doi: https://doi.org/10.15407/microbiolj83.01.032

SYMBIOTIC PROPERTIES OF *SINORHIZOBIUM MELILOTI* AND ETHYLENE PRODUCTION BY ALFALFA PLANTS AT THE EARLY STAGES OF THE SYMBIOSIS FORMATION UNDER DIFFERENT WATER SUPPLY AND SEED TREATMENT BY LECTIN

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The symbiotic properties of bacteria significantly influence on the effectiveness of symbiosis and the yield capacity of plants. Therefore, it is important and relevant to study the features of micro- and macrosymbionts interactions, in particular under stressful conditions, and to find ways to improve the productivity of symbiotic systems. ***Aim.*** *The investigation of the nodulation and nitrogen-fixing activities of Sinorhizobium meliloti as well as the ethylene production by alfalfa plants at the early stages of the formation of symbiotic system under conditions of both optimal and insufficient water supply and the pre-sowing treatment of seeds by lectin.* ***Methods.*** *Microbiological (cultivation of bacteria culture, seed inoculation), physiological (pot experiment), biochemical (nitrogenase activity and ethylene production determination).* ***Results****. It was observed a decrease of the nodulation activity of Sinorhizobium meliloti under drought as well as under lectin application, which was accompanied by an increase in the amount of produced by macrosymbiont ethylene at the early stages of the symbiosis formation. At the same time, the nitrogen fixing activity was inhibited at the insufficient water supply only. The use of lectin promoted the symbiotic apparatus functioning under optimal and insufficient water supply. After renewal of irrigation an increase of the nodule weight and nitrogen fixing activity as well as the aboveground mass of alfalfa plants were noted under lectin treatment.* ***Conclusions.*** *It was identified the role of ethylene as a negative regulator of the nodulation processes at pre-sowing treatment of alfalfa seeds with lectin. The formation of the effective nitrogen-fixing system capable of full recovery after drought under lectin application confirms the prospects for further research in the use of lectins to create optimal conditions for the realization of the symbiotic potential of rhizobia and to increase the symbiotic system´ resistance to the action of stress factors.*

Keywords: Symbiosis, rhizobia, alfalfa, nodulation activity, nitrogen fixation, ethylene production, lectin, water supply.

As a result of the interaction between nodule bacteria and legume plants the specialized structures – nodules are formed on the roots. Ones are the sites, where rhizobia in the form of bacteroides continue to live. A number of compounds, including hormonal, are involved in this process. Among them, ethylene plays a significant role. It is the central regulator of the nodulation process [1–3] and is involved in various stages of symbiosis formation. In particular, it is considered [4] that ethylene negatively controls the number of nodules formed, acting at different stages of their development: at the level of calcium oscillations caused by the influence of Nod factors produced by rhizobia; during deformations of root hairs stimulated by Nod factors; the growth of the infection thread; the development of the nodule primordia. It is noted, that ethylene is a powerful negative regulator of nodulation in legumes with indeterminate nodules [5]. At the same time in legumes with determinate nodules, the relationship between ethylene and this process is more complex [6, 7]. Some data confirm the positive effect of ethylene at the early stages of symbiosis [8]. Therefore, the ethylene production by legumes at the interaction with nodule bacteria as well as its connection with physiological processes in rhizobia requires further study involving various research objects and conditions.

Ethylene is also well known as a participant of the plants' response to biotic and abiotic stresses [9]. In this case, the release of this hormone as a mediator of the response to an external factor is an example of stress-induced morphogenetic changes in plants. The ethylene-signaling pathway consists of a number of elements that ultimately lead to the activation of ethylene-induced transcription factors that alter the expression of certain genes, including those involved in the nodulation process and plant defense reactions [2, 10].

To date, one of the important ways to increase the symbiotic systems resistance to stress is to use biologically active compounds. In particular, it was shown [11] that the use of homologous lectin promotes better forming of soybean-rhizobia symbiosis, stimulates one functioning and increases the plants productivity under insufficient water supply. The authors assume that this is due to the ability of this protein to increase the symbiotic system's resistance to drought through the forming of plant adaptation reactions. It has been found [12] that the introduction of soybean lectin into the inoculation suspension of *Sinorhizobium meliloti* improves nodule formation on plant roots, increases the chlorophylls and carotenoids content in leaves, and the nitrogen-fixing activity during budding and flowering-seedpod stages under optimal and insufficient water supply.

The **aim** of the presented research is the study of the nodulation and nitrogen fixing activities of *Sinorhizobium meliloti* as well as the ethylene production by alfalfa plants at the early stages of the formation of symbiotic system under conditions of both optimal and insufficient water supply and the pre-sowing treatment of seeds by lectin.

Materials and methods. The studies were conducted at the Institute of Plant Physiology and Genetics NAS of Ukraine using high effective nodule bacteria *S. meliloti* strain 441 from Museum Collection of Strains of Symbiotic and Associative Nitrogen Fixing Microorganisms of the IPPG NAS of Ukraine and alfalfa (*Medicago sativa* L.) of Nadezhda cultivar.

Bacteria were cultivated at 28 ºC to the stationary growth phase on 79 culture medium, which contained, per liter: KH_2PO_4 – 0.5 g, $MgSO_4 \times 7H_2O$ – 0.2 g, NaCl – 0.1 g, $CaCO_3$ – traces, yeast extract – 0.5 g, mannitol – 10 g, pH 7.0. To prepare inoculum suspension biomass of bacteria was washed off from agar substrate with water and was carefully suspended. The number of microorganisms was 10^9 cells/ml. The duration of the bacterization of the seeds was 60 minutes.

Alfalfa plants were grown in 4-kilogram pots (10 plants each) on washed river sand under natural lighting, temperature and air humidity. The sand humidity 60 % (normal) and 30 % (drought) of total moisture capacity (TMC) was maintained by controlled irrigation. The drought was created on the 10^{th} day after germination. The drought continued 15 days. The Helrigel's mixture containing 0.25 of nitrogen norm was a source of mineral nutrition. Before sowing, the seeds were sterilized with concentrated sulfuric acid and washed in running tap water. In the corresponding variants the seeds were treated with a solution (100 μg/ml) of commercial soybean seed lectin ("Lectynotest", Lviv) for 20 hours after sterilization, and in the control variants – water was used. After that, seeds were inoculated with *S. meliloti* nodule bacteria. Plants for analysis were selected during the vegetative stages (18 and 25 days after germination) at a background of different water supply, as well as at the early flower stage, after irrigation restoration.

The nitrogen-fixing activity was determined by the acetylene method. Plant roots were placed in hermetically sealed glass vials where acetylene was injected to its final concentration of 10 %. After 1 hour of incubation in the dark, the gas mixture was analyzed on an "Agilent Technologies 6850" (USA) gas chromatograph with a flame ionization detector. The gas separation was performed on a column (Supelco Porapak N) at the thermostat´s temperature of +55 °C and the detector´s – +150 °C. The carrier gas was nitrogen (50 ml per 1 min). The volume of the gas mixture analyzed sample was 1 ml. Pure ethylene was used as the standard ("Sigma-Aldrich", USA). The amount of ethylene formed from acetylene in 1 hour under the action of nitrogenase of the incubated sample was expressed in molar units of the formed ethylene per plant in 1 hour (nmol C_2H_4/plant×h).

The specific activity of ethylene production by whole plants was determined by placing them in hermetically sealed glass vials. After 24 hours of plants incubation in the dark, the content of ethylene in the vials was measured using an "Agilent Technologies 6850" gas chromatograph, the data were calculated per unit of the plants dry mass and expressed in molar units of the formed ethylene per gram of dry mass (nmol C_2H_4/g of dry mass).

Statistical processing of experimental data was carried out in accordance with generally accepted methods with the use of Microsoft Excel 2010 software package. In the Tables and Figures the means and standard errors (M ± SE) are presented. Values of $p<0.05$ were considered to be significant.

Results. The study of the nodulation activity of rhizobia (Table 1) showed that under optimal water supply, the use of lectin did not significantly affect nodulation and nodule mass, while under the insufficient water supply, it led to a significant decrease in these indices. On the 18th day after germination, under insufficient water supply at the background of lectin application, the number of nodules was almost 2 times less compared to control, whose indices were the same as under optimal water supply. On the 25th day after germination, this difference decreased – the depression in the number and weight of nodules on average was 1.5 times compared to control.

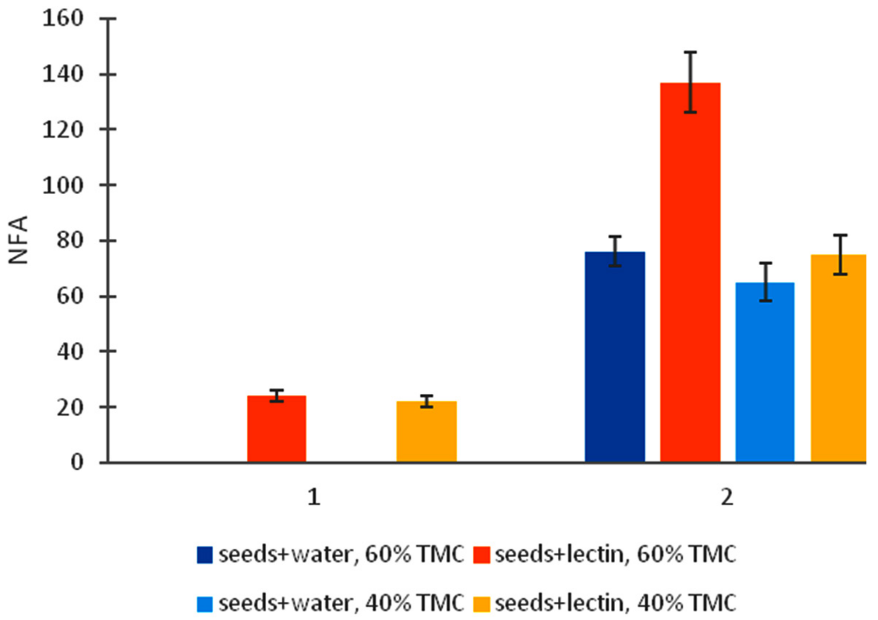
Table 1

The nodulation activity of *Sinorhizobium meliloti* under different water supply and lectin treatment. Plants at the vegetative stages

Variant	Nodules		
	number per plant		dry mass, mg/plant
	day after germination/day of drought		
	18/8	25/15	
60 % TMC			
Seeds + water	3.87 ± 0.37	12.75 ± 1.07	6.2 ± 0.56
Seeds + lectin	3.75 ± 0.32	12.25 ± 1.19	5.6 ± 0.55
30 % TMC			
Seeds + water	3.88 ± 0.39	7.0 ± 0.63	6.0 ± 0.59
Seeds + lectin	1.98 ± 0.19*	4.5 ± 0.24*	4.1 ± 0.40*

Note: Here and in Tables 2 and 3 (M ± SE), * – $p < 0.05$ vs. variant without lectin use under respective water supply. In this Table n=8.

At the same time, the study of nitrogen-fixing activity revealed (Fig. 1), that in the variants in which lectin was used, the symbiotic apparatus began to function earlier, and later it was noted also an increased activity of nitrogen fixation by nodules in comparison with the variants without treatment, especially under the optimal water supply.



F i g. 1. Nitrogen fixing activity (NFA, nmol C_2H_4/(plant × h)) of *Sinorhizobium meliloti* in symbiosis with alfalfa plants under different water supply and the treatment of seeds by lectin: 1 – 18th day after germination (8th day of drought), 2 – 25th day after germination (15th day of drought); n = 5

It has been revealed (Table 2) that the use of lectin for pre-sowing seed treatment activates the ethylene production by alfalfa plants on the 18th day after germination, both under optimal (by 90 %) and insufficient (by 16 %) water supply. On the 25th day, this effect remained only under drought conditions, while it was more pronounced compared to the previous sample selection – an increase of 31 % to control. Under the optimal water supply, alfalfa plants whose seeds were treated with lectin produced the same amount of ethylene as without treatment. It should be stressed that with the development of plants, the volume of ethylene released by them decreased.

After the resumption of irrigation, in the symbiotic systems formed under exogenous lectin treatment the indices of nodule mass on plants and their nitrogen-fixing activity increased, as well as an increase in the aboveground mass of alfalfa was revealed (Table 3).

Table 2

Ethylene production (nmol C_2H_4/g of dry mass) by inoculated alfalfa under the use of lectin and different water supply. Plants at the vegetative stages

Variant	Day after germination/day of drought	
	18/8	25/15
60 % TMC		
Seeds + water	1.07 ± 0.11	0.27 ± 0.03
Seeds + lectin	2.03 ± 0.26*	0.28 ± 0.02
30 % TMC		
Seeds + water	1.37 ± 0.13	0.39 ± 0.02
Seeds + lectin	1.58 ± 0.10	0.51 ± 0.03*

Note: In this Table n = 10.

Table 3

The effectiveness of *Sinorhizobium meliloti* in symbiosis with alfalfa under different water supply conditions and lectin treatment. Plants at the early flower stage, irrigation renewal

Variant	Nodule dry mass, mg/plant	Nitrogen fixing activity, nmol C_2H_4/(plant × h)	Aboveground dry mass, mg/plant
60 % TMC			
Seeds + water	5.34 ± 0.31	88 ± 7	119 ± 10
Seeds + lectin	14.2 ± 0.86*	180 ± 9*	153 ± 11*
30 % TMC			
Seeds + water	4.55 ± 0.25	54 ± 3	70 ± 6
Seeds + lectin	9.71 ± 0.75*	100 ± 7*	118 ± 9*

Note: In this Table: nodule mass – n = 8, nitrogen fixing activity – n = 5, aboveground mass – n = 10.

At the same time, it was noted that at the post-stress period, the indices of nodule mass and their activity on plants of the variant with the use of lectin exceeded those for plants that were constantly under optimal water supply conditions but without lectin treatment – by 82 and 14 %, respectively. The aboveground mass of plants of these variants was the same.

Discussion. It is well known, that the habitat determines the features of manifestation of the bacteria´ properties. In the case of symbiosis, it is necessary to take into account the mutual influence of macro- and microsymbionts, in addition to the direct influence of environmental conditions, such as climatic conditions or soil properties and so on. Analysis of the results obtained in our experiments showed that both lectin treatment and reduced water supply inhibited nodulation activity of rhizobia as well as caused an increase in the level of ethylene released by alfalfa plants. Considering that exogenous lectin can provide a protective effect at the early stages of plant ontogenesis [13], we assume that pre-sowing treatment of alfalfa seeds with this protein activated the plants protective mechanisms and thus caused increased production of ethylene in response to *S. meliloti* inoculation or lack of moisture. It is noted [3, 4] that ethylene synthesis in inoculated roots is triggered by Nod factors, herewith being activated plant defense responses that lead to restriction of the number of nodules formed, as far as nodule formation is associated with significant energy costs. It should be noted that under optimal water supply increased ethylene release due to the action of lectin was observed only on the 18th day after germination. On the 25th day there was no difference in this index between the plants of the control and experimental variants. Under the insufficient water supply, at the influence of lectin ethylene production occurred on the 25th day more intensively than in control plants. Obviously, the additional influence of insufficient water supply increased the duration of this process and led to a significant decrease in the number of nodules on plant roots, because it is known that ethylene negatively affects nodulation in the legume-rhizobial symbiosis, hindering the Nod factor signaling [14].

It is shown [10] that bacteria can modulate the level of ethylene in the plant. This is due to their production of either the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme [15] or the rhizobitoxin vinylglycine component [16]. ACC-deaminase encoded by the acdS gene decomposes the precursor of ethylene synthesis ACC into ammonium and alpha-ketobutyrate [15]. Respectively, rhizobia that produce ACC-deaminase reduce the ethylene negative effect on the nodulation process [17]. Rhizobitoxin acts as an inhibitor of plant ACC-synthase [18], and *Bradyrhizobium* strains with impaired rhizobitoxin production form fewer nodules on the host plant roots [19] and are less competitive than their parent forms [20, 21].

It can be assumed, that the lack of moisture, acting directly on rhizobia, affected their reaction to ethylene released by the plant, which contributed to a longer hormone production by alfalfa under drought conditions. On the other hand, we should not underestimate the influence of water stress directly on the plant, in particular on those processes that are associated with ethylene-producing activity [22].

It is known that symbiotic nitrogen fixation is one of the physiological processes that is inhibited under conditions of water shortage. Recent studies [8] have confirmed the involvement of ethylene in the regulation of nitrogen fixation under drought. In our experiments, under the influence of water stress, the rate of nitrogen fixation decreased, while the use of lectin contributed to an increase in the acetylene reduction activity of rhizobia in symbiosis with alfalfa plants both under the optimal and insufficient water supply.

Thus, the decrease in the rhizobia nodulation activity was accompanied by an increased release of ethylene by alfalfa plants due to both the effects of drought and lectin, while the nitrogen-fixing activity of rhizobia in symbiosis with plants decreased only under the influence of insufficient water supply.

Conclusions. In our research, we have identified the role of ethylene as a negative regulator of the nodulation process at pre-sowing treatment of alfalfa seeds with lectin. At the same time, the relationship between the processes of N_2 fixation by *S. meliloti* in symbiosis with alfalfa and ethylene production needs further detailed study.

It should be noted that, despite the identified negative impact of alfalfa seeds treatment with lectin on nodulation activity of rhizobia and nodule growth under the lack of moisture at the initial stages of symbiosis formation, in the future, after the resumption of irrigation to the optimal level (60 % TMC), there was observed an increase in the nodule mass and nitrogen-fixing activity, as well as in the aboveground mass of alfalfa plants (to the level of control plants that were not exposed to stress factor). This indicates that pre-sowing treatment of seeds with lectin promotes the formation of effective symbiotic systems capable of full recovery after the termination of water stress, and confirms the prospects for further research on the use of lectins to increase the symbiotic systems resistance to the action of adverse factors.

СИМБІОТИЧНІ ВЛАСТИВОСТІ *SINORHIZOBIUM MELILOTI* ТА ПРОДУКУВАННЯ ЕТИЛЕНУ РОСЛИНАМИ ЛЮЦЕРНИ НА РАННІХ ЕТАПАХ ФОРМУВАННЯ СИМБІОЗУ ЗА РІЗНОГО ВОДОЗАБЕЗПЕЧЕННЯ ТА ОБРОБКИ НАСІННЯ ЛЕКТИНОМ

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Резюме

Симбіотичні властивості бактерій суттєво впливають на ефективність симбіозу та урожайність рослин. Тому важливим та актуальним є вивчення особливостей взаємодії мікро- й макросимбіонтів, зокрема за стресових умов, і пошук шляхів підвищення продуктивності симбіотичних систем. **Мета.** Дослідження нодуляційної та азотфіксувальної активностей *Sinorhizobium meliloti*, а також продукування етилену рослинами люцерни на ранніх етапах формування симбіотичних систем за умов оптимального та недостатнього водозабезпечення і передпосівної обробки насіння лектином. **Методи**. Мікробіологічні (вирощування бактеріальної культури та проведення інокуляції насіння), фізіологічні (вегетаційний експеримент), біохімічні (визначення нітрогеназної активності та інтенсивності продукування етилену). У дослідженнях використано бульбочкові бактерії *Sinorhizobium meliloti* високоефективного штаму 441 та люцерну посівну *Medicago sativa* (L.) сорту Надєжда. Для приготування інокуляційної суспензії використано бактерії, що культивувалися на поживному середовищі 79 при 28 ºС за стаціонарної фази росту. Кількість мікроорганізмів становила 10^9 кл/мл. Рослини вирощували на піщаному субстраті з внесенням поживного середовища Гельрігеля, що містило 0,25 норми азоту. Посуху тривалістю 15 діб створювали, починаючи з 10 доби після появи сходів. **Результати**. На ранніх етапах формування симбіотичної системи люцерна – *S. meliloti* виявлено зниження нодуляційної активності ризобій і активізацію продукування етилену за умов недостатнього водозабезпечення та передпосівної обробки насіння лектином. При цьому відзначено, що його використання стимулювало функціонування симбіотичного апарату за оптимального і недостатнього водозабезпечення. Таким чином, збільшення кількості продукованого рослинами етилену супроводжувалось інгібуванням нодуляційної активності ризобій за дії посухи і лектину, тоді як азотфіксувальна активність зменшувалась лише під впливом недостатнього водозабезпечення. Після поновлення поливу на фоні використання лектину відзначено збільшення маси бульбочок та азотфіксувальної активності, а також підвищення надземної маси рослин люцерни. **Висновки.** Результати представленої роботи можуть бути свідченням ролі етилену як негативного регулятора нодуляції за передпосівної обробки насіння люцерни лектином. Водночас зв´язок процесів продукування етилену та фіксації молекулярного азоту ризобіями в симбіозі з рослинами потребує подальшого детального вивчення. Виявлене після поновлення поливу підвищення показників ефективності симбіотичної системи, сформованої на фоні використання лектину, зокрема збільшення надземної маси люцерни до рівня рослин контрольного варіанту, що не зазнали впливу стресового фактору, показують, що передпосівна обробка насіння лектином сприяє ефективній взаємодії мікро- та макросимбіонтів, забезпечує формування симбіотичної системи, здатної до повноцінного відновлення після припинення дії водного стресу, і підтверджує перспективність подальших досліджень щодо використання лектинів для створення оптимальних щодо реалізації симбіотичного потенціалу ризобій умов і підвищення стійкості бобово-ризобіального симбіозу до дії несприятливих чинників.

Ключові слова: Симбіоз, ризобії, люцерна, нодуляційна активність, азотфіксація, продукування етилену, лектин, водозабезпечення.

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Received 23.03.2020