

**INFLUENCE OF *BACILLUS AMYLOLIQUEFACIENS* SUBSP.
PLANTARUM IMV B-7404 STRAIN EXOMETABOLITES
ON PHENYLALANINE AMMONIA-LYASE ACTIVITY
IN WINTER WHEAT SEEDLINGS**

I. V. DRAGOVOZ, Yu. V. KORZH, N. O. LEONOVA,
V. M. ILIASH, L. V. AVDEEVA

*Zabolotny Institute of Microbiology and Virology,
National Academy of Sciences of Ukraine, Kyiv;
e-mail: igordragovoz@ukr.net*

Influence of Bacillus amyloliquefaciens subsp. plantarum IMV B-7404 strain exometabolites on phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) activity in winter wheat seedlings has been studied. A significant increase of PAL activity at 4-6 hours after treatment of plant roots with Bacillus amyloliquefaciens subsp. plantarum IMV B-7404 strain exometabolites and in case of leaves infection with Bipolaris sorokiniana plant pathogen has been shown. It was established that PAL activity changed along with a decrease of the infected surface area of the leaves evidenced for the induction of response in winter wheat seedlings induced by IMV B-7404 strain exometabolites. It was concluded that the studied exponents could be used as model systems in the research of phytoimmunity induction mechanisms.

Key words: Bacillus amyloliquefaciens subsp. plantarum IMB B-7404, exometabolites, winter wheat, Bipolaris sorokiniana, phenylalanine ammonia-lyase, phytoimmunity induction.

Aerobic spore-forming bacteria of *Bacillus* genus are considered as perspective agents of biological control, since they are antagonists to numerous phytopathogenic fungi and bacteria owing to synthesis of a broad range of secondary metabolites with expressed bactericidal and fungicidal properties [1]. One should note not only antibiotic substances among *Bacillus* genus bacteria exometabolites but also compounds characterized by phytostimulating and immunomodulating activity. The latter, in its turn, determines the expediency of using the *Bacillus* strains when creating efficient biological preparations of broad-range effect for plant growing needs [2].

Bacillus amyloliquefaciens subsp. *plantarum* IMB B-7404 strain with antifungal activity as to phytopathogens *Fusarium graminearum* and *Bipolaris sorokiniana* [3] was selected by the results of screening conducted by the authors. The nature of IMB B-7404 strain exometabolites, which determines its phytostimulating and antagonistic activity [4, 5], was studied. Using MALDI-TOF mass-spectrometry and molecular genetic methods of analysis, it was also established that lipopeptide antibiotic exometabolites of the studied strain belonged to the family of fengicins [6].

Phytoimmunity development is a complex biochemical process induced by stress factors of biotic and abiotic nature. It is known that activation of phenol metabolism and, in particular, an increase of activity of the basic anti-stress enzymes – phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) and peroxidase is one of defense responses of plants to the effect of stressors. The enzyme PAL is one of recognized biochemical markers of the induced plant resistance both to diseases caused by phytopathogens and to the effect of various stressor factors of biotic and abiotic nature. PAL performs deamination of phenylalanine with formation of *trans*-cinnamic acid and ammonia, takes part in synthesis of salicylic acid precursors, phytoalexins, lignin monomers, which strengthen mechanical and chemical barriers of plant cells and thus prevent their infection by phytopathogenic fungi [7, 8]. PAL activity can change depending on the phase of plant development, its genotype and external conditions [9]. The work [10], where the enzyme activity was depressed by specific inhibitors, evidences for the important role of PAL in phytoimmunity induction. Under treatment by these inhibitors, the pathogenic lesion increased even in genetically resistant plants, as a result of the cessation of forming phenols and lignin precursors.

It is also known that phenols, especially in oxidized form, are toxic for phytopathogenic microorganisms, and thus they can limit their penetration to plant cells [11].

The work's purpose was to study the change of PAL activity in winter wheat seedlings, during the formation of a response to the leaf disease caused by *B. sorokiniana* under the effect of *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 strain exometabolites.

Materials and Methods

The research object was *B. amyloliquefaciens* subsp. *plantarum* IMB B-7404 strain kept in the depository of Zabolotny IMV of NAS of Ukraine. The fungal strain *B. sorokiniana* from the collection of the Department of Antibiotics of IMV of NAS of Ukraine was also used in the work. Micromycete *B. sorokiniana* is an agent of brown spot disease (helminthosporiosis) of cereals. The experiments were performed in the model system created on the basis of Smuglyanka variety of winter wheat seedlings treated with *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 strain exometabolites and/or infected with micromycete *B. sorokiniana*.

The strain IMV B-7404 was cultivated in periodic conditions presented in the works [3, 4]. The wheat seedling leaves were infected with the agent of *B. sorokiniana* by the method described in the work [12]. Investigation of the influence efficiency of *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 strain exometabolites as to brown spot leaves disease of Smuglyanka variety of winter wheat was performed under condition of a single introduction of cell-free cultural liquid (3-5 ml per 50 ml vial). In 2, 4, 6 and 8 hours after plants treatment with exometabolites or phytopathogen and at the end of cultivation (the 7th day after treatment with pathogen) the winter wheat roots and seedlings were fixed in liquid nitrogen and kept at -24 °C for further analysis.

Buffer extracts from the winter wheat roots and seedlings were used as the source of enzymes. Protein content was determined by Bradford method [13].

PAL activity was estimated by spectrophotometric method at 290 nm under formation of *trans*-cinnamic acid [14].

The disease development was evaluated visually by the extent of the lesion of plant seedlings (0-4 points) after symptoms appearance on the 4-7th day after the infection [15].

The obtained results were processed statistically (mean values, mean value errors, root-mean-square deviations for $n = 5$ under the significance level $P = 0.95$) and analyzed using a computer program packet STATISTICA 6.0 and Microsoft Excel.

Results and Discussion

The winter wheat seedlings were treated from the first leaf appearance (stage 11 by Zadoks scale) with supernatant fluid of *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 containing exometabolites including antibiotic compounds of lipopeptide nature, by watering of the sand. The data obtained evidenced that the basic (control) level of the enzyme activity in roots was 5 times higher than in the above-ground part of plants (Fig. 1). Such a high level of PAL activity in the wheat roots evidences for the important barrier function of roots in the early phases of ontogeny; they serve for delivering nutritional elements and microbe exometabolites, which include antibiotic compounds, phytohormones, etc., from the soil.

It is known that PAL activity in the plant cells can change considerably during some hours under the effect of different environmental stressor factors [8, 16]. The results obtained evidence for the considerable increase of PAL activity both in the roots and above-ground part at the 4-6th hour after treatment with exometabolites of the studied strain. It is possible that such a character of PAL activity increase (displaced by 2 hours) during several hours after seedlings watering may evidence for the development of the winter wheat seedlings response caused by the treatment with extracellular metabolites of *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 strain. A quick change of the enzyme activity may be connected with synthesis of phenol compounds, which takes part in formation of defense mechanisms of plant organism in response to the environmental stressor factor (influence of phytopathogenic fungus).

PAL activity after treatment with pathogen conidia after the appearance of the second leaf (stage 12 by Zadoks scale) evidences for the same regularities noted under plants treatment with cultural fluid of *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 (Fig. 2). Thus, the increase of PAL activity at the 4th hour after pathogen treatment of control plants has been established, the enzyme activation being simultaneously observed both in the above-ground part and in roots. Thus, no difference was noted in time between the enzyme activation in the

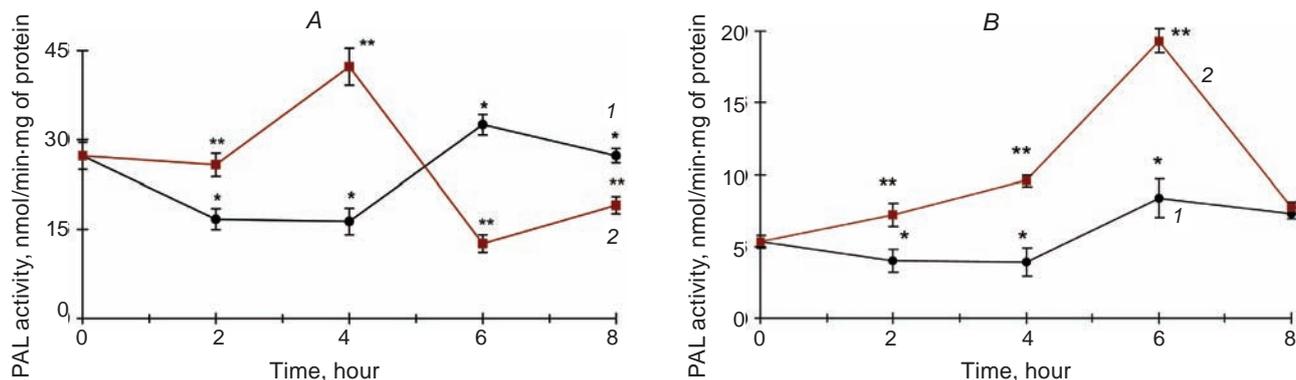


Fig. 1. Change of PAL activity in roots (A) and seedlings (B) of the winter wheat under the effect of *B. amylo-liquefaciens* subsp. *plantarum* IMV B-7404 exometabolites: 1 – control; 2 – exometabolites ($M \pm m$, $n = 5$). Difference between indices of curve 1 (*) and curve 2 (**) is probable at $P < 0.05$

leaves and roots. It is probable that the plant signal of infection by pathogen (the elicitor action) is comparatively quickly distributed to different organs; this determines almost simultaneous inclusion of response mechanisms. The obtained results confirm this supposition.

It was of interest that the level of PAL activity remained at the level of control in seedlings treated preliminarily with exometabolites of the strain IMV B-7404 after the leaves were infected with phytopathogen. It is known that the catalytic potential of PAL in many plants (including monocotyledonous) is much higher than it is required for synthesis of polyphenols [17]. That is why even short-term activation of PAL is sufficient for accumulating a necessary pool of phenol compounds in seedlings [18]. On the other hand, phenol compounds, espe-

cially their monomeric forms, can inhibit PAL activity [19], which also occurs under treatment of the winter wheat leaves with conidia of *B. sorokiniana*. Thus, the preliminary treatment of seedlings with the strain exometabolites and their further infection with phytopathogen did not change PAL activity. The results obtained evidence for differentiated (different) change of PAL activity during accumulation of phenol compounds and lignin under the effect of the strain exometabolites or phytopathogen. The data concerning the change of PAL activity, total content of phenols and lignin under the effect of salicylic acid, depending on different barley genotypes' resistance to fusariose, count in favor of such a supposition [20].

The use of the model systems “plant-phytopathogen”: tomatoes – *Fusarium*, beans –

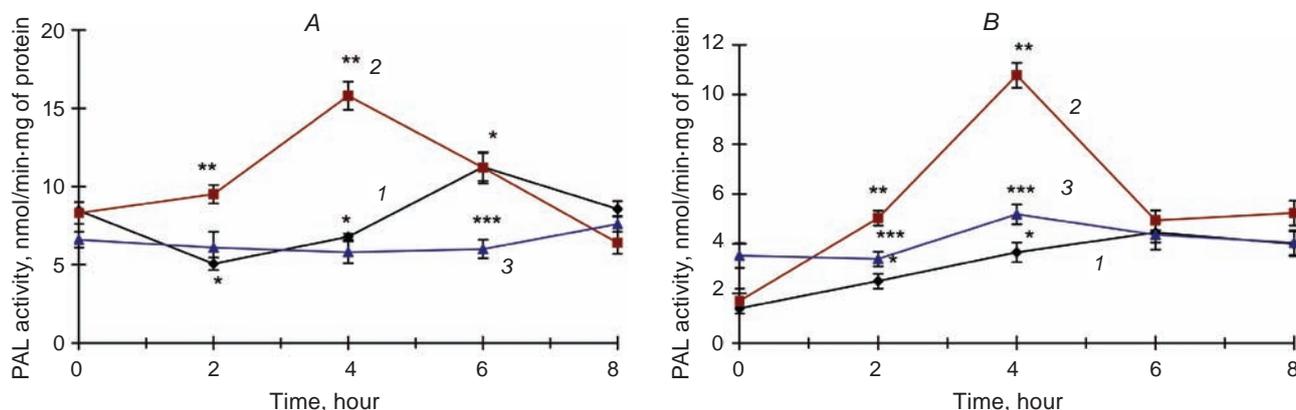


Fig. 2. Change of PAL activity in the roots (A) and seedlings (B) of the winter wheat under the effect of *B. amylo-liquefaciens* subsp. *plantarum* IMV B-7404 exometabolites and infection with *B. sorokiniana*: 1 – control (water); 2 – phytopathogen conidia; 3 – exometabolites and phytopathogen conidia ($M \pm m$, $n = 5$). Difference between indicators of curve 1 (*), curve 2 (**) and curve 3 (***) is probable under $P < 0.05$

Disease development, biological efficiency of B. amyloliquefaciens subsp. plantarum IMV B-7404 exometabolites and PAL activity under infection of the wheat seedlings with B. sorokiniana

Experiment variants	Disease development, points (0-4)*		Biological efficiency, % **		PAL activity, nmol/min·mg of protein*	
	on the 4 th day	on the 7 th day	on the 4 th day	on the 7 th day	roots	seedlings
Control (water)	2.2 ± 0.6	1.6 ± 0.3	–	–	81.5 ± 5.7	5.3 ± 0.2
<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> IMV B-7404 exometabolites	1.2 ± 0.3	1.2 ± 0.2	45.0	20.0	21.0 ± 1.0	7.5 ± 0.3

* PAL activity and disease development level were determined on the 7th day after the plant treatment with *B. sorokiniana* pathogen. ** Difference between the disease development in the control and research variants (presented in percents).

Botrytis, sugar beet – *Cercospora* [21] is the commonly accepted approach to studying the induction of protective responses in plants by certain bacilli strains. In the subsequent work we used the model system “wheat – *B. sorokiniana*” modified from the pathosystem “rice – *Cochliobolus miyabeanus*” [12] to investigate a possible mechanism of the effect of extracellular secondary metabolites of the *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 strain.

The results of investigations conducted on the model system “wheat – phytopathogenic fungus (*B. sorokiniana*)”, have shown a credible decrease of the disease development under the effect of a complex of metabolites of the strain IMV B-7404 (Table). So, when the wheat roots were treated with the cell-free cultural liquid of the studied strain and further infection of leaves with phytopathogenic microorganism, the real decrease of the infected leaf area was observed after 4-7 days. As this took place, the degree of the disease development and, correspondingly, the biological efficiency of the treatment with exometabolites was at the level of 20-45% (Table). Therewith, PAL activity was essentially different both by its level in the winter wheat seedlings and roots, and by the character (stimulation or inhibition) of activity compared with the control variant. That evidences for the complex and mediated system of regulation of phenylpropanoid way of biosynthesis of phenol compounds.

Thus, the considerable increase of PAL activity in the above-ground part and roots first proceeded under the effect of *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404 exometabolites and further infection of wheat seedlings with brown spot disease

agent *B. sorokiniana*. Then a credible decrease of the leaf surface of the seedlings affected by phytopathogen could be observed. The change of PAL activity along with a decrease of the surface area of winter wheat leaves affected by phytopathogenic fungus evidence for the response induction caused by exometabolites of the studied bacilli strain. The change of PAL activity and decrease of the affected winter wheat leaves’ surface area may be used as indicators in model systems when investigating modulator preparations – modulators of phytoimmunity and the mechanisms of its induction.

ВПЛИВ ЕКЗОМЕТАБОЛІТІВ ШТАМУ *BACILLUS AMYLOLIQUEFACIENS* SUBSP. *PLANTARUM* ІМВ В-7404 НА АКТИВНІСТЬ ФЕНІЛАЛАНІН-АМОНІЙ-ЛІАЗИ В ПРОРОСТКАХ ОЗИМОЇ ПШЕНИЦІ

I. V. Драговоз, Ю. В. Корж, Н. О. Леонова, В. М. Іляш, Л. В. Авдєєва

Інститут мікробіології і вірусології
ім. Д. К. Заболотного НАН України, Київ;
e-mail: igordragovoz@ukr.net

Досліджено вплив екзометаболітів штаму *Bacillus amyloliquefaciens* subsp. *plantarum* ІМВ В-7404 на активність фенілаланін-амоній-ліази (ФАЛ, 4.3.1.24) в проростках озимої пшениці. Показано значне підвищення активності ФАЛ через 4–6 год після обробки екзометаболітами штаму ІМВ В-7404 коренів рослин та у разі інфікування листків фітопатогеном *Bipolaris sorokiniana*. Встановлено, що зміна активності ФАЛ поряд зі зменшенням площі ураженої

грибним патогеном поверхні листків свідчать про індукцію системної стійкості у проростків озимої пшениці, зумовлену екзометаболітами штаму. Зроблено висновок, що ці показники можуть бути використані як модельні системи при вивченні механізмів активації фітоімунітету.

Ключові слова: *B. amyloliquefaciens* subsp. *plantarum* IMB B-7404, екзометаболіти, проростки озимої пшениці, *Bipolaris sorokiniana*, фенілаланін-амоній-ліаза, індукція фітоімунітету.

ВЛИЯНИЕ ЭКЗОМЕТАБОЛИТОВ ШТАММА *BACILLUS AMYLOLIQUEFACIENS* SUBSP. *PLANTARUM* ИМВ В-7404 НА АКТИВНОСТЬ ФЕНИЛАЛАНИН-АММОНИЙ-ЛИАЗЫ В ПРОРОСТКАХ ОЗИМОЙ ПШЕНИЦЫ

И. В. Драговоз, Ю. В. Корж, Н. О. Леонова, В. М. Иляш, Л. В. Авдеева

Институт микробиологии и вирусологии
им. Д. К. Заболотного НАН Украины, Киев;
e-mail: igordragovoz@ukr.net

Исследовано влияние экзометаболитов штамма *Bacillus amyloliquefaciens* subsp. *plantarum* IMB B-7404 на активность фенілаланін-аммоній-ліази (ФАЛ, 4.3.1.24) в проростках озимої пшениці. Показано значительное повышение активности ФАЛ через 4–6 ч после обработки экзометаболитами штамма IMB B-7404 корней растений и при инфицировании листьев фитопатогеном *Bipolaris sorokiniana*. Установлено, что изменение активности ФАЛ наряду с уменьшением площади пораженной грибным патогеном поверхности листьев свидетельствуют об индукции ответной реакции у проростков озимої пшениці, вызванной экзометаболитами штамма. Сделан вывод, что эти показатели могут быть использованы в качестве модельных систем при изучении механизмов активации фитоимунитета.

Ключевые слова: *Bacillus amyloliquefaciens* subsp. *plantarum*, екзометаболіти, озимая пшеница, *Bipolaris sorokiniana*, фенілаланін-аммоній-ліаза, індукція фітоімунітету.

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