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ADAPTIVE REACTIONS OF WINTER WHEAT (*TRITICUM AESTIVUM* L.) AFFECTED BY EYESPOT CAUSAL AGENT UNDER THE ACTION OF *BACILLUS SUBTILIS* BACTERIAL ISOLATES

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Aim. *To investigate the effect of Bacillus subtilis 537/B1 bacterial isolates to the lectin activity changes and generated malonic dialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) content of winter wheat seedlings of varieties with different susceptibility – Myronivska 808 and Renan were infected by eyespot causal agent Pseudocercosporella herpotrichoides (Fron) Deighton.* ***Methods.*** *Microbiological, immunological and biochemical methods.* ***Results.*** *It is shown that an oxidative explosion developed at the early stages of interaction between plant and fungi, which resulted in the formation of the reactive oxygen species (ROS) in increased quantities and enhanced lipid peroxidation reaction. Under conditions of pathogenesis, the suspension of Bacillus subtilis 537/B1 bacterial isolates revealed tread effect induced the activity of the antioxidant enzymes (superoxide dismutase, catalase), activity of PR-proteins (lectins), which led to the development of induced resistance.* ***Conclusion.*** *The investigated strain Bacillus subtilis can be considered as promising for create preparations on its basis to increase the resistance of plants to stress biotic nature.*

Keywords: adaptation, Bacillus, bacteria, catalase, induced resistance, lectins, peroxide lipid oxidation, superoxide dismutase, winter wheat.

The loss of crop due to the influence of various biotic and abiotic factors is one of the relevant problems of modern agricultural production. The global trend of reducing the doses of agrochemicals determines the need to increase the use in plant growth of new, additional sources of mineral nutrition and biological means of plant protection. The use of bacteria for seed or plant treatment, in order to increase their yields and disease resistance, considered to be as a related to environmentally friendly food production technologies direction [1]. It has been found that bacteria of the genus *Bacillus* Cohn. are the promising agents for plant protecting from biotic stress [2]. They are also considered as producers of metabolites with hormonal and signaling functions (auxins, cytokinins, gibberellins, abscissae, salicylic and jasmonic acids) [3]. However, the treatment with *Bacillus* in agricultural practice often turns out to be ineffective, because the interaction in the "plant-bacteria" system is not studied sufficiently [4].

The problem of increasing the plant resistance to pathogens is the most important and requires detailed study. The solution of this problem relates not only to the study of the plant physiology and pathogenic agent, but also with their interaction. It will create a more effective system of plant protection.

Thus, the purpose of our research was to study the effect of *Bacillus subtilis* 537/B1 bacterial isolates on seeds and seedlings of wheat, affected by eyespot causal agent.

Materials and methods. Two winter wheat (*Triticum aestivum* L.) varieties with different susceptibility to eyespot causal agent (*Pseudocercosporella herpotrichoides*), i.e.: Myronivska 808 variety – a sensitive to pathogen and Renan variety – a resistant to pathogen were used.

The seedlings were grown on the sand in chemically neutral containers under controlled laboratory conditions (16-h photoperiod, light intensity 15 000 lx, air temperature 25/20 °C (day/night), air humidity 60%); 25–35 ml of the Hoagland-Arnon nutrient solution was added to each container. The moisture level of the substrate was maintained at a constant (70%) level using an additional nutrient solution.

Conidial suspension of fungus *Pseudocercosporella herpotrichoides* (Fron) Deighton was used to the infection. According to the International Catalog of Fungal Nomenclature (Index Fungorum), the current name of this fungus is *Oculimacula yallundae* (Wallwork & Spooner) Crous & W. Gams (2003), an eyespot agent [5].

The eye-shaped elliptical lesions which give eyespot its name form on lower stem bases near to the soil surface. The lesions are straw yellow, often with black pupil-like dots in the centre, and are bordered by greenish-brown to dark-brown rings. In cases of severe infection stems are weakened at the point of infection which makes the host susceptible to lodging. Tiny black microsclerotia are formed on the spots surface by the end of the vegetation. The disease does not appear on the roots. Affected stems ripen early, causing white ears [6].

The fungus conidia were grown in a potato-glucose medium according to generally accepted methods.

Bacillus subtilis 537/B1 bacteria were isolated from Antarctic soil of Galindes Island [7]. Bacteria *Bacillus subtilis* 537/B1 were cultivated at 30°C for 16h (overnight). Cell culture medium was centrifuged at 10.000 g for 5 min and washed twice by 0.85% NaCI. The supernatant was discarded and bacterial cells were resuspended in sterile 0.85% NaCI adjusting to the final concentration 3x108 CFU/mL 10 (OD=0.3).

Live cell suspension of bacterial isolates was applied on seedlings immediately after preparing.

The used experimental variants were the following: 1) control; 2) 7-day-old wheat seedlings infected with a conidia suspension of pathogenic fungi; 3) 7-day-old wheat seedlings inoculated with a suspension of *Bacillus subtilis* 537/B1 bacterial isolates; 4) 7-day-old wheat seedlings infected with a conidia suspension of pathogenic fungi and suspension of *B. subtilis* 537/B1 bacterial isolates; 5) 7-day-old wheat seedlings from seeds inoculated with a suspension of *B. subtilis* 537/B1 bacterial isolates; 6) 7-day-old wheat seedlings from seeds inoculated with suspension of *B. subtilis* 537/B1 bacterial isolates and infected with conidia suspension of pathogenic fungi. The control was sprayed with distilled water.

The high virulent strain *Pseudocercosporella herpotrichoides* was used, which was kindly provided by the V.M. Remeslo Myronivka Institute of wheat National Academy of Agrarian Sciences of Ukraine.

Selection of plant material for biochemical studies was carried out 4, 24, 48 hours after infection.

Lectin-like proteins of cell walls and cell organelles fractions were isolated according to the method described by Lutsyk [8]. Lectin activity was determined by erythro-agglutination assay [9]. It was calculated as reversed value to a minimum protein concentration, which caused the agglutination of rat erythrocytes $(\mu g/ml)^{-1}$:

LA = Titer/Protein concentration

The protein content in the obtained extracts was determined by the method of Bradford spectrophotometrically at 595 nm wave length [10].

The level of generated malonic dialdehyde (MDA) as a product of lipid peroxidation was estimated according to Dhindsa and Matowe [11].

The concentration of MDA was determined in its unit equivalent using a coefficient of molar extinction 155×105 $mM^{-1}cm^{-1}$. The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assessed by inhibition of the nitroblue tetrazolium photoreduction spectrophotometrically at 560 nm wavelength [12]. Catalase (CAT, EC 1.11.1.6) activity was measured by determining the rate of H_2O_2 decomposition during 1 min, spectrophotometrically at 240 nm wave length [13].

Each experiment was performed in triplicate. The data were subjected to analysis of variance (ANOVA) with subsequent Student's t-test or Duncan's multiple range test at $P<0.05$. Data are expressed as means of replicates ± standard deviation.

Results. The reactive oxygen species (ROS) production is one of the first plant reactions to stress factors which can be the mediators of the signal transmission that contribute to the formation of plant resistance. The displacement of equilibrium to the pro-oxidants direction is the informative indicator for assessing the degree of stress factors influence to the organism [14]. The analysis of the obtained results showed the oxidative stress development in wheat seedlings as well as in wheat seedlings from seeds infected with a conidia suspension of pathogenic fungi and also with the suspension of *Bacillus subtilis* 537/B1 bacterial isolates. This is confirmed by the enhancement of the intensity of lipid peroxidation (LPO) processes characterized by the level of generated malonic dialdehyde (MDA). The genetic features of each variety and the duration of exposure greatly affect the intensity of lipid peroxidation. The MDA content in photosynthetic tissues was no significant difference with the control in winter wheat seedling of Myronivska 808 variety in 4 hours after inoculated with the suspension of *B. subtilis* 537/B1 bacterial isolates. (Fig. 1a).

But in winter wheat seedling from seeds inoculated with the suspension of *B. subtilis* 537/B1 bacterial isolates, the MDA content slightly increased (by 6%), in wheat seedlings from seeds inoculated with the suspension of *B. subtilis* 537/B1 bacterial isolates and infected with the conidia suspension of pathogenic fungi this parameter significantly decreased (by 23%). A tendency

to decrease of MDA content in all experimental variants was observed after 24 hours of exposure (Fig. 1b).

It should be admitted a significant reduction of MDA content in variants of seedlings and seed treatment with suspension of bacterial isolates (by 14% and 20% respectively). Exposure prolongation to 48 hours conducted the fluctuations in the MDA content (Fig. 1c).

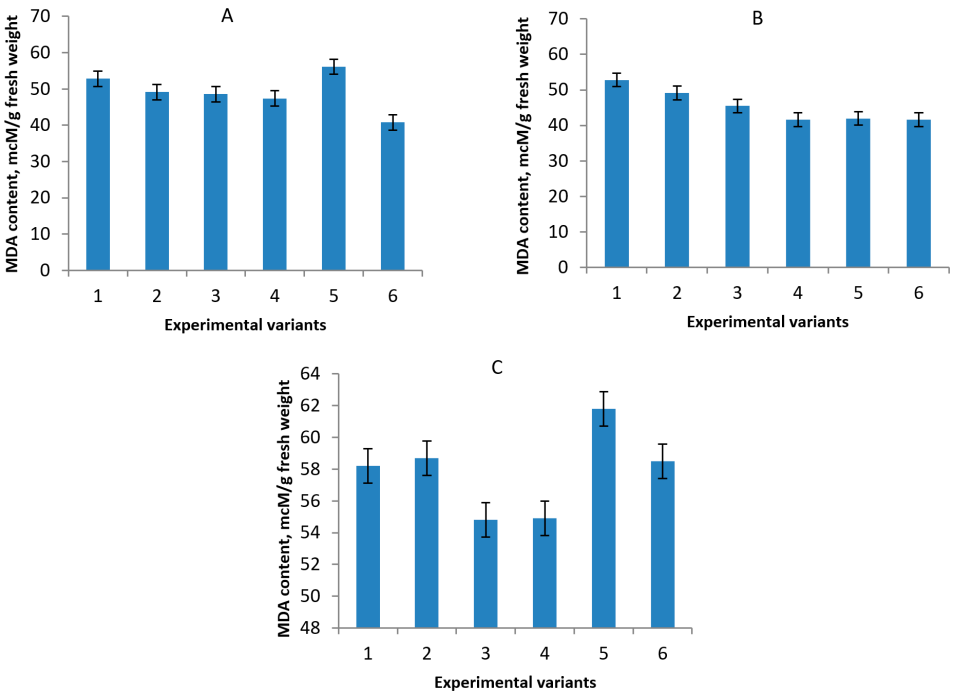

Fig. 1. MDA content in winter wheat seedlings of Myronivska 808 variety
Abbreviations: a – after 4 hours, b – after 24 hours, c – after 48 hours of exposure: 1) control; 2) 7-day-old wheat seedlings infected with a conidia suspension of pathogenic fungi; 3) 7-day-old wheat seedlings inoculated with a suspension of *B. subtilis* 537/B1 bacterial isolates; 4) 7-day-old wheat seedlings infected with a conidia suspension of pathogenic fungi and suspension of *B. subtilis* 537/B1 bacterial isolates; 5) 7-day-old wheat seedlings from seeds inoculated with a suspension of *B. subtilis* 537/B1 bacterial isolates; 6) 7-day-old wheat seedlings from seeds inoculated with suspension of *B. subtilis* 537/B1 bacterial isolates and infected with the conidia suspension of pathogenic fungi.

Treatment with the suspension of *B. subtilis* 537/B1 bacterial isolates and treatment with a conidia suspension of pathogenic fungi and suspension of bacterial isolates *B. subtilis* 537/B1 caused the reduction of the LPO intensity in contrast of the control over 48 hours of exposure.

Winter wheat seedlings of Renan variety had a lower basal level of MDA. This result characterizes Renan as resistant variety. Response reactions developed from the 4th hour of exposure (Fig. 2a). MDA content decreased compare with the control variant in seedlings and seedlings from seeds treated with a suspension of *B. subtilis* 537/B1 bacterial isolates. Inoculation of infected seedlings and seeds with the suspension of *B. subtilis* 537/B1 bacterial

isolates caused reduction of MDA content in photosynthetic tissues. Oxidation processes developed more intensively in the variants infected with the *P. herpotrichoides* after 24 hours of exposure (Fig. 2b).

The protective effect of *Bacillus subtilis* 537/B1 bacterial isolates under *P. herpotrichoides* infection in seedlings and seedlings from seeds inoculated with *Bacillus subtilis* 537/B1 was more clearly at 48 hours of exposure. MDA content in these variants had been manifested in lower or at the level of control values (Fig. 2c).

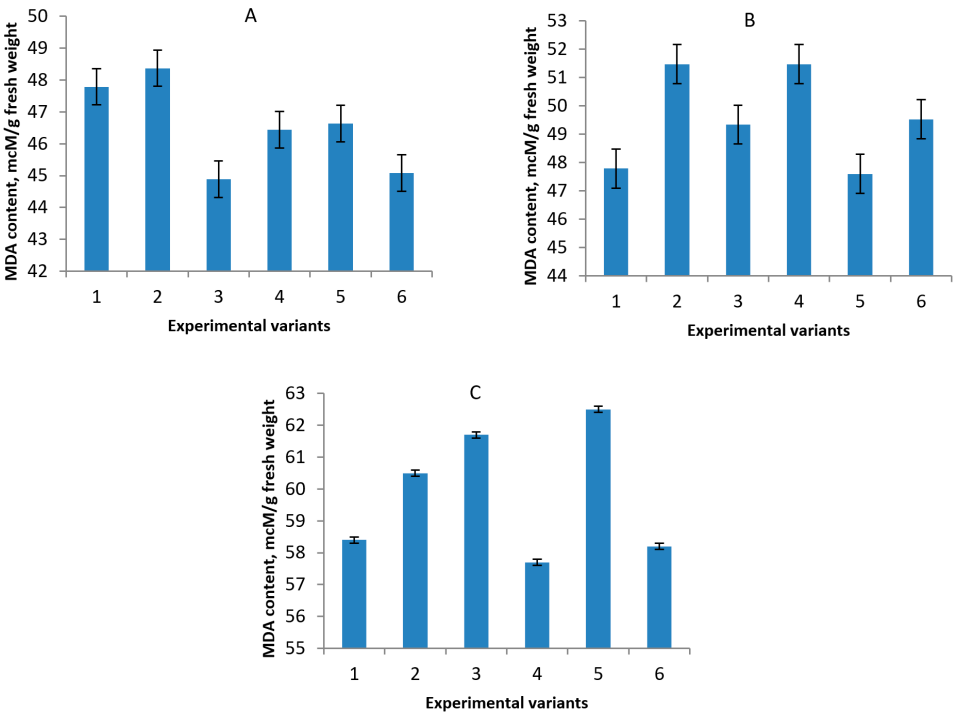

Fig. 2. MDA content in winter wheat seedlings of Renan variety
Abbreviations as in Fig. 1.

Consequently, the activation of LPO processes under treatment with bacterial preparations related to the fact that interaction between plant and fungi in early stages characterized by oxidative burst with ROS formation in increased quantities.

Enzyme complex is involved in the ROS content regulation. The key enzyme in this process is SOD – the only antioxidant enzyme that provides the chains breakdown of oxygen-dependent free radical reactions of the aerobic organism's cells [15].

CuZn-SOD is attached to the thylakoid membrane stromal surface. CuZn-SOD is resistant to denaturation and can withstand temperatures to 80° C. Determination of SOD activity in mesophilic cells showed that treatment of seedlings and seeds of Myronivska 808 variety with *B. subtilis* 537/B1 bacterial isolates contributed the SOD activation after 48 hours of exposure (Fig. 3a).

A maximum increase of the SOD activity was observed in the variant of seedlings infected with a conidia suspension of pathogenic fungi (5 times).

SOD activity increased by 2 times in seedlings infected with a conidia suspension of pathogenic fungi and suspension of *B. subtilis* 537/B1 bacterial isolates, in seedlings from seeds inoculated with the suspension of *B. subtilis* 537/B1 bacterial isolates and infected with the conidia suspension of pathogenic fungi – by 2.4 times.

SOD activity of the Renan variety seedlings was no difference with the control in most experimental variants. The activity of SOD was lower in variants of seedlings from seeds inoculated with a suspension of *B. subtilis* 537/B1 bacterial isolates and seedlings infected with a conidia suspension of pathogenic fungi and suspension of *B. subtilis* 537/B1 bacterial isolates (Fig. 3b).

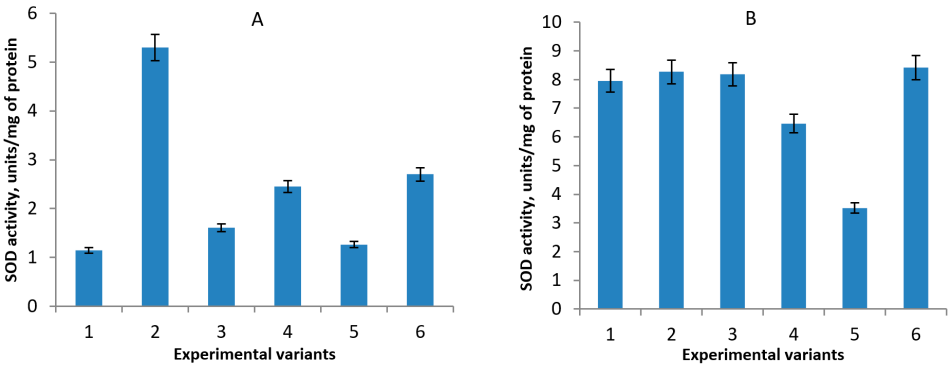

Fig. 3. SOD activity in winter wheat seedlings after 48 hours of exposure: a – Myronivska 808 variety, b – Renan variety
Abbreviations as in Fig. 1.

Functioning of the antioxidant enzymes complex provides the utilization of excessive amount of ROS under stress factors conditions. Catalase plays an important role in maintaining the physiological level of H2O2. This enzyme functions as a reducing agent. Heme-containing catalase reacts with H2O2 with a constant of 107 M-1s-1 [16]. Catalase activity slightly reduction after 48 hours of exposure of the Myronivska 808 variety seedlings infected with a conidia suspension of pathogenic fungi, wheat seedlings infected with a conidia suspension of pathogenic fungi and suspension of *B. subtilis* 537/B1 bacterial isolates and seedlings from seeds inoculated with a suspension of *B. subtilis* 537/B1 bacterial isolates may be related to the enzyme substrate depletion (Fig. 4a).

The increase of catalase activity or no difference with the control was observed after 48 hours of exposure (after inoculation with bacterial suspension) for Renan variety. But the activity of catalase decreased by 31% in seedlings infected with a conidia suspension of pathogenic fungi (Fig. 4b).

The changes of enzymes activity that dismutate the H_2O_2 formed as a result of plant infections are adaptive since this is the first response to the oxidative burst.

Consequently, a suspension of *Bacillus sp.* bacterial isolates showed protective effect and induced the activity of ROS utilizers under pathogenesis.

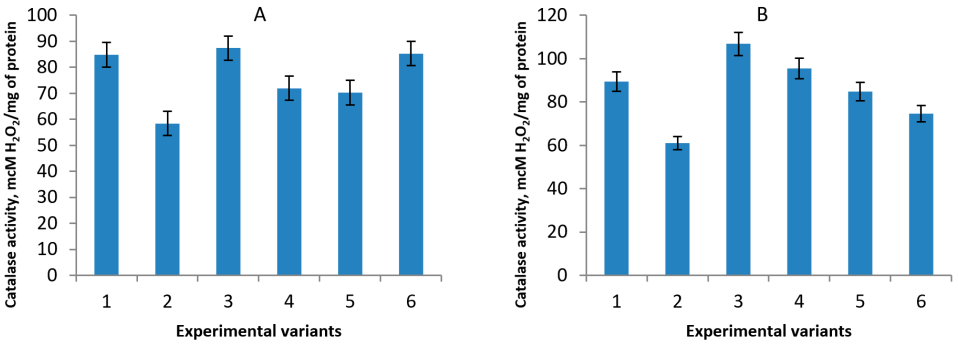

Fig. 4. Catalase activity in winter wheat seedlings after 48 hours of exposure: a – Myronivska 808 variety, b – Renan variety
Abbreviations as in Fig. 1.

It should be noted that *B. subtilis* 537/B1 bacterial isolates inhibit the development of various diseases in plants not only through the synthesis of various anti-fungal metabolites but also indirectly through the mechanism of the induced-resistance system [2]. It is regulated by produced hormones such as salicylic acid, abscisic acid, jasmonic acid, ethylene [17] and by cyclic lipopeptides [18].

The pathogen-induced systemic acquired resistance is accompanied by a coordinated activation of pathogenesis-related genes (PRs, from pathogenesis-related). These genes encode proteins with antimicrobial activity [19] and controlled by the redox-regulated protein NPR1 (from non expressor of PR genes 1), which is activated by salicylic acid, is its receptor [2] and functions as a transcriptional co-activator of a large number of PR genes [17].

Chitin-binding proteins (PR-4 group) belong to a large group of proteins – the lectins that are the PR-proteins. Lectins are proteins that can selectively bind to polysaccharides, glycoproteins and glycolipids without causing their chemical transformation [20]. The increase of lectin activity compared to control was observed in all variants of the experiment for the Myronivska 808 variety (Fig. 5a).

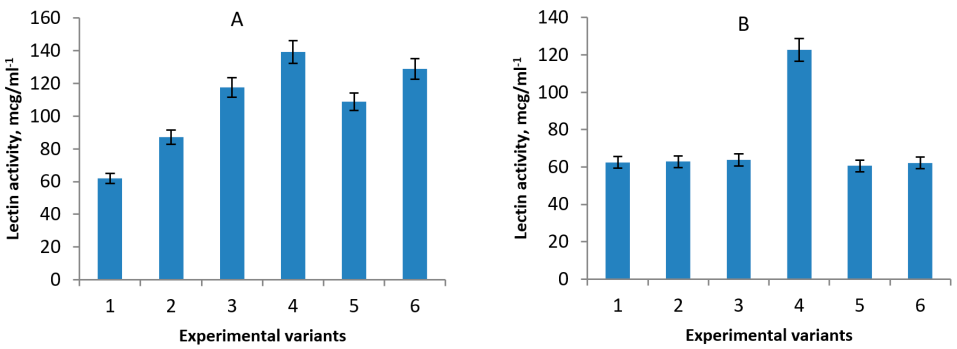

Fig. 5. Lectin activity in winter wheat seedling after 48 hours of exposure: a – Myronivska 808 variety, b – Renan variety
Abbreviations as in Fig. 1.

Lectin activity of the Renan varieties was no significant difference with the control after 48 hours of suspension inoculation. But lectin activity increased significantly (by 2 times) in the seedlings infected with a conidia suspension of pathogenic fungi and suspension of *B. subtilis* 537/B1 bacterial isolates (Fig. 5b).

Discussion. The using of *B. subtilis* 537/B1 bacterial isolates to plant protection, firstly, usually associated with their competition with pathogenic microflora for nutrients and colonization niche [21]. Secondly, it is associated with the synthesis of various metabolites with antibiotic activity – antibiotics, biosurfactants, siderophores, hydrogen cyanide, etc. Thirdly, it is related to the synthesis of hydrolytic enzymes such as chitinase, glucanase, proteases, and lipase. They can destroy pathogenic fungi cells and a number of pathogen effectors compounds [22]. Fourthly, it is associated with elicitor activity and the initiation of the induced and acquired systemic resistance [23]. These processes are carried out with the help of bacterial determinants (MAMPs - microbe-associated molecular pattern) such as flagellin, lipopolysaccharides (LPS), siderophores, antibiotics, biosurfactants and volatile organic compounds [24].

The results analysis suggests that the distinctive feature of induced systemic resistance, mediated with the *B. subtilis* 537/B1 bacterial isolates, is the resistance development under the mechanism of sensitization, which is called priming [25]. The phenomenon of priming with bacterial agents is the increase of plant cells sensitivity to the foreign substances influence and is characterized by faster and more powerful activation of cellular mechanisms of plant protection to pathogen attacks. It can last for a long time, which conducts to increase plants resistance. Induced systemic resistance develops by priming mechanism and is observed at different stages of plant-pathogen interaction. It begins with early responses, which are controlled by the hormonal system. It is activated by various signaling and protective proteins. The process ends with the long-term response reactions that involve the controlled chromatin changes and DNA methylation. Protective reactions that developed from induced systemic resistance under the action of bacterial isolates are characterized by rapid and early ROS accumulation (including H_2O_2) which activates the redox-sensitive transcription factors and PR-protein genes [26] and regulates the interaction of salicylic, jasmonic and ethylene signaling pathways. The induced systemic resistance mechanism can also be associated with the barrier formation to penetrate the pathogen by the callose deposition, strengthening of plants cell walls and the production of metabolites with antimicrobial activity (phytoalexins) [27].

ROS generation in plants that developed from induced systemic resistance under the action of bacterial isolates can play a critical role in the priming effect formation. Early ROS accumulation was observed in seedlings inoculated with the suspension of bacterial isolates after infection with a conidia suspension of pathogenic fungi. It is assumed this is due to their ability to produce the determinants [2].

ROS generation related to the expression of protective genes could be associated with the protective effect of lipopolysaccharides [2]. It has been found a direct correlation between H_2O_2 generation and a surfactin concentration of different strains of *B. subtilis* 537/B1 which inoculate the

tobacco cell suspensions [28]. The role of elicitors can also be performed by phytohormones at systemic resistance [2]. ROS also can activate transcription factors and regulate ROS-sensitive genes through them [29].

Investigation of plant transcriptome showed the transcription factors after inoculation of *B. subtilis* 537/B1 accumulate in plants and remain to be inactive until the pathogen infection. But they are supposed to give the plant ability to faster reaction to the pathogen attack. Hence, they provide a priming effect [2].

Conclusion. Consequently, our research suggests the feasibility of using *B.subtilis* 537/B1 bacterial isolates as promising and effective tools for the activation of plant protective reactions under biotic stressor. It might be a key to the next stage of "green revolution".

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АДАПТИВНІ РЕАКЦІЇ ОЗИМОЇ ПШЕНИЦІ (*TRITICUM AESTIVUM* L.), УРАЖЕНОЇ ЗБУДНИКОМ ОЧКОВОЇ ПЛЯМИСТОСТІ, ЗА ДІЇ БАКТЕРІАЛЬНИХ ІЗОЛЯТІВ *BACILLUS SUBTILIS*

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Резюме

Ціль. Дослідити вплив бактеріальних ізолятів *Bacillus subtilis* 537/Б1 на зміни лектинової активності та вмісту малонового диальдегіду (МДА), супероксиддисмутази (СОД) та каталази у проростках озимої пшениці різних за стійкістю сортів – Миронівська 808 та Renan, інфікованих збудником очкової плямистості *Pseudocercosporella herpotrichoides* (Fron) Deighton. **Методи**. Роботу виконано з використанням мікробіологічних, імунологічних та біохімічних методів. **Результати.** Показано, що на ранніх стадіях взаємодії рослини та гриба розвивався оксидний вибух, що призвело до утворення активних форм кисню (АФК) у підвищених кількостях та розвитку реакції перекисного окиснення ліпідів. В умовах патогенезу суспензія бактеріальних ізолятів *Bacillus subtilis* 537/Б1 виявила ефект протектора, що індукував активність антиоксидантних ферментів (супероксиддисмутази, каталази), а також активність PR-білків (лектинів), що призвело до розвитку індукованої стійкості. **Висновок**. Досліджуваний штам *Bacillus subtilis* є перспективним для створення на його основі препаратів для підвищення стійкості рослин до стресів біотичної природи.

Ключові слова: адаптація, *Bacillus*, бактерії, каталаза, індукована стійкість, лектини, перекисне окиснення ліпідів, супероксиддисмутаза, озима пшениця.

АДАПТИВНЫЕ РЕАКЦИИ ОЗИМОЙ ПШЕНИЦЫ (*TRITICUM AESTIVUM* L.), ПОРАЖЕННЫХ ВОЗБУДИТЕЛЕМ ОЧКОВОЙ ПЯТНИСТОСТИ, ПРИ ДЕЙСТВИИ БАКТЕРИАЛЬНЫХ ИЗОЛЯТОВ *BACILLUS SUBTILIS*

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Резюме

Цель. Исследовать влияние бактериальных изолятов *Bacillus subtilis* 537/Б1 на изменения лектиновой активности и содержания малонового диальдегида (МДА), супероксиддисмутазы (СОД) и каталазы в проростках озимой пшеницы разных по устойчивости сортов – Мироновская 808 и Renan, инфицированных возбудителем очковой пятнистости *Pseudocercosporella herpotrichoides* (Fron) Deighton. **Методы.** Работа выполнена с использованием микробиологических, иммунологических и биохимических методов. **Результаты.** Показано, что на ранних стадиях взаимодействия растения и гриба развивался оксидный взрыв, что привело к образованию активных форм кислорода (АФК) в повышенных количествах и развитию реакции перекисного окисления липидов. В условиях патогенеза суспензия бактериальных изолятов *Bacillus subtilis* 537/Б1 проявила эффект протектора, который индуцировал активность антиоксидантных ферментов (супероксиддисмутазы, каталазы), а также активность PR-белков (лектинов), что привело к развитию индуцированной устойчивости. **Вывод.** Исследуемый штамм *Bacillus subtilis* является перспективным для создания на его основе препаратов для повышения устойчивости растений к стрессам биотической природы.

Ключевые слова: адаптация, *Bacillus*, бактерии, каталаза, индуцированная устойчивость, лектины, перекисное окисление липидов, супероксиддисмутаза, озимая пшеница.

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