The intensity of ethylene release by soybean plants under the influence of fungicides in the early stages of legume-rhizobial symbiosis

T. P. Mamenko, S. Y. Kots, Y. O. Khomenko

Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kyiv, Ukraine

Article info
Received 24.12.2019
Received in revised form 21.01.2020
Accepted 22.02.2020

Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kyiv, Ukraine
Tel.: +38-050-964-17-89.
E-mail: t_mamenko@ukr.net

Mamenko, T. P., Kots, S. Y., & Khomenko, Y. O. (2020). The intensity of ethylene release by soybean plants under the influence of fungicides in the early stages of legume-rhizobial symbiosis. Regulatory Mechanisms in Biosystems, 11(1), 98–104. doi:10.15421/022014

The effect of pre-sowing treatment of soybean seeds with fungicides on the intensity of ethylene release, the processes of nodulation and nitrogen fixation in different symbiotic systems in the early stages of ontogenesis were investigated. The objects of the study were selected symbiotic systems formed with the participation of soybean (Glycine max (L.) Merr.) Diamond variety, strains Bradyrhizobium japonicum 634b (active, virulent) and 604k (inactive, highly virulent) and fungicides Maxim XL 035 PS (lactoxonitril, 25 g/L, metalaxyl, 10 g/L), and Standak Top (fipronil, 250 g/L, thiophanate methyl, 225 g/L, pinaclorostrobin, 25 g/L). Before sowing, the seeds of soybean were treated with solutions of fungicides, calculated on the basis of one rate of expenditure of the active substance of each preparation indicated by the producer per ton of seed. One part of the seeds treated with fungicides was inoculated with rhizobium culture for 1 h (the titre of bacteria was 10⁷ cells/mL). To conduct the research we used microbiological, physiological, biochemical methods, gas chromatography and spectrophotometry. It is found that, regardless of the effectiveness of soybean rhizobial symbiosis, the highest level of ethylene release by plants was observed in the stages of primordial leaf and first true leaf. This is due to the initial processes of nodulation – the laying of nodule primordia and the active formation of nodules on the roots of soybeans. The results show that with the participation of fungicides in different symbiotic systems, there are characteristic changes in phytohormone synthesis in the primordial leaf stage, when the nodule primordia are planted on the root system of plants. In particular, in the ineffective symbiotic system, the intensity of phytohormone release decreases, while in the effective symbiotic system it increases. At the same time, a decrease in the number of nodules on soybean roots inoculated with an inactive highly virulent rhizobia 604k strain due to the action of fungicides and an increase in their number in variants with co-treatment of fungicides and active virulent strain 634b into the stage of the second true leaf were revealed. It was shown that despite a decrease in the mass of root nodules, there is an increase in their nitrogen-fixing activity in an effective symbiotic system with the participation of fungicides in the stage of the second true leaf. The highest intensity of ethylene release in both symbiotic systems was recorded in the stage of the first true leaf, which decreased in the stage of the second true leaf and was independent of the nature of the action of the active substances of fungicides. The obtained data prove that the action of fungicides changes the synthesis of ethylene by soybean plants, as well as the processes of nodulation and nitrogen fixation, which depend on the efficiency of the formed soybean-rhizobial systems and their ability to realize their symbiotic potential under appropriate growing conditions.

Keywords: Bradyrhizobium japonicum; Glycine max; l-aminocyclopropane-l-carboxylic acid; symbiotic system.

Introduction

The phytohormone ethylene has been known as a negative regulator of the nodulation process for almost four decades. Since then, significant progress has been made in understanding both the transduction of ethylene signaling pathways and the nodulation process. Its negative effect is manifested in the early stages of symbiosis development – before or during the release of calcium ions induced by the action of Nod factors. It has been proven (Olaker, 2011) that in Medicago truncatula it regulates the expression of early nodulation genes ENODI1, RIP1, and thus may affect the processes of calcium ion release. Ethylene treatment effectively inhibits Nod factor-induced calcium ion release and expression of early ENOD nodulation genes by disrupting Nod factor signaling pathways (Hayashi et al., 2010; Ju et al., 2012). Finding out exactly how ethylene signaling pathways regulate calcium release will provide a clear understanding of ethylene-mediated local inhibition of nodulation in its early stages (Kots & Hryshchuk, 2019). It is proved that two ethylene regulatory pathways are established for inoculation with rhizobia of legumes (Larrainzar et al., 2015). The first path is fast (1 hour), is temporary, independent of Nod factors and positively controlled by ethylene. The second occurs more slowly (6 hours), depends on the perception of Nod factors and is negatively controlled by this phytohormone (Larrainzar et al., 2015). The first pathway is probably part of a defensive reaction, while the latter is responsible for activating nodulation programs and negatively affects several hormonal signaling pathways. This led the authors to propose a major negative regulatory role for ethylene. After inoculation of legume rhizobia, it was found that the key enzymes in the biosynthesis of ethylene with l-aminocyclopropane-1-carboxylic acid, aminocyclopropane-1-carboxylic acid, aminocyclopropane-1-carboxylic acid, aminocyclopropane-1-carboxylic acid synthase and aminocyclopropane-1-carboxylic acid oxidase are produced, as a result of which it forms in the nodulation zone (Larrainzar et al., 2015). These results are confirmed in the works of many authors who noted the induction of ethylene biosynthesis by Nod factors (Miyata et al., 2013; van Zeijl et al., 2015) and increasing its emission at early stages of legume-rhizobial symbiosis formation (Suganuma et al., 1995; Lopez-Gomez et al., 2012). The negative effect of ethylene on the processes of nodulation and nitrogen fixation of root nodules was found in the study of pea plants (Pisum sativum L.), beans (Phaseolus vulgaris L.), clover (Trifolium repens L.) (Tamimi & Timko, 2003; Ferguson et al., 2011; Khatibi & Schäfer, 2012). Thus, treatment of the roots of alfalfa plants (Medicago
sativa L.) with an inhibitor of ethylene synthesis by aminooxyaceticglycine led to an increase in the number of nodules formed, which tested to form its participation in the regulation of nodulation in legumes (Prayitno et al., 2006; Prayitno, 2010). In addition, in inoculated pea plants (P. sativum L.), nodule primordia are laid opposite to the xylem poles, whereas the enzyme aminocyclopropanecarboxylic acid oxidase, which controls the final stage of phytohormone biosynthesis, is localized opposite the phloem poles (Prayitno, 2010). Treatment of pea roots with inhibitors of phytohormone synthesis by aminooxyaceticglycine or silver led to the formation of nodules opposite the phloem poles (Lee & LaRue, 1992a, 1992b). This indicates its participation in the processes associated with determining the location of the nodules on the roots of plants. Ethylene controls the site of the initiation of nodules, their number and the growth of nodule primordia (Lohar et al., 2009). Ethylene blocks the development of nodules at different stages of development, and its action is manifested in blocking one or more of the early stages of nodule development independently (Oldroyd et al., 2001). Thus, it has been established that in inoculated plants of Sinorhizobium meliloti, there is an intensification of ethylene synthesis, which can participate in the regulation of successful infection and formation of root nodules by inhibiting further infection or blocking part of already developed infectious filaments (Oldroyd et al., 2001; Oldroyd, 2011). In addition, it blocks root hair deformity and the initiation of infectious filament growth in M. truncatula (Guerra et al., 2010; Guan et al., 2013).

In the last decade, a great deal has been found through the use of ethylene signaling pathway mutants and transgenic plants carrying ethylene-related genes. However, there is still far from a complete understanding of the effect of the hormone on the processes of nodulation. Particularly useful for such studies has been the supernodulating mutant M. truncatula Mtskl, which is hypersensitive to Nod factors (Penmetsa & Cook, 1997; Oldroyd & Downie, 2008; Prayitno & Mathesius, 2010; Oldroyd, 2011). This makes it useful for research in the field of transcriptomics, since gene expression induced by nod factors is more regulated in skl than in wild-type plants (Breakspear et al., 2014; Larrainzar et al., 2015). The Mtskl M. truncatula mutant is insensitive to ethylene because it does not exhibit the classic “triple response” by treatment with aminocyclopropanecarboxylic acid or ethylene (Penmet- sa & Cook, 1997). It has been proven that Mtskl is the AtEIN2 ortholog – an integral membrane protein comprising an N-terminal sequence similar to proteins belonging to the macrophage protein family associated with natural resistance (NRAMP) (Penmetsa et al., 2008). Its hydrophobic nucleus consists of 10 transmembrane domains, which are located inside its N-terminal end. A study of transgenic plants confirmed the results obtained using Mtskl that ethylene suppresses nodulation. This is due to a mutation of the EIN2 gene (ethylene insensitive 2), a key component of the regulation of ethylene signaling. As a result, the supernodulatory mutant of M. truncatula becomes insensitive to the influence of this phytohormone and produces 10 times more nodules compared to wild-type plants (Penmetsa & Cook, 1997). To date, one EIN2 gene was found in M. truncatula (Penmetsa et al., 2008), pea and chickpea (Weller et al., 2015), while two were found in common beans (Weller et al., 2015), soybean (Miyata et al., 2013) and in L. japoni- cus (Chân et al., 2013). Moreover, two genes L. japonicus LjEIN2a and LjEIN2b are expressed in all studied organs, including roots and nodu- les (Miyata et al., 2013). The presence of two EIN2 genes in common beans, soybeans and L. japonicus, as well as in all plants that form determinate nodules, may partly explain the mystery of why soybean nodules are formed in the presence of ethylene (Lee & LaRue, 1992a) and ethylene-insensitive mutants (Schmidt et al., 1999). These observations led to the hypothesis that the formation of indeterminate and determinate nodules may be regulated differently by this phytohormone. It was proved that, in contrast to pea and alfalfa, in soybean, ethylene does not play a significant role in the regulation of nodulation, which is associated with various types of formed nodules – indeterminate in the first case and determinate in the second (Schmidt et al., 1999). This led to a detailed study of the role of this phytohormone in nodulation of soybean plants using mutant lines that were insensitive to its action, in particular, T119N54 with a mutation in the etr-1 gene encoding one of its receptors (Suganuma et al., 1995; Schmidt et al., 1999). In the treat-ment of aminooxyaceticglycine in plants of wild-type Hobbit 87, in contrast to the mutant etr-1, a decrease in the number of formed nodules on soybean roots was observed, which explained the influence of ethylene on the development of the root system of plants, reduction of root length. At the same time, no difference was observed in the wild-type Hobbit 87 and the ethylene-insensitive mutant etr-1 in the number of nodules formed on soybean roots, both in treated and non-treated silver thiosulfate (ethylene inhibitor) plants. In addition, it was found that ethylene also did not participate in the negative regulation of nodulation by nitrate (Xia et al., 2017).

It is known that some legumes that form determinate nodules respond to ethylene. The sensitivity of the nodulation to phytohormone was shown for a bean plant with determinate nodules – pea (P. sati- vum), in which treatment with aminooxyaceticglycine and cobalt ions increased the number of nodules on the roots, while ethephon reduced it (Nukui et al., 2000; Tamini & Timko, 2003). Differences in the response of ethylene are observed in L. japonicus transgenic plants insensitive to it, carrying the vector with mutated etr-1 from Arabidopsis (Lohar et al., 2009). The various lines of Lotus japonicus were obtained and classified according to their hypocotyl responses to treatment with aminocyclopropanecarboxylic acid: the “hypersensitive” lines have a number of nodules similar to the number in wild-type plants, while the lines are “hypersensitive” with signs of the absence of ethylene responses and with a large number nodules (Lohar et al., 2009). Therefore, it has been suggested that the insensitivity of the nodulation in soybeans to ethylene is not related to the type of nodules formed, since in other legumes the development of the nodules is a process sensitive to the phytohormone (Xie et al., 1996; Nukui, 2000). This issue requires fur- ther careful study.

Scientists have determined that rhizobia can control the level of ethylene released, reducing its negative role in nodulation (Ma et al., 2002). Thus, Bradyrhizobium elkanii strains produce rhizobitoxin, an inhibitor of aminocyclopropanecarbonyl-synthase, which reduces the intensity of phytohormone secretion and increases the number of nodules on the roots of the host plant. Another mechanism for reducing ethylene release is the presence in the rhizosphere of the enzyme aminocyclopropanecarboxylic acid: the “hypersensitive” lines have a number of nodules similar to the number in wild-type plants, while the lines are “hypersensitive” with signs of the absence of ethylene responses and with a large number nodules (Lohar et al., 2009). Therefore, it has been suggested that the insensitivity of the nodulation in soybeans to ethylene is not related to the type of nodules formed, since in other legumes the development of the nodules is a process sensitive to the phytohormone (Xie et al., 1996; Nukui, 2000). This issue requires fur- ther careful study.

Scientists have determined that rhizobia can control the level of ethylene released, reducing its negative role in nodulation (Ma et al., 2002). Thus, Bradyrhizobium elkanii strains produce rhizobitoxin, an inhibitor of aminocyclopropanecarbonyl-synthase, which reduces the intensity of phytohormone secretion and increases the number of nodules on the roots of the host plant. Another mechanism for reducing ethylene release is the presence in the rhizosphere of the enzyme aminocyclopropanecarboxylic acid: the “hypersensitive” lines have a number of nodules similar to the number in wild-type plants, while the lines are “hypersensitive” with signs of the absence of ethylene responses and with a large number nodules (Lohar et al., 2009). Therefore, it has been suggested that the insensitivity of the nodulation in soybeans to ethylene is not related to the type of nodules formed, since in other legumes the development of the nodules is a process sensitive to the phytohormone (Xie et al., 1996; Nukui, 2000). This issue requires fur- ther careful study.

Scientists have determined that rhizobia can control the level of ethylene released, reducing its negative role in nodulation (Ma et al., 2002). Thus, Bradyrhizobium elkanii strains produce rhizobitoxin, an inhibitor of aminocyclopropanecarbonyl-synthase, which reduces the intensity of phytohormone secretion and increases the number of nodules on the roots of the host plant. Another mechanism for reducing ethylene release is the presence in the rhizosphere of the enzyme aminocyclopropanecarboxylic acid: the “hypersensitive” lines have a number of nodules similar to the number in wild-type plants, while the lines are “hypersensitive” with signs of the absence of ethylene responses and with a large number nodules (Lohar et al., 2009). Therefore, it has been suggested that the insensitivity of the nodulation in soybeans to ethylene is not related to the type of nodules formed, since in other legumes the development of the nodules is a process sensitive to the phytohormone (Xie et al., 1996; Nukui, 2000). This issue requires fur- ther careful study.

Scientists have determined that rhizobia can control the level of ethylene released, reducing its negative role in nodulation (Ma et al., 2002). Thus, Bradyrhizobium elkanii strains produce rhizobitoxin, an inhibitor of aminocyclopropanecarbonyl-synthase, which reduces the intensity of phytohormone secretion and increases the number of nodules on the roots of the host plant. Another mechanism for reducing ethylene release is the presence in the rhizosphere of the enzyme aminocyclopropanecarboxylic acid: the “hypersensitive” lines have a number of nodules similar to the number in wild-type plants, while the lines are “hypersensitive” with signs of the absence of ethylene responses and with a large number nodules (Lohar et al., 2009). Therefore, it has been suggested that the insensitivity of the nodulation in soybeans to ethylene is not related to the type of nodules formed, since in other legumes the development of the nodules is a process sensitive to the phytohormone (Xie et al., 1996; Nukui, 2000). This issue requires fur- ther careful study.
of external factors. In this regard, our aim was to study the effect of presowing treatment of soybean seeds with fungicides on the intensity of ethylene release in plants at early stages of the legume-rhizobial symbiosis formation.

Materials and methods

The objects of the study are chosen symbiotic systems, formed with participation of soybean (Glycine max (L.) Merr.) Diamond variety, strains of various effectiveness of Bradyrhizobium japonicum 634b (active, virulent) and 604k (inactive, highly virulent) and fungicidal Maxim XL 035 PS (thiophanate-methyl, 10 g/L) and Standak Top (thiophanate-methyl, 225 g/L, piraclostrobin, 25 g/L). We used B. japonicum strains from the museum collection of the symbiotic nitrogen fixation department of the Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine. Before sowing, soybeans were treated with solutions of fungicides, calculated on the basis of one rate of expenditure of the active substance of each preparation indicated by the producer per ton of seeds.

For research, fungicides were used that differed in the spectrum of action of active substances. In particular, the active ingredients (thiophan, thiophanate-methyl, pyraclostrobin), which are part of Standak Top, combine fungicidal and insecticidal action. Each of these substances has a special mechanism of action, as well as the duration of protective action. In particular, the action of thiophan consists in blocking gamma-aminobutyric acid, which regulates the passage of the nerve impulse through chlorine channels in the membranes of the nerve cells, causing a violation of the function of the nervous system of insects. Piraclostrobin interferes with mitochondrial breathing, blocking the transport of electrons, and violates the energy exchange in a fungus cell. Thiophanate-methyl suppresses the formation of ergosterol, as well as the biosynthesis of nucleic acids in fungal cells (www.demetra-agra.com.ua).

The fungicide Maxim XL contains two active ingredients (thiophanoxide and metalaxyl), one of which, thiophanoxide, is an analogue of a natural antibiotic and is excreted by soil bacteria Pseudomonas pyrocinia, which inhibit the growth of pathogenic fungi. Influence of substances of the subclass of triazolinfuron on the growth and reproduction of the pathogen is associated with a violation of the function of cell membranes (www.demetra-agra.com.ua).

One part of the seeds treated with fungicides was inoculated with suspension of rhizobium culture 1 hour. The culture of rhizobium was grown on solid mannitol – yeast medium for 9 days at 26–28 °C (the titer of bacteria was 10^7 cells/mL). The other part of fungicide-treated seeds was not inoculated by rhizobium culture.

Plants were grown in sand pots in the application of a nutritional mixture of Gelrigel with 0.25 nitrogen rates from natural light and optimal water supply. For research, soybean roots were selected in the early stages of ontogenesis – seedlings leaves, primordial leaves, first true leaf, second true leaf, and root nodules in the stage of third true leaf.

The control options were: non-inoculated plants without fungicide treatment, and inoculated with strains 634b and 604k without using fungicide treatment.

The nodulation ability of B. japonicum was determined by counting the number and mass of root nodules. Nitrogen fixation activity (NFA) (acetylene reduction activity) of intact plants was measured on gas chromatograph “Agilent GC system 6850” (USA) with flame-ionization detector (Hardy et al., 1968). The separation of gases was carried out on a column (Supelco Porapak N) at a thermostat temperature of 55 °C and a detector – 150 °C. The carrier gas was helium (20 mL per 1 minute).

As a result, pure ethylene (Sigma-Aldrich, USA) was used. The amount of ethylene formed from acetylene in 1 hour by the action of nitrogenase, that is, the total nitrogen fixation activity was expressed in molar units of ethylene formed per 1 hour (μmol C_2H_4/pot • h). The specific nitrogen fixation activity was calculated on the mass of root nodules from one plant and expressed in molar units of ethylene formed in 1 hour (μmol C_2H_4/g nodules • h).

To determine the intensity of ethylene evolution the plant samples were placed in 75 mL glass vials, which were sealed immediately and left in the dark for 24 h (Guzmán & Ecker, 1990). After incubation, the gas mixture containing ethylene was analyzed on gas chromatograph “Agilent GC system 6850” (USA). The volume of the analyzed sample gas mixture was 1 cm^3. As a standard, pure ethylene (Sigma-Aldrich, USA) was used. The amount of ethylene evolution from the incubated sample was expressed in nmoles at the rate of one plant per hour (nmol C_2H_4/plant • h).

Materials and methods

The objects of the study are chosen symbiotic systems, formed with participation of soybean (Glycine max (L.) Merr.) Diamond variety, strains of various effectiveness of Bradyrhizobium japonicum 634b (active, virulent) and 604k (inactive, highly virulent) and fungicidal Maxim XL 035 PS (thiophanoxide-methyl, 10 g/L) and Standak Top (thiophanoxide-methyl, 225 g/L, piraclostrobin, 25 g/L). We used B. japonicum strains from the museum collection of the symbiotic nitrogen fixation department of the Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine. Before sowing, soybeans were treated with solutions of fungicides, calculated on the basis of one rate of expenditure of the active substance of each preparation indicated by the producer per ton of seeds.

For research, fungicides were used that differed in the spectrum of action of active substances. In particular, the active ingredients (thiophan, thiophanate-methyl, pyraclostrobin), which are part of Standak Top, combine fungicidal and insecticidal action. Each of these substances has a special mechanism of action, as well as the duration of protective action. In particular, the action of thiophan consists in blocking gamma-aminobutyric acid, which regulates the passage of the nerve impulse through chlorine channels in the membranes of the nerve cells, causing a violation of the function of the nervous system of insects. Piraclostrobin interferes with mitochondrial breathing, blocking the transport of electrons, and violates the energy exchange in a fungus cell. Thiophanate-methyl suppresses the formation of ergosterol, as well as the biosynthesis of nucleic acids in fungal cells (www.demetra-agra.com.ua).

The fungicide Maxim XL contains two active ingredients (thiophanoxide and metalaxyl), one of which, thiophanoxide, is an analogue of a natural antibiotic and is excreted by soil bacteria Pseudomonas pyrocinia, which inhibit the growth of pathogenic fungi. Influence of substances of the subclass of triazolinfuron on the growth and reproduction of the pathogen is associated with a violation of the function of cell membranes (www.demetra-agra.com.ua).

One part of the seeds treated with fungicides was inoculated with suspension of rhizobium culture 1 hour. The culture of rhizobium was grown on solid mannitol – yeast medium for 9 days at 26–28 °C (the titer of bacteria was 10^7 cells/mL). The other part of fungicide-treated seeds was not inoculated by rhizobium culture.

Plants were grown in sand pots in the application of a nutritional mixture of Gelrigel with 0.25 nitrogen rates from natural light and optimal water supply. For research, soybean roots were selected in the early stages of ontogenesis – seedlings leaves, primordial leaves, first true leaf, second true leaf, and root nodules in the stage of third true leaf.

The control options were: non-inoculated plants without fungicide treatment, and inoculated with strains 634b and 604k without using fungicide treatment.

The nodulation ability of B. japonicum was determined by counting the number and mass of root nodules. Nitrogen fixation activity (NFA) (acetylene reduction activity) of intact plants was measured on gas chromatograph “Agilent GC system 6850” (USA) with flame-ionization detector (Hardy et al., 1968). The separation of gases was carried out on a column (Supelco Porapak N) at a thermostat temperature of 55 °C and a detector – 150 °C. The carrier gas was helium (20 mL per 1 minute).

As a result, pure ethylene (Sigma-Aldrich, USA) was used. The amount of ethylene formed from acetylene in 1 hour by the action of nitrogenase, that is, the total nitrogen fixation activity was expressed in molar units of ethylene formed per 1 hour (μmol C_2H_4/plant • h). The specific nitrogen fixation activity was calculated on the mass of root nodules from one plant and expressed in molar units of ethylene formed in 1 hour (μmol C_2H_4/g nodules • h).

To determine the intensity of ethylene evolution the plant samples were placed in 75 mL glass vials, which were sealed immediately and left in the dark for 24 h (Guzmán & Ecker, 1990). After incubation, the gas mixture containing ethylene was analyzed on gas chromatograph “Agilent GC system 6850” (USA). The volume of the analyzed sample gas mixture was 1 cm^3. As a standard, pure ethylene (Sigma-Aldrich, USA) was used. The amount of ethylene evolution from the incubated sample was expressed in nmoles at the rate of one plant per hour (nmol C_2H_4/plant • h).

The results were statistically analyzed in the Statistica 6.0 (Statsoft Inc., USA) program pack. The tables and figures show the arithmetic mean values and their standard errors (x ± SE). The reliability of the differences between the samples was evaluated using the single-factor dispersion analysis (ANOVA), using the Mann-Whitney U-criterion. Differences were considered to be significant at P < 0.05.

Results

The pre-sowing treatment of soybean seeds with fungicides without inoculation with rhizobia did not lead to significant changes in the intensity of ethylene release by plants into the the seedlings leaves stage (Fig. 1). In the following stages of ontogenesis – primordial leaves, the first and second true leaves – there was an increase in the release of phytohormone by soybean plants. In particular, with the action of the fungicide Maxim its production increased by 41.7% in the primordial leaves stage and by 30.5% in the first true leaves stage. With pre-sowing seed treatment with fungicide Standak Top, ethylene synthesis by soybean plants increased by 52.8% in the primordial leaves stage, but its largest production is recorded in the first true leaf stage by 99.8% of the level of non-treatment plants. It was found that in the stage of the second true leaf the intensity of phytohormone release by plants exceeded the level of unprocessed plants by 18.1% for Maxim and 44.9% for Standak Top.

![Fig. 1. Influence of fungicides on the intensity of ethylene (C_2H_4) release by non-inoculated soybean plants in the early stages of ontogeny: I – seedlings leaves, II – primordial leaves, III – first true leaf, IV – second true leaf (x ± SE, n = 8); data compared to control (without fungicides) are reliable at * – P < 0.05, ** – P < 0.01, *** – P < 0.001; here and in Fig. 2 and 3 the reliability of the differences between the samples was evaluated using the single-factor dispersion analysis (ANOVA), using the Mann-Whitney U-criterion](image-url)
Maxim fungicide was accompanied by a decrease of phytohormone synthesis by 29.1% (Fig. 2). An increase in the level of phytohormone release in this symbiotic system involving fungicides was observed in the primordial leaf stage. In particular, the intensity of ethylene production by plants increased by 24.2% with the action of Maxim fungicide and by 26.6% with the action of Standak Top. The highest level of ethylene release was observed in the stage of the first true leaf in all variants of the experiment using pre-sowing seed treatment with fungicides and inoculation with the active strain of B. japonicum.

Despite the fact that with the action of fungicides its production was slightly reduced in this symbiotic system, the results are still within the margin of error of the experiment. In the stage of the second true leaf with the participation of fungicides Maxim and Standak Top together with inoculation of rhizobia of the active strain, phytohormone secretion by soybean plants was reduced by 28.4% and 19.4% respectively.

It was determined that the reduction of the weight of nodules on the roots of plants in the stage of the second true leaf did not significantly affect the indices of specific and total nitrogen fixing activity (Fig. 3).

### Table 1

| Fungicides                | Stage of first true leaf | Stage of second true leaf |
|---------------------------|--------------------------|---------------------------|
|                           | number, pieces/ plant | weight, mg/ plant | number, pieces/ plant | weight, mg/ plant |
| Strain 634b               | 11.12 ± 0.78           | 22 ± 1.5                      | 16.51 ± 1.12            | 69 ± 2.8          |
| Maxim XL + strain 634b   | 11.92 ± 0.72           | 18 ± 1.1                       | 18.62 ± 1.32             | 56 ± 2.2           |
| Standak Top + strain 634b| 12.81 ± 0.84           | 18 ± 1.4                       | 23.14 ± 1.64             | 47 ± 2.1           |
| Strain 604k               | 17.88 ± 1.21           | 31 ± 1.8                       | 33.41 ± 2.36             | 61 ± 2.3           |
| Maxim XL + strain 604k   | 18.72 ± 1.34           | 33 ± 1.8                       | 24.62 ± 1.52             | 37 ± 1.6           |
| Standak Top + strain 604k| 17.35 ± 1.27           | 23 ± 1.4                       | 26.11 ± 1.71             | 55 ± 1.8           |

Note: * – data compared to the control are reliable at $P < 0.05$; ** – $P < 0.01$ (‘’ and ‘’ – relative to the variants with inoculation with strains 634b and 604k, respectively); the reliability of the differences between the samples was evaluated using the single-factor dispersion analysis (ANOVA), using the Mann-Whitney U-criterion, here and in Fig. 3 presents the average values for two years.

---

**Fig. 2.** Influence of fungicides on the intensity of ethylene release by soybean plants during inoculation of seeds with with inactive strain 604k (A) and active strain 634b (B) of B. japonicum in the early stages of ontogenesis: I – seedlings leaves, II – primordial leaves – first true leaf, IV – second true leaf (s = SE, n = 8); data compared with control are reliable at $* *$ – $P < 0.05$, $** *$ – $P < 0.01$ (‘’ and ‘’ – relative to the variants with inoculation of strains 634b and 604k, respectively); see Fig. 1.

**Fig. 3.** Influence of fungicides on the total (TNA) and specific (SNA) nitrogenase activity of soybean plants after inoculation of seeds with active strain 634b of B. japonicum in the early stages of ontogenesis: I – seedlings leaves, II – primordial leaves – first true leaf, IV – second true leaf (s = SE, n = 8); data compared with control (634b) are reliable at $* *$ – $P < 0.01$; see Fig. 1.

With the use of fungicidal preparations in an ineffective symbiotic system formed with the participation of an inactive highly virulent rhizobium 604k strain, we observed a decrease in the number and weight of nodules on the plant roots in the stage of the second true leaf. In particular, for the actions of Maxim fungicide by 26.3% and for the actions of Standak Top by 22.1% (Table 1). In an effective symbiotic system formed with the participation of the active virulent strain 634b, the use of pre-sowing treatment of soybean seeds with fungicides led to an increase in the number of nodules on the roots and a decrease in their weight in the stage of the second true leaf. In particular, the action of Maxim fungicide increased the number of root nodules by 12.7%, and under the action of Standak Top by 40.1%, whereas the weight of nodules decreased by 10.8 and 31.8%, respectively.

In particular, we observed an increase in these soybean indices by co-inoculation with the rhizobia of the active strain 634b and fungicides in the stage of the second true leaf compared to the variant without using them. Thus, in the stage of the second true leaf the total nitrogen fixing activity of...
Ethylene plays a leading role in determining the type of nodules (determinate vs. indeterminate) and affects nodule meristem activity, which is crucial for the development of legume-rhizobial symbiosis. The formation of indeterminate nodules in soybean plants, which form nodules with a determinate type of meristem, has been studied extensively. Ethylene synthesis in the roots of legume plants is a key factor in the formation of indeterminate nodules (Schmidt et al., 1999).

We have shown that the pre-sowing treatment of soybean seeds with fungicides affects ethylene synthesis. Fungicidal drugs inhibit ethylene synthesis, leading to a decrease in ethylene release into the primordial leaves stage, which is crucial for the formation of legume-rhizobial symbiosis. The action of fungicides in non-inoculated soybeans intensifies the release of ethylene by plants during the early stages of ontogenesis, the first true leaf, and the intensity of phytohormone release by soybean intensifies. These findings are consistent with previous studies (Khatabi & Schäfer, 2012).

Ethylene plays a positive role in legume-rhizobial symbiosis as it regulates transcription processes at certain stages (Larminaz et al., 2015).

We have shown that the action of fungicides in non-inoculated soybeans intensifies the release of ethylene by plants during the early stages of ontogenesis, which affects nodule meristem activity, leading to a decrease in ethylene release into the primordial leaves stage. The action of fungicides in non-inoculated soybeans intensifies the release of ethylene by plants during the early stages of ontogenesis, the first true leaf, and the intensity of phytohormone release by soybean intensifies. These findings are consistent with previous studies (Khatabi & Schäfer, 2012).

It is believed that the formation of “stress” ethylene is one of the fastest reactions to external influences (Urao et al., 2002; Wang et al., 2002). Its release is realized only in the presence of oxygen and indicates the transition of cellular metabolism to a stress state (Wang et al., 2002). We have found that the action of fungicides in non-inoculated soybeans intensifies the release of ethylene by plants during the early stages of ontogenesis – primordial leaves, the first and second true leaves, especially in the Standak Top treatment. This supports our view that pre-sowing soybean seeds with fungicides for plants is an additional stress factor affecting its metabolism. In the presented studies, this is manifested in the increased production of “stress” phytohormone of ethylene by plants of non-inoculated soybeans, the seeds of which were pre-treated with fungicides.

When forming an effective symbiotic system, formed with the participation of soybeans and the active strain of rhizobia 634b, the pre-soaking treatment of soybean seeds with fungicides for plants is an additional stress factor affecting its metabolism. In the presented studies, this is manifested in the increased production of “stress” phytohormone of ethylene by plants of non-inoculated soybeans, the seeds of which were pre-treated with fungicides.

It is accepted that active ethylene synthesis in the roots of legumes is initiated as a result of the action of nod factors produced by rhizobia (Tsyganova & Tsyganov, 2015). At the same time, studies conducted in recent years have shown the adverse effect of drugs with fungicidal activity on the effectiveness of legume-rhizobial symbiosis. This is manifested in the violation of the regulatory signaling system between macro- and microsymbionts, blocking the activity of nodulation genes and reducing the level of the rhizobial Nod factor (Bikrol et al., 2005; Nason et al., 2007). Fungicidal drugs affect the inhibition of flavonoid Nod receptor, which induces inhibition of the synthesis and secretion of flavonoid substances produced by the plant. Thus, they violate legume-rhizobial signaling (Bikrol et al., 2005; Fox et al., 2007; Standish et al., 2018).

We have shown that the pre-sowing treatment of soybean seeds with fungicides produces a significant effect on the formation of microsymbionts – the formation of microbial-plant relationships is the action of fungicides as a stress factor, which also affects the ability of the formed symbiotic systems to realize their adaptive potential. This indicates the feasibility of further research in this direction.
plants is reduced by co-treatment with fungicides and the active rhizobia strain 634b. It is noted that in the effective symbiotic system with the participation of fungicides there was an increase in the number of root nodules, but there was a decrease in their weight in the stage of the second true leaf. It should be noted that this did not affect negatively the indicators of total and specific nitrogen fixation activity as their values increased. We have observed a similar tendency to increase nitrogen fixation in this symbiotic system due to fungicides in previous studies (Marmenko et al., 2019). However, the intensity of total nitrogen fixation activity was lower than in previous studies, and the specific nitrogen fixation activity on the contrary increased, especially with the action of Maxim fungicide.

**Conclusions**

Regardless of the effectiveness of soybean rhizobial symbiosis, the highest levels of ethylene release by plants are observed in the primordial leaves’ stage and the first true leaf, which is associated with the initial processes of nodulation—the laying of nodules primordia and the active formation of nodules on soybean roots. Pre-sowing treatment of soybean seeds with fungicides together with inoculation with an inactive rhizobia 604k strain leads to a decrease in ethylene synthesis by plants in the primordial leaves’ stage and inhibition of nodulation processes into the second true leaf stage. The use of soybean seed fungicides with the participation of the active rhizobium strain 634b induces an intensification of ethylene release by plants into the primordial leaves’ stage, which is accompanied by an increase in the number of root nodules, as well as their total and specific nitrogen-fixing activity.

**References**

Bikrol, A., Saxena, N., & Singh, K. (2005). Response of Glycine max in relation to nitrogen fixation as influenced by fungicide seed treatment. African Journal of Biotechnology, 4(7), 667–671.

Bredehorst, A., Liu, C., Roy, S., Stacey, N., Rogers, C., Trick, M., Morieri, G., Mysore, K. S., Torres-Jerez, I., Vernié, T., Ju, C., Yoon, G. M., Sheminrich, J. M., Liu, D. Y., Ying, Z. L., Jiang, J., Garret, W. M., Kessenebrock, M., Groth, G., Tucker, L. M., Cooper, B., Kieber, J. J., & Chang, C. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proceedings of the National Academy of Sciences, 109(47), 19486–19491.

Kotb, B. A., & Schiller, P. (2012). Ethylene in mutualistic symbioses. Plant Signaling and Behavior, 7(12), 1634–1638.

Kosnier, C., Winou, T., Pitschke, A., Mukter, L., Sato, S., Kanoke, K., Tabata, S., Sandal, N., Stougard, J., Webb, K. J., Szyszkojowski, K., & Parniske, M. (2005). Seven Lotus japonicus genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. Plant Cell, 17(8), 2217–2228.

Kots, S. Y., & Hryshchuk, O. (2019). Fihoehormonalna refutaltiasia bovobyo-zhibialnoho simbiozu [Phytohormonal regulation of legume-rhizobial symbiosis]. Fiziologiya Rastenii i Genetika, 51(1), 3–27 (in Ukrainian).

Larranzá, E., Reyle, B. K., Kim, S. C., Cepas, A., Guerra, J. C. P., Coussens, G., De Keyser, A., De Rycke, R., De Bodt, S., Van de Walle, J., Van der Linden, S., & Velde, W. (2010). Comparison of development and function of peripheral vasculature in nodules of Medicago truncatula. Plant Physiology, 152(3), 1574–1584.

López-Gómez, M., Sandal, N., Stougard, J., & Boller, T. (2012). Interplay of flg22-induced defence responses and nodulation in Lotus japonicus. Journal of Experimental Botany, 63(1), 393–401.

Lee, K. H., & LaRue, T. A. (1992a). Exogenous ethylene inhibits nodulation of Pisum sativum L. cv. Sparkle. Plant Physiology, 100(3), 1759–1763.

Ma, W., Pruegel, D., & Glick, B. R. (2003). Rhizobium leguminosarum biovar viciae 1-aminocyclopropane-carboxylate deaminase promotes nodulation of pea plants. Applied and Environmental Microbiology, 69(8), 4396–4402.

Ma, W., Peng, X., & Glick, B. R. (2002). Strategies used by rhizobia to lower plant ethylene levels and increase nodulation. Canadian Journal of Microbiology, 48(1), 947–954.

Marmenko, T. P., Khomenko, Y. O., & Kots, S. Y. (2019). Influence of fungicides on activities of enzymes of phenolic metabolism in the early stages of formation and functioning of soybean symbiotic apparatus. Regulatory Mechanism in Biosystems, 10(1), 111–116 (in Ukrainian).

Miyata, K., Kawaguchi, M., & Nakagawa, T. (2013). Two distinct EIN2 genes cooperatively regulate ethylene signaling in Lotus japonicus. Plant Cell Physiology, 54(9), 1469–1477.

Murset, V., Hernecke, H., & Pessi, G. (2012). Disparate role of rhizobial ACC deaminase in root-nodule symbioses. Symbiosis, 57(1), 43–50.

Nakagawa, T., Kaku, H., Shimodori, Y., Sugiyama, A., Shimamura, M., Takahashi, K. (2011). From defense to symbiosis: Limited alterations in the kinase domain of LysM receptor-like kinases are crucial for evolution of legume-Rhizobium symbiosis. Plant Journal, 65(2), 169–180.

Nason, M. A., Farrar, J., & Bartlett, D. (2007). Strobilurin fungicides induce changes in photosynthetic gas exchange that do not improve water use efficiency of plants grown under conditions of water stress. Pest Management Science, 63(12), 1191–1200.

Nakai, N., Ezura, H., Yuhoshi, K.-I., Yasutata, T., & Minamiwaka, K. (2000). Effects of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in Lotus japonicus and Macropetrum atropurpureum. J. Phytopathology, 148(6), 189–197.

Oldroyd, G. E. D., & Downie J. A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. Annual Review of Plant Biology, 59(1), 519–546.

Oldroyd, G. E. D., Engstroem, E. M., & Long, S. R. (2001). Ethylene inhibits the Nod factor signal transduction pathway of Medicago truncatula. Plant Cell, 13(9), 1835–1849.

Oldroyd, G. E. D., Murray, J. D., Poole, P. S., & Downie, J. A. (2011). The rules of bacterial and fungal endosymbionts. Plant Journal, 63(1), 141–154.
Penmetsa, V. P., Uribe, P., Anderson, J., Lichtenzveig, J., Gish, J.-C., Nam, Y. W., Engstrom, E., Xu, K., Sckisiel, G., Pereira, M., Baek, J. M., Lopez-Meyer, M., Long, S. R., Harrison, M. J., Singh, K. B., Kiss, G. B., & Cook, D. R. (2008). The Medicago truncatula ortholog of Arabidopsis EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations. Plant Journal, 55(40), 580–595.

Prayitno, J. (2010). Root and nodulation phenotypes of the ethylene-insensitive sickle mutant of Medicago truncatula. Hayati Journal of Biosciences, 17(3), 131–136.

Prayitno, J., & Mathesius, U. (2010). Differential regulation of the nodulation zone by silver ions, L-α-(2-amino-ethoxyvinyl)-glycine, and the skl mutation in Medicago truncatula. Hayati Journal of Biosciences, 17(1), 15–20.

Prayitno, J., Inmin, N., Rolfe, B. G., & Mathesius, U. (2006). Identification of ethylene-mediated protein changes during nodulation in Medicago truncatula using proteome analysis. Journal of Proteome Research, 5(11), 3084–3095.

Prayitno, J., Rolfe, B. G., & Mathesius, U. (2006b). The ethylene-insensitive sickle mutant of Medicago truncatula shows altered auxin transport regulation during nodulation. Plant Physiology, 142(1), 168–180.

Schmidt, J. S., Harper, J. E., Hoffman, T. K., & Bent, A. F. (1999). Regulation of soybean nodulation independent of ethylene signaling. Plant Physiology, 119(3), 951–960.

Standish, J. R., Brenneman, T. B., & Stevenson, K. L. (2018). Dynamics of fungicide sensitivity in Venturia effusa and fungicide efficacy under field conditions. Plant Disease, 102(8), 1606–1611.

Saganuma, N., Yamauchi, H., & Yamamoto, K. (1995). Enhanced production of ethylene by soybean roots after inoculation with Bradyrhizobium japonicum. Plant Science, 112(2), 163–168.

Tamura, S. M., & Timko, M. P. (2003). Effects of ethylene and inhibitors of ethylene synthesis and action on nodulation in common bean (Phaseolus vulgaris L.). Plant Soil, 257(1), 125–131.

Uras, T., Zaman-Shinozaki, K., & Shinozaki, K. (2000). Two-component systems in plant signal transduction. Trends in Plant Science, 5(2), 67–73.

van Zeijl, A., Op den Camp, R. H. M., Deinum, E. E., Chamikhova, T., Franssen, H., Op den Camp, H. J. M., Bouwmeester, H., Kohlen, W., Bisseling, T., & Geurts, R. (2015). Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinin in Medicago truncatula roots. Molecular Plant, 8(8), 1213–1226.

Wang, K. L., Li, H., & Ecker, J. R. (2002). Ethylene biosynthesis and signaling networks. The Plant Cell, 12(1), 131–151.

Weller, J. L., Foo, E. M., Hecht, V., Ridge, S., Vander Schoor, J. K., & Reid, J. B. (2015). Ethylene signaling influences light-regulated development in pea. Plant Physiology, 169(1), 115–124.

Xia, X., Ma, C., Dong, S., Xu, Y., & Gong, Z. (2017). Effects of nitrogen concentrations on nodulation and nitrogenase activity in dual root systems of soybean plants. Soil Sciences and Plant Nutrition, 63(5), 470–482.