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NEGATIVE HORMONAL REGULATION OF SYMBIOTIC NODULE DEVELOPMENT. I. ETHYLENE

(review)

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Abstract

The process of symbiotic nodule formation resulting from interaction between legume plants and rhizobia is controlled by both partners. From the plant side the important role belongs to a system of hormonal regulation, involving all classes of phytohormones identified in plants. Negative regulation of nodulation is very important for the plant since the symbiotic nodule formation is highly energy-consuming process. Moreover, nodules lacking nitrogen fixation might be formed during interaction with ineffective strain of rhizobia, and it is disadvantageous for the plant. Up to now, there are data about involving of four phytohormones into negative regulation of nodule formation: ethylene, abscisic, jasmonic and salicylic acids. In this review, the role of ethylene in negative regulation of nodulation is discussed. Ethylene negatively regulates the number of developing symbiotic nodules at different stages of their formation. The first negative effect of ethylene appears at the level of calcium spiking, triggered by Nod actors produced by rhizobia. Further, ethylene negatively influences deformations of roots hairs, stimulated by Nod factors, infection thread growth, as well as nodule primordium development. In tropical legume *Sesbania rostrata* Bremek. & Oberm. ethylene represses the activity of nodule meristem, leading to formation of determinate type of nodule (with temporal meristem activity), while at the absence of ethylene indeterminate nodules (with prolonged meristem activity) are formed. At the same time, it was found that in soybean *Glycine max* (L.) Merr., ethylene is not involved in regulation of nodulation. It seems that ethylene involvement into regulation of nodule formation is not strictly dependent on the type of nodules, since in the other legume plants, forming determinate nodules, number of nodules is negatively affected by ethylene. It is suggested that ethylene synthesis in inoculated roots is triggered by Nod factors, which activate plant defense responses, leading to restriction of number of forming nodules. Hypernodulating mutant of *Medicago truncatula* Gaertn. *sickle*, carrying a mutation in the gene *MtEIN2*, which is the key component in ethylene signal transduction pathway, is characterized by decreased level of defense response activation, as it was shown by proteomic analysis. It is interesting that not only the plants, but rhizobia as well can control ethylene level in rhizosphere and therefore influence nodule number. One of such mechanisms is the synthesis of rhizobitoxin by some rhizobial strains, which has structural similarity with inhibitor of ethylene synthesis aminoethoxyvinylglycine (AVG). The other mechanism is more widespread among rhizobia and it deals with synthesis of ACC deaminase, an enzyme, which cleaves the precursor of ethylene synthesis 1-aminocyclopropane-1-carboxylic acid (ACC). Thus, regulation of ethylene level may be important for practical application, potentially allowing to increase plant's ability to nodulation. However, it should be taken into account that nodule number is precisely regulated by the plant because nodule formation is very energy-consuming process. Even more, it is necessary to remember that ethylene stimulates development of root hairs and decrease of their level may influence an intake ability of root and lead to deficiency of nutrient elements.

Keywords: plant-microbe interactions, legume-rhizobial symbiosis, symbiotic nodule, phytohormones, ethylene, rhizobia, plant defense, ACC deaminase, rhizobitoxin.

Soil bacteria named rhizobia may be involved in symbiotic interaction with legumes and induce new root organs, nitrogen-fixing nodules [1, 2]. A signaling dialogue between rhizobia and legumes is the basis for the formation of nitrogen-fixing nodules [3].

Flavonoids excreted by legume roots induce the expression of nodulation genes [4, 5]. As a result, rhizobia synthesize specific signaling molecules—lipochitooligosaccharides called Nod factors [5]. In the last decade, genes of legumes involved in the initial stages of Nod factor reception and signaling have been identified [3, 6, 7]. The signal triggers a variety of plant responses: membrane polarization, calcium oscillations in root hairs, and reorganization of their cytoskeleton elements [8]. As a result, root hair deformation and typical curling occur. Simultaneously, cell divisions in the inner layers of root cortex and pericycle are reactivated, which results in nodule primordium formation [9]. Rhizobia infect symbiotic nodule tissue with tubular structures named infection threads [9]. This leads to the bacterial release into the cytoplasm of plant cells and to their differentiation induced by plant nodule-specific cysteine-rich peptides [10] into specialized forms, bacteroids [9, 11], able to fix atmospheric nitrogen to ammonium in plant host cells [12].

Phytohormones are important signaling molecules involved in the majority of physiological processes in plants. Obviously, they play an important role in the initiation, development and functioning of symbiotic nodules. To date, all phytohormone groups (auxin, cytokinins, ethylene, abscisic acid, jasmonic acid, salicylic acid, and gibberellins) have been shown to participate in the development of symbiotic nodules [13-19].

In this review, the role of ethylene in negative regulation of the development and functioning of symbiotic nodules is discussed.

Effect of ethylene on nodulation. The negative effect of ethylene on the number and nitrogen-fixing activity of developing symbiotic nodules was identified over 40 years ago while studying various plants: pea (*Pisum sativum* L.) [20, 21], bean (*Phaseolus vulgaris* L.) [22], and clover (*Trifolium repens* L.) [21]. At the same time, treatment of alfalfa (*Medicago sativa* L.) roots with the ethylene inhibitor aminoethoxyvinylglycine (AVG) has been shown to result in a 2-fold increase in the number of nodules formed, indicating ethylene involvement in regulation of nodule development in legumes [23]. Subsequently, it was confirmed that the inhibitors of ethylene synthesis and action stimulate nodule development [24].

Ethylene was also suggested to mediate the negative effect of nitrates on nodulation, since treatment of alfalfa (*M. sativa*) with AVG increased the number of nodules in plants exposed to nitrates [25]. At the same time, the studies of nitrate and light effect on nodule formation in pea (*P. sativum*) cv. Sparkle have demonstrated that nitrates block nodulation at earlier stages (before infection thread development) compared with treatment with ethylene or light (the majority of infections were blocked in root hairs or cells of the outer cortex layers) [26]. Moreover, in plants grown under the exposure of nitrate (in contrast to exogenous ethylene), treatment with the inhibitors of ethylene action (Ag^+) restored the number of nodules on the main root only, not on lateral roots [26].

The effect of ethylene on nodule formation in young plants has been described in pea (*P. sativum*), sweetclover (*Melilotus alba annua* Desr.), and soybean (*Glycine max* (L.) Merr.) [27]. When treated with ethylene (0.07 $\mu\text{l/l}$), the number of nodules formed reduced two-fold in Sparkle and Rondo pea varieties. Increasing the amount of ethylene (0.45 $\mu\text{l/l}$) resulted in complete inhibition of nodulation. Sweetclover plants showed similar sensitivity, while the number of nodules in soybean has not changed. Treatment with exogenous ethylene did not result in the decrease in the number of infections in pea and clover plants but stopped their development in root hair cells or outer cortex layers. Thus, it was shown that ethylene may affect more than one stage of nodulation blocking both the

growth of infection threads from epidermal cells to the outer cortex layers, and nodule primordium initiation [27].

Experiments with pea (*P. sativum*) have demonstrated nodulation opposite to xylem poles [28]. Later, localization of ACC oxidase, an enzyme that controls the final stage of ethylene biosynthesis from 1-aminocyclopropane-1-carboxylic acid (ACC), has been shown. Treatment of roots with AVG or Ag⁺ resulted in formation of a part of nodules opposite to phloem poles. Thus, it was concluded that ethylene provides the location of symbiotic nodule initiation [29].

Inhibiting of nodulation at different developmental stages by ethylene has been demonstrated using a model legume *Medicago truncatula* Gaertn. [30]. Moreover, the negative effect of ethylene was manifested since the earliest stages of symbiosis (before or during calcium oscillation caused by Nod factors secreted by rhizobia). Also, ethylene inhibited root hair deformation and initiation of infection thread growth. Earlier, ethylene has been demonstrated to inhibit infection thread growth after its growth initiation in *M. truncatula* [31]. It has been suggested that multiple ethylene effects may be either due to the existence of inhibition of nodule formation at one early stage of development, or due to independent inhibiting of several stages of nodule development [30]. Activation of ethylene synthesis induced by plant inoculation with *Sinorhizobium meliloti* plants may regulate the number of successful infections leading to formation of nodules by inhibiting further initiation of infections or by blockage of the emerging infection threads [30].

A tropical legume *Sesbania rostrata* Bremek. & Oberm. is known to develop various types of nodules, i.e. determinate nodules (with limited meristem activity), or indeterminate nodules (with prolonged meristem activity) depending on the growth conditions. Moreover, ethylene plays the leading role in the determining of nodule type: adding of its inhibitor (Ag⁺) to the substrate resulted in formation of indeterminate nodules, adding of ethephon (2-chloroethylphosphonic acid which decomposes with the conversion to ethylene) resulted in formation of determinate ones [32]. Thus, the negative effect of ethylene on the nodule meristem functioning has been shown.

The plants of *Vicia sativa* ssp. *nigra* (L.) Ehrh. inoculated with a strain of *Rhizobium leguminosarum* bv. *viciae* appeared to be an interesting model to study the role of ethylene in the regulation of nodulation. When growing in the light, they formed aberrant thick short roots (a Tsr-phenotype) with abnormal arrangement of nodules [33]. In the roots growing in light, rhizobial Nod factors have been shown to induce an increased response expressed in overproduction of ethylene, with which the described phenotype is associated [34]. The Tsr-phenotype was accompanied by the changes of the transverse (relative to the longitudinal axis of the cell) microtubule organization to longitudinal one resulting in the increase in cell diameter and thickened roots. Also, the Tsr-phenotype could be obtained by treating the roots with ethephon, and its manifestation was inhibited by treatment of plants with AVG, wherein nodulation of the main root was restored [34].

Genetic analysis of the role of ethylene in nodulation. Further, the studies of the role of ethylene in nodule formation developed with adequate genetic models—symbiotic gene mutants exhibiting increased sensitivity or insensitivity to ethylene, transgenic plants with increased or decreased production of ethylene.

Ethylene sensitive mutants in the genes *Pssym5* и *Pssym16* have been described in pea (*P. sativum*) [35, 36]. A *Pssym5* mutant forms just few root nodules, but their number is significantly increased after treatment with synthetic inhibitors of ethylene action or synthesis, as well as at culturing of mutant plant

root systems at low temperatures. At this, ethylene production of mutant plants did not differ from wild type plants, which indicates the *Pssym5* mutants increased sensitivity to ethylene [35]. E2 mutant in the gene *Pssym5* demonstrated abortion of infection threads and premature stopping of cell divisions in root cortex resulting in a greatly reduced number of nodule primordia and nodules compared with the wild type (37).

In R50 mutant (*Pssym16*), the number of nodules is lower than that of wild type, and infection threads grow not towards the center of the root but twisted greatly in enlarged cortical cells. However, only a small proportion of these threads are related to cell divisions and formation of nodule primordia. Moreover, in case of its appearance primordium has abnormal structure and a flattened shape, being formed by the cells that had undergone essentially only anticlinal divisions. Inhibitors of ethylene synthesis and action restored the R50 mutant ability to nodulation (36). In pea mutant E132 (*Pssym21*) with a strongly reduced ability to nodulation, treatment with cobalt or silver ions increases the number of root nodules formed, but it was considerably inferior to that in wild type roots (cv. Sparkle) [38].

Pea R82 mutant in the gene *Pssym17* was characterized by reduced and thickened roots as well as with shortened stems and reduced number of nodules (as compared with wild type). In addition, it produced an increased amount of ethylene, however, the inhibitors of ethylene synthesis and action did not cause the the normal phenotype restoration [39].

A *M. truncatula* insensitive to ethylene *sickle* mutant was obtained developing a 10-fold number of nodules than the initial line [31]. This phenotype was the result of the successful completion of numerous infections (nodule formation), while the majority of the wild type infections have been aborted. At the same time, in contrast to the wild type, the *sickle* mutant was insensitive to treatment with ethylene and ACC, which suggested that it had a defected ethylene signal transduction. Later, *sickle* was shown to have a mutation in the *Arabidopsis thaliana* (L.) Heynh. *ETHYLENE-INSENSITIVE (EIN2)* ortholog, the key component in the signal transduction pathway activated by ethylene [40]. The studies of root proteomes of the wild type the *sickle* mutant exposed to treatment with ACC and rhizobial inoculation revealed the proteins differentially synthesized during nodulation [41]. Thus, ACC treatment induced the synthesis of stress pprg-2 proteins (protein family PR10), ACC oxidase, Kunitz-type proteinase inhibitor, ascorbate peroxidase, and heat shock proteins in wild type roots. In inoculated mutant roots, the synthesis of pprg-2, Kunitz-type proteinase, and ACC oxidase was reduced which indicates that the stress-activating proteins are regulated by ethylene in the process of nodulation. Probably, their reduced synthesis in mutant as compared to wild type allows the development of a significantly greater number of infections and nodules. Thus, the negative effect of ethylene on nodule formation may be mediated by stress-activated protein synthesis [41].

Transgenic *Lotus japonicus* (Regel) K. Larsen plants with *Cm-ERS1/H70A* mutated gene encoding the ethylene receptor of muskmelon (*Cucumis melo* L.) that demonstrated insensitivity to ethylene were used to study its role in nodule development in deervetch. There was a significant increase in the number of infection threads and nodule primordia, and the number of mature nodules in wild type plants and in the three transgenic lines analyzed did not differ [42]. In roots of transgenic lines, the increased expression of the *NIN* gene necessary for the formation of infection threads and nodule primordia, has been shown [43]. Obviously, the early stages of symbiotic nodule development in *L. japonicus* including formation of infection threads and induction of nodule primordia are nega-

tively regulated by ethylene (probably indirectly through the *NIN* gene) [42]. Transgenic plants of *L. japonicus* with the *A. thaliana* gene with a dominant *AtETR1* mutation insensitive to ethylene, showed an increase in the number of nodules (at decreasing the number of nodules formed opposite xylem poles) and an increase in the number of bacteroids surrounded with a common symbiosome membrane [44].

A recent screening of deervetch (*L. japonicus*) mutants revealed three allelic *enigma* mutants insensitive to ethylene and demonstrating its increased production (compared to wild type) [45]. In *LjEIN2a* mutants, the number of nodules is reduced (unlike *M. truncatula sickle* with a mutation in the orthologous *MtEIN2* gene and with a hypernodulation phenotype) [31]. At the same time, the majority of nodules in *enigma* mutants were formed opposite to proto-phloem poles thus proving the effect of the modified ethylene status on nodule initiation. This unexpected contradiction can probably be explained by the presence of the second gene copy in the *L. japonicus* genome, *LjEIN2b* [45]. In an independent study, two copies of the *EIN2—EIN2-1* and *EIN2-2* genes have been identified in *L. japonicus* and switching of both genes by RNAi resulted in an increase in the number of nodules formed [46].

Analysis of legume mutants revealed close relationship between ethylene and auxin signal transduction pathways. Thus, a pea (*P. sativum*) SGE_{cr}t mutant (*curly roots*) forming a compact root system with curled roots in high density substrates was characterized by increased auxin synthesis [47] and reduced nodulation [48]. Treatment of plants with AGV restored the nodule number up to the wild type value [49].

In a *L. japonicus rel3* mutant with defective ta-siRNAs synthesis (trans-acting small interfering RNA) regulating *ARF3a*, *ARF3b* and *ARF4* (Auxin Response Factors) activity, the number of nodules was reduced compared to that of wild type, and adding of AVG restored this value to the level characteristic of the wild type. Probably, changes in auxin signal transduction pathway in the mutant results in the increase in ethylene production or in a higher sensitivity to it which, ultimately, results in the reduction of the nodule number [50].

Role of ethylene in nodulation in soybean and other legumes with determinate type of symbiotic nodules. In soybean (*G. max*), rhizobia inoculation in 4 weeks resulted in the activation of ethylene synthesis and accumulation of ACC—ethylene synthesis precursor—in plants. The main increase was associated with nodule functioning, and the quantity of excreted ethylene was not dependent on the efficacy of the strain used. Unlike legumes developing indeterminate nodules, physiologically active concentrations of ethephon had no inhibitory effect on nodule formation in soybean [51]. In later studies, induction of ethylene synthesis by soybean roots was observed in 3 days after inoculation with effective *Bradyrhizobium japonicum* strain, but there was no induction in case of a heterologous *R. leguminosarum* bv. *viciae* strain or in case of a mutant with blocked root hair curling (as a result, nodules were not formed) [52]. The AVG ethylene synthesis inhibitor caused a decrease in the amount of ethylene, but had no effect on the number of nodules, which raised a question about the role of ethylene in nodule formation in soybean [52]. In this connection, mutant soybean lines insensitive to ethylene were used, including T119N54 with a mutation in the *etr-1* gene which encodes one of ethylene receptors [53]. It has been shown that in the Hobbit 87 wild type and the *etr1-1* ethylene insensitive mutant, the number of nodules in plants, both treated and untreated with ethylene action inhibitor (silver thiosulfate), did not differ. Furthermore, ethylene is also not involved in the negative regulation of nodulation by nitrates. After treatment with ACC, a decrease in the number of nodules

was observed in Hobbit 87 wild type plants (unlike the *etr-1* mutant). The authors attribute this to the influence of ethylene on root development in Hobbit 87 wild type plants, resulting in a decrease in the root length. It was concluded that, unlike pea (*P. sativum*) and alfalfa (*M. truncatula*), ethylene does not play an important role in nodulation regulation in soybean which is associated with different types of nodules formed—indeterminate in the first case and determinate in the second case [53]. In other experiments using inhibitors of ethylene biosynthesis (cobalt chloride and AVG), no ethylene effect on the inhibition of nodulation and nitrogen fixation has been shown in soybean as well [54].

At the same time, the above-described studies of transgenic *L. japonicus* plants that revealed nodulation sensitivity to ethylene in this type of legumes with determinate nodules refuted the hypothesis of the association of soybean insensitivity to ethylene and the nodule type. Also, nodulation sensitivity to ethylene has been shown for another legume with determinate nodules, the beans (*P. vulgaris*) in which treatment with AVG and cobalt ions increased the number of nodules, and treatment with ethephon decreased it [55].

Effect of rhizobia on the ethylene content in the roots of legumes. Considering the negative role of ethylene in nodulation, it is not surprising that rhizobia use various strategies to reduce the ethylene content [56]. Thus, the *Bradyrhizobium elkanii* strains produce rhizobitoxin, an ACC synthase inhibitor (one of the key enzymes in ethylene biosynthesis). It is noteworthy that rhizobitoxin has structural similarities to AVG. Rhizobitoxin synthesis by the USDA94 strain reduced the amount of ethylene excreted by inoculated roots and increased the number of root nodules in *Macropodium atropurpureum* (DC.) Urb. host plants. At the same time, the mutant with defective rhizobitoxin synthesis formed a reduced (versus the original strain) number of nodules [57]. Inoculation of mung bean (*Vigna radiata* (L.) R. Wilczek) with mutant *B. elkanii* strain defected in the rhizobitoxin synthesis decreased the number of mature nodules relative to inoculation with the original USDA61 strain. At the same time, the mutant formed a significant number of immature nodules, indicating the negative impact of ethylene at the late stages of nodulation [58].

Another mechanism for reducing the amount of ethylene is the presence of ACC deaminase enzyme sequestering ACC in rhizobia [59]. The presence of ACC deaminase activity may contribute to the development of symbiosis in stressful conditions (for example, soil pollution with heavy metals) [60]. Meanwhile, disabling of ACC deaminase in different rhizobia species leads to different results, and the decrease of nodulation is observed not in all species [61].

Thus, the active synthesis of ethylene in legume roots is considered to be initiated as a result of the activity of Nod factors produced by rhizobia. Ethylene negatively regulates nodulation both at the earliest (in calcium oscillations caused by the action of Nod factor) and at later (growth infection thread, formation of nodule primordium and maintenance of nodule meristem) stages of nodulation. Soybean plants are an exception, and it should be noted that soybean nodulation insensitivity is probably not related to the type of nodule formed, since nodulation in other legumes is a sensitive to ethylene process. Activation of defense reactions by ethylene in plant roots has been shown, which is probably the mechanism of realization of the negative effect of ethylene on nodulation. Synthesis of ethylene opposite to phloem poles determines the place nodule initiation opposite to xylem poles. Various mechanisms aimed at reducing the ethylene content in the rhizosphere (synthesis of certain rhizobitoxin strains and widespread of ACC deaminase activity among strains of rhizobia) can be regarded as an interesting example of adaptive evolution of rhizobia.

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