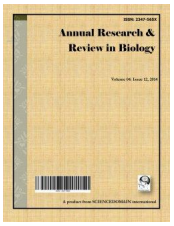

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The Mechanism of Action of Rhizobacteria *Enterobacter nimipressuralis* 32-3 on the Mineral Nutrition and Productivity of Soybean

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Authors' contributions

This work was carried out in collaboration between both authors. Author LAC designed the study, managed the analyses of the study, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Author MIB managed the analyses of the study, wrote the protocol, performed the statistical analysis and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The aim of the research was to study the mechanism of action of soil rhizobacteria *E. nimipressuralis* 32-3 on the elements of mineral nutrition of soybean (content of N and P_2O_5 in shoot, productivity of plants), qualitative and quantitative content of phytohormones in cultural liquid of this strain. Pot experiments were carried out in greenhouse during two seasons (2014 and 2015) to evaluate the influence of the strain *Enterobacter nimipressuralis 32-3* on the growth, productivity of soybean (cultivar Krepysh) and accumulation P_2O_5, total N in shoot. The results revealed that pre sowing seed inoculated by *E. nimipressuralis 32-3* increased the content of N and P_2O_5 in shoot of soybean by 28-46% and 12-14% respectively compared to the control (against control accordingly). It was established that improvement of mineral nutrition of plants with inoculation contributed to increasing their productivity and height. So, biomass of dry shoot soybean increased by 30% and 14% against control, amount of beans on the plants with inoculation exceeded control dates by 64-

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70%. It was showed that the height of plants with inoculation increased by 15-17% compared to the control. The determination of quantitative (methods of thin-layer chromatography) compound of phytohormones, which are produced by *E. nimipressuralis 32-3* was carried out in the experiments. It has been established that in the cultural liquid of this strain there are physiologically active substances of phytohormones: auxin, gibberellins and cytokinin line.

Keywords: Enterobacter nimipressuralis 32-3; phytohormones' soybean; productivity.

1. INTRODUCTION

The main aspect of contemporary biological Agriculture is the use of microorganisms with complex useful properties needed by plants. The creation of microbial preparation is ecologically safe and effective for growth of agricultural plants. It is known that inoculation increases the growth processes of plants and their productivity [1-4]. Such Biopreparations as Rhizobophyt, Diazophyt and others were created in the department of microbiology of our Institution [5]. As is well known, the production of phytohormones is one of the most important properties of symbiotic, rhizosphere and epiphytic bacteria, which stimulate and improve plant growth and development. They may belong to different classes: auxins, gibberellins and cytokinins [6]. It was established that 42% - 97% of bacteria strains has the ability to produce phytohormones of auxin nature (in particular, β-indolyl-3-acetic acid: IAA). The producers of phytohormones were revealed among such bacteria species as *Azospirilllum, Azotobacter, Fgrobacterium, Enterobacter, Klebsiella, Pseudomonas, Clostridium, Bacillus* [7-9]. Moreover, many free-living microorganisms, which are not associated with the plants during their own life, have the ability to synthesis of phytohormones [10]. So, approximately 80 species of soil bacteria are capable of producing IAA, which can be accumulated in the soil [11,12]. In our previous experiments the selection of active strains soils bacteria *E. nimipressuralis* 32-3, which transformed of hard soluble phosphates has been conducted and studied their properties [13,14]. During the study of physiological and biochemical properties of this strain, it was found that its cultural liquid has a high physiological activity [15,16].

The aim of our research was to study the mechanism of action of soil rhizobacteria *E. nimipressuralis* 32-3 on the elements of mineral nutrition of soybean (content of N and P_2O_5 in shoot), productivity of plants, qualitative and quantitative content of phytohormones in cultural liquid of this strain.

2. MATERIALS AND METHODS

The experiments were conducted in the green house during two seasons: April - July 2014 and 2015. Soybean plants were grown in polyethylene pots (container volume 1000 ml), substratum – sterilized sand, in five replications. Experiments were carried out on two backgrounds of hard soluble phosphorus compounds: 1) phytin, 2) Ca_3 (PO_4). Phytin or $Ca_3(PO_4)_2$ were added to the sand as the only source of phosphorus (organic and mineral phosphates respectively). The plants were fertilized with Prianishnikov`s phosphate-free solution (every 10 days). Seeds were inoculated with bacterial suspension of *E. nimipressuralis* 32-3 (4.5×10^6 cells/ml) be for pre sowing and, for the control variant, others were moistened in water. These plants were grown for 75 days.

Content of total N and P_2O_5 in shoot of soybean was determined according to the standard methods of colorimetry [17]. Determination of content total N in plants was determined by using of Nessler reactive and P_2O_5 – by addition of molybdenum acid.

Determination of the activity of phytohormones synthesis by *E. nimipressuralis* 32-3 was conducted under conditions of laboratory experiment. Cultivation of the strain was carried out on the glucose-asparagine (GA) medium under dynamic conditions (240 r/min) for 48 hours at 28°C. Inoculate of seeds was used a 24-hour broth culture, grown on a GA medium. A native culture liquid of *E. nimipressuralis* 32-3 was used for the analysis. Prior to extraction, the culture liquid was centrifuged (20 minutes) at 6000 r/min. Extraction of phytohormones was carried out in separating watering cans: three times for 15 minutes with dynamic shaking of the mixture. The combined extracts were dried with Na_2SO_4 (anhydrous), filtered, evaporated and washed with 3 ml of 70% ethanol. To extract IAA, diethyl ether (1:1 by volume) was used at pH 3, acidification was carried out with 2n HCl. Extraction of gibberellins was carried out with ethyl acetate (1:3 by volume) at pH 2.5,

acidification - 1 n HCl; extraction of cytokinins was carried out with n-butanol (1:1 at pH 8), alkalization – 1n NaOH [18]. Determination of the quantitative content of phytohormones in the culture liquid of the *E. nimipressuralis* 32-3 was carried out by quantitative spectrodensitometric thin-layer chromatography [19]. For the extraction was used 96% ethanol. Preliminary purification and concentration of the ethanol extract was carried out on chromatographic plates of the Silufol UV254. Express purification included the following steps of chromatography: 1) in chloroform, 2) in NH3 12.5%, 3) in a solvent system: ethyl acetate - acetic acid (20:1). The zones, coinciding with Rf of the applied standard solutions, were scraped off and eluted with ethanol. The IAA eluate was re-chromatographed on plates with silicon oxide (Merck No. 5715) in a solvent system: chloroform - ethyl acetate - acetic acid (100:100:1). For chromatography of cytokinins and gibberellins plates with aluminium oxide ("Merck" No. 5713) were used. System of solvents for cytokinins: chloroform - acetic acid (19:1), for gibberellins: n-butanol-acetic acid-water (4:1:5). For quantitative determination of phytohormones scanning spectrodensitometr "Camag TLC Scanner" used. The statistical treatment of researches results were conducted by the standard methods [20] and Programme Statistica 7.0.

3. RESULTS AND DISCUSSION

The results of our experiments showed positive influence of inoculation on the content of P_2O_5 in shoot of soybean. So, on the background of $Ca_3(PO_4)_2$ in plants with inoculation contained 5.1mg/g P_2O_5 against 3.6 mg/g in control (supplement 46%) and 5.9 mg/g P_2O_5 against 4.6 mg/g in control (supplement 28%) by using phytin (Table 1). Similar trend was observed for accumulation of total N: its content increased in shoot of soybean to 1.75% and 1.90% against 1.56% and 1.67% in the control by using phytin or $Ca_3(PO_4)_2$ respectively.

Improvement of mineral nutrition of plants with inoculation contributed to increasing of soybean productivity (Table 2). So, on the background of phytin the biomass of dry shoot in plants with inoculation increased to 2.6 g against 2.0 g in control (supplement 30%) and 2.4 g (supplement 14%) against 2.1 g in the control by using $Ca_3(PO_4)_2$ (Fig. 1). Also the amount of beans on the plants with inoculation exceeded control dates: by 64% and 70% compared to the control by using phytin and $Ca_3(PO_4)_2$, respectively (Fig. 2).

Table 1. The influence of inoculation on the content of N and P_2O_5 in the shoot of soybean (middle for 2014 and 2015)

Variant	N (%)	P_2O_5 (mg/g)
Phytin		
Control (without inoculation)	1.56	4.6
E. nimipressuralis 32-3	1.75	5.9
LSD_{05}	0.09	0.25
$Ca_3(PO_4)_2$		
Control (without inoculation)	1.67	3.6
E. nimipressuralis 32-3	1.90	5.1
LSD_{05}	0.11	0.36

Note: LSD_{05} – least significance difference

Our results showed the increase of inoculated plants height compared to the control. So, on the background of $Ca_3(PO_4)_2$ the height of plants with inoculation amounted 53.7 cm against 45.9 cm in control (supplement 17%). Similar results were marked by using phytin: the height of plants with inoculation amounted 50.6 cm against 43.8 cm in control (supplement 16%) (Fig. 3).

So, the research made it possible to reveal that pre sowing seed inoculation with *E. nimipressuralis* 32-3 promoted to increase of accumulation N and P_2O_5 in shoot, the height of plants and their productivity while growing soybean on the background of hard soluble compounds of phosphorus: ($Ca_3(PO_4)_2$ or phytin). These data indicated that *E. nimipressuralis* 32-3 promoted the assimilation of hard soluble compounds of phosphorus by soybean. So, our research showed one aspect of the mechanism of action of soil bacteria *E. nimipressuralis* 32-3 on plants. The possibility of using phosphate-mobilizing bacteria to eliminate phosphorus deficiency in soils of agricultural lands and improve plant growth is also discussed by other researchers [21,22].

The results of our chromatographic study confirmed previous: in the cultural liquid of *E. nimipressuralis* 32-3 revealed the available of IAA and compounds of gibberellic nature. Moreover, it was established that *E. nimipressuralis* 32-3 produces phytohormones of cytokinin nature: in free active shape (zeatin) and

in bound form (zeatin-riboside). Quantitative determination of phytohormones in the cultural liquid showed, that strain *E. nimipressuralis* 32-3 produces auxins, which are presented by IAA: β-indolyl-3-acetic acid (Table 3). Thus, its amount in the cultural liquid was composed 449 μkg/l.

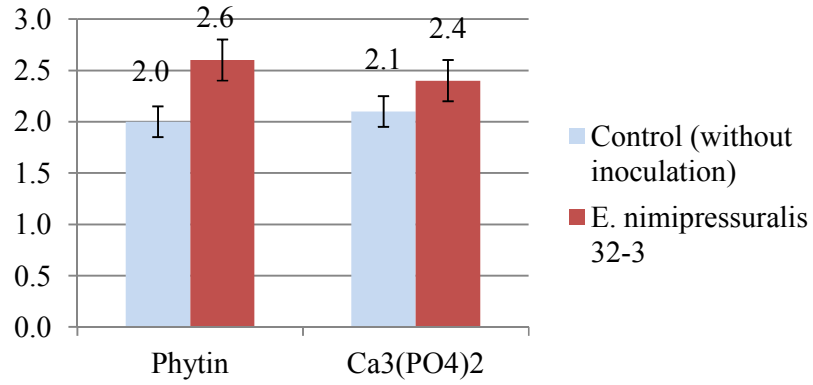

Fig. 1. The influence of inoculation on the dry biomass of 1 soybean plant, g (middle for 2014-2015)

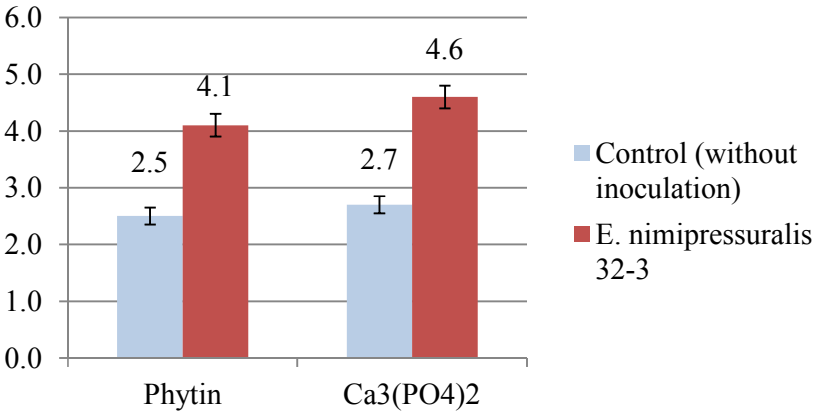

Fig. 2. The influence of inoculation on the amount of bean from 1 soybean plant (middle for 2014-2015)

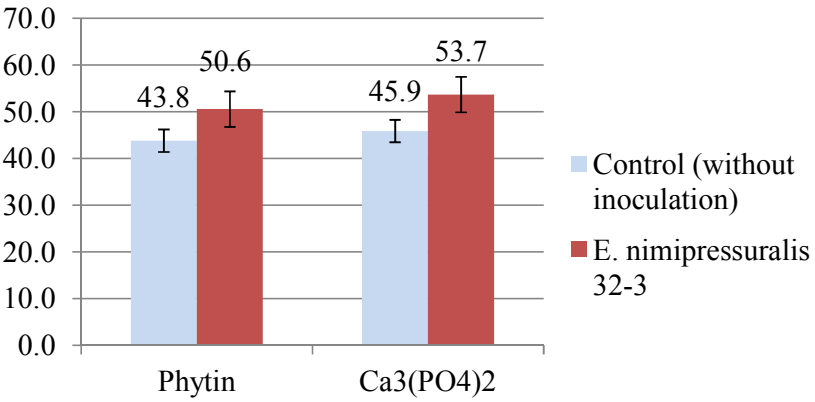

Fig. 3. The influence of inoculation on the height of 1 soybean plant, sm (middle for 2014-2015)

Table 2. The influence of inoculation on the productivity and height of soybean plants (middle for 2014 and 2015)

Variant	Dry biomass of 1 plant, g	Amount of bean/ 1 plant	Height of 1 plant, sm
Phytin			
Control (without inoculation)	2.0	2.5	43.8
E. nimipressuralis 32-3	2.6	4.1	50.6
LSD_{05}	0.15	0.30	2.40
$Ca_3(PO_4)_2$			
Control (without inoculation)	2.1	2.7	45.9
E. nimipressuralis 32-3	2.4	4.6	53.7
LSD_{05}	0.20	1.00	3.80

Note: LSD_{05} – least significance difference

Table 3. Contents of phytohormones in the cultural liquid of *E. nimipressuralis* 32-3

Phytohormones		Quantity, µkg/l
Auxin	β-indolyl-3-acetic acid	449.0±12.33
Cytokinin	Zeatin	254.0±9.35
	Zeatin-riboside	366.0±9.67
Gibberellins	Gibberellic acid	18781.0±123.50

Also, the studies were carried out allowed to revealed and identify in cultural liquid of *E. nimipressuralis* 32-3 physiological active substances, which belong to the cytokinines. It was showed, that they are presented in forms of free (zeatin) and related (zeatin-riboside) compounds. The quantity of zeatin in the cultural liquid was 254 µkg/l and zeatin-riboside – 366 µkg/l. Total content of cytokinines amounted 620 µkg/l of cultural liquid. According to our results in the cultural liquid of this strain were detected not identifying compounds of giberrelic substance in high amount. The total content of compounds giberrelic substance amounted 18781 µkg/l of cultural liquid.

Thus, quantitative determination of phytohormones in the cultural liquid showed, that strain *E. nimipressuralis* 32-3 produces auxin (IAA), cytokinines (zeatin and zeatin-riboside), compounds of gibberellin substance. It is founded, that by cultivation of *E. nimipressuralis* 32-3 during 48 hours, in cultural liquid contained up to 449 µkg/l IAA, 254 µkg/l zeatin and 18781 µkg/l of gibberellin substance.

4. CONCLUSIONS

Conducted surveys and investigations made it possible to establish the mechanism of the action of rhizobacterium *E. nimipressuralis* 32-3 on plants. It has been established that pre sowing seed inoculation contributes to an increase of N and P_2O_5 content in soybean shoots due to assimilation of hard soluble phosphates. The ability of *E. nimipressuralis* 32-3 to produce phytohormones (auxins, gibberellins and cytokinins) has been revealed. The quantitative determination of phytohormones was carried out by the method of thin-layer chromatography in the cultural liquid of this strain.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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