in variant no. 3 upon combined inoculation of *Rhizobium* and *Azospirillum* [6, 21]. As we demonstrated earlier, inoculation of legumes with a binary preparation comprising root nodule bacteria and free-lining diastrophism led to the establishment of an efficient symbiosis with good nodulation and high nitrogenase activity [6]. In turn, this results in an increase in plant biomass, accumulation of protein, and increase in the leaf area [21, 22]; however, denitrification activity may also elevate under certain conditions.

As virtually all nitrates were reduced over 2 days to free nitrogen, we had to add nitrates again at a dose of 10 mg/vial to continue the experiment. When doing so, we evacuated the gas phase containing evolved $N_2O$. The vials were washed with sterile air, and the gas phase identical to that at the beginning of experiment (sterile air and 5% $C_2H_2$) was formed. Further measurements after 2 days demonstrated that the reduction of nitrate to nitrous oxide still continued in variant no. 3 (coinoculation with *S. meliloti* and *A. lipoferum*): about 8.5% nitrogen was reduced by both cultures during 2 days after the second addition of nitrate. In the case of separate inoculation with *A. lipoferum* (variant no. 4), about 7.5% nitrogen was reduced; and no reduction of $NO_3^-$ were observable in the case of separate inoculation with *S. meliloti.* Consequently, only *Azospirillum* plays the role of denitrifier on day 4 of the experiment.

The presence of nitrite in the agar medium was determined on day 4 of the experiment. The maximal amount of nitrites, 40.5% nitrogen transformed from $NO_3^-$ to $NO_2^-$, was observed in variant no. 4 (inoculation with *Azospirillum*). The nitrate reductase activity was slightly lower—29.2% nitrogen of nitrates reduced to nitrites—in variant no. 3 (inoculation with the binary preparation).

**Nitrogen fixation.** Nitrogen-fixing activity was determined concurrently with denitrification. The nitrogen-fixing activity in the experiments with coinoculation of *Azospirillum* and *Sinorhizobium* was severalfold higher than in the variant with *Azospirillum* monoinoculation. On the average, the total nitrogenase activity corresponded to 38.9 nmol ethylene per vial on day 2 after inoculation and 43.1 ethylene per vial on day 4 after the second addition of nitrate (variant no. 3). *S. meliloti* lacked the nitrogenase activity (variant no. 2); the nitrogenase activity of *A. lipoferum* (variant no. 4) amounted to 19.5 and 13.8 nmol ethylene per vial on days 2 and 4, respectively. Consequently, this means that only free-living nitrogen fixer *A. lipoferum* fixed molecular nitrogen. It is not at all surprising, as the symbiotic system is not yet formed at such an early stage (4 days); in addition, the plants were depressed by a small volume of closed vials (100 $cm^3$) and acetylene, introduced almost immediately. As for the symbiotic nitrogen fixation, the conditions were nonoptimal for its manifestation.

Note that these data on NG activity correlate with the results obtained on the NG activity of *Spirillum* sp. culture with exudates of alfalfa roots [23].

It is known that *Azospirillum* are capable of accumulating a storage compound, poly-β-oxybutyrate (**POB**). The cells rich in POB require no sources of energy and carbon and fix nitrogen in the absence of an exogenous carbon source [24]. Even assuming that the cells used for inoculation were rich with POB (usually, 20–30% of cell dry weight) accumulated when growing the introduced culture, it is nonetheless natural that the cells on day 4 are exhausted and, certainly, already use the energy of root exudates of alfalfa seedlings.

Thus, during the study of the effect of combined and separate inoculations of alfalfa seedlings with *Azospirillum* and *Sinorhizobium* cultures in the short experiment (4 days), we found that in the case of monopreparation of *Azospirillum* or *Sinorhizobium* in the presence of nitrate no more than 20% nitrate was reduced on day 2 (variants nos. 2 and 4); however, in the case of coinoculation with symbiotic nitrogen fixer *Sinorhizobium* and free living *Azospirillum*, a drastic $N_2O$ discharge was observed. Thus, a separate inoculation is preferable and leads to preservation of nitrate in the medium.

**Long-term multivariant experiment (30 days).** The results of experiments on combined and separate inoculations of alfalfa seedlings during a short exposure (4 days) formed the background for the second set of experiments (Table 2). This set was represented by a long-term (30 days) experiment (12 variants) on the optimization of the time for nitrate application as well as separate or combined inoculation with *A. lipoferum* and *S. meliloti* to find the optimal conditions for an active nitrogen fixation on the background of a minimal loss of nitrogen due to denitrification. After 14 days, when alfalfa plants developed the first nodules, nitrates

**Table 2.** Long-term 30-day microvegetation experiment. Variants of the experiment on optimization of the time for adding nitrate and mono- or binary *S. meliloti* and *A. lipoferum* inoculum to alfalfa plants

| No. | Alfalfa plants | *Sinorhizobium meliloti* | *Azospirillum lipoferum* | $KNO_3$ |
|---|---|---|---|---|
| 1 | Plants (control) | | | |
| 2 | Plants with additions | + day 1 | | + day 1 |
| 3 | ″ | | | + day 1 |
| 4 | ″ | + day 1 | | |
| 5 | ″ | + day 1 | | + day 14 |
| 6 | ″ | + day 14 | | + day 1 |
| 7 | ″ | Coinoculation + day 1 | | + day 1 |
| 8 | ″ | | + day 1 | + day 1 |
| 9 | ″ | Coinoculation + day 14 | | + day 1 |
| 10 | ″ | Coinoculation + day 1 | | + day 14 |
| 11 | ″ | | + day 14 | + day 1 |
| 12 | ″ | | + day 1 | + day 14 |

Note: + day 1, $KNO_3$ or bacterial inoculum are added simultaneously with planting seedlings and + day 14, $KNO_3$ or bacterial inoculum are added 14 days after the beginning of the experiment.

were added depending on the particular variant (500 mg/l or 35 mg per 70 ml of the Thornton medium).

**Development of nodules (long-term experiment).** The results of this experiment demonstrate (Table 2 and 3, figure) that the moment when nitrates were added had a most pronounced effect on the development of nodules. For example, we observe a complete inhibition of nodule formation in variant no. 2, when nitrates and rhizobial inoculum were added simultaneously. Moreover, the simultaneous addition of *Azospirillum* in this case did not activate the development of nodules (variant no. 7). Thus, nitrates almost completely inhibit nodule development. Variant no. 5 (plants were inoculated with *Sinorhizobium* when planted, i.e., immediately, and nitrates were added later) and variant no. 4 (plants were inoculated with *Sinorhizobium*; no nitrates) display similar activity of nodule formation (on the average, 20–22 nodules per flask), i.e., a later addition of nitrates (after 2 weeks) does not inhibit the development of legume–rhizobial symbiosis. The most abundant development of nodules was observed in variant no. 10 (coinoculation and late addition of nitrates; on the average, 32 nodules per flask). Evidently, we observe an illustrative picture of how a free-living nitrogen fixer *Azospirillum* assists a more active development of nodules, which complies with the literature data on a positive effect of combined inoculation on the nodule development by legumes [7].

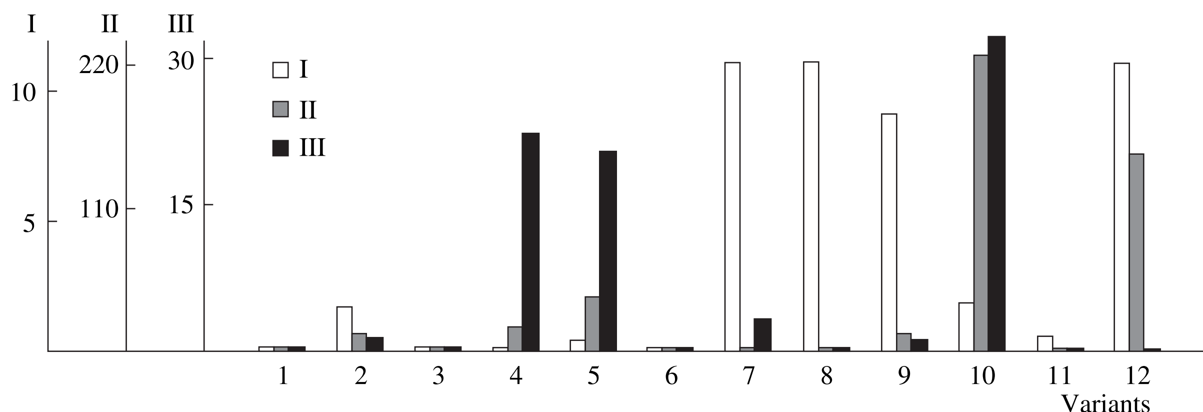
**Table 3.** The activities of nitrogen fixation and denitrification and the number of formed nodules in alfalfa seedlings after combined and separate inoculations with *A. lipoferum* and *S. meliloti* and addition of nitrates at different times

| Variant no. | After 20 days | | After 30 days |
|---|---|---|---|
| | Denitrification, $N_2O$ μmol/flask · 24 h | Nitrogen fixation, $C_2H_4$ nmol/flask · 24 h | Mean number of nodules/flask |
| 1 | 0 | 0 | 0 |
| 2 | 1.6 | 10.1 | 1 |
| 3 | 0 | 0 | 0 |
| 4 | 0 | 16.3 | 22 |
| 5 | 0.3 | 39.6 | 20 |
| 6 | 0 | 0 | 0 |
| 7 | 11.0 | 0.2 | 3 |
| 8 | 11.0 | 0 | 0 |
| 9 | 9.0 | 11.7 | 1 |
| 10 | 1.8 | 224.7 | 32 |
| 11 | 0.5 | 0 | 0 |
| 12 | 11.0 | 148.8 | 0 |

**Denitrification (long-term experiment).** The activity of denitrification was measured on day 20 of the experiment. The most active denitrification was observed in variant no. 12 (nitrate was added on day 14 after inoculation of plants with *Azospirillum*). Denitrification is similarly active in variants nos. 7–9. These variants are different but similar in that the inoculation was performed with *Azospirillum* and nitrate was added at the very beginning of the experiment.

**Nitrogen fixation (long-term experiment).** A nonsymbiotic nitrogen fixation was active in variant no. 12, where *Azospirillum* inoculum was added at the beginning of the experiment and nitrates, after 14 days (148.8 $C_2H_4$ nmol/flask · 24 h). Presumably, active associative interactions of *Azospirillum* with alfalfa roots were established in this variant due to rich exudation. Possibly, invasion of the roots provided the adequate conditions for this process. In variant no. 5, where *Sinorhizobium* inoculum was added simultaneously with planting seedlings and nitrates were applied after 14 days, we recorded only the symbiotic nitrogen-fixing activity; the nitrogenase activity amounted to 39.6 $C_2H_4$ nmol/flask · 24 h. Thus, we observed a more active associative nitrogen fixation in this experiment compared with the symbiotic. The maximal nitrogen fixation was recorded in variant no. 10, when the plants were inoculated with a mixture of *Azospirillum* and *Sinorhizobium* simultaneously with planting seedlings and nitrates were added 14 days later. The nitrogen-fixing activity in this case was 224.7 $C_2H_4$ nmol/flask · 24 h, i.e., both associative and symbiotic nitrogen fixations took place due to a large number of developed nodules. Presumably, a coinoculation with *Sinorhizobium* and *Azospirillum* in the absence of the inhibiting effect of nitrates enhanced rhizobial invasion of alfalfa roots and induced plant nodulation. Addition of nitrates 14 days later had no inhibiting effect on the already formed legume–rhizobial symbiosis. In this variant (no. 10), plants actively assimilated the applied nitrates and the nitrate losses due to denitrification were minimal (1.8 μmol/flask · 24 h).

Thus, it is demonstrated that the exudates of alfalfa seedlings with the first pair of cotyledonary leaves in the short (4 days) experiment are able to provide a high denitrification activity of diazotrophic bacteria in the rhizosphere. This fact is another confirmation of our




(I) Denitrification activity ($N_2O$ μmol/flask · 24 h), (II) nitrogen-fixing activity ($C_2H_4$ nmol/flask · 24 h), and (III) the average number of formed nodules (per flask) in alfalfa seedlings after combined and separate inoculations with *A. lipoferum* and *S. meliloti* and the addition of nitrates at different times (see Table 2).

earlier results [24] showing that it is necessary to take into account, not only the nitrogen-fixing activity, but also the denitrification activity of diazotrophic bacteria when evaluating the contribution of these bacteria to soil nitrogen balance.

Analysis of the results obtained in 30-day experiment suggests that the inoculation with bacterial preparations and application of mineral nitrogen fertilizers should be separated in time. According to our data, it can be recommended to inoculate alfalfa with the binary preparation of *A. lipoferum* strain 137 and *S. meliloti* simultaneously with plating and add nitrates 2 weeks later.

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