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ACCUMULATION OF PHYTOHORMONES BY SOIL BACTERIA *AZOTOBACTER VINELANDII* AND *BACILLUS SUBTILIS* UNDER THE INFLUENCE OF NANOMATERIALS

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ABSTRACT

Azotobacter vinelandii IMV B-7076 was shown to be capable of biosynthesis and accumulation of various biologically active substances, including high amounts of 3-indoleacetic acid (IAA) in free and bound forms, in the cultural medium. Cultivation of these bacteria with vermiculite particles had increased accumulation of abscisic acid (ABA) in 2.3 times compared to the control. Addition of silica dioxide nanoparticles to the nutrient medium had resulted in higher cytokinin content. Thus, the detected amount of zeatin was 2.4 times higher; zeatin-riboside – higher in 3.6 times; while the content of zeatin-glucoside had a slight increase by 3%.
High amounts of cytokinins, 3-indoleacetic and abscisic acids were also detected in the cultural medium of *Bacillus subtilis* IMV B-7023. Bacteria cultivation in the presence of vermiculite particles in nutrient medium had stimulated an increase of isopentyl adenine content in 4.0 times compared to control. Substantial (in 4.5 times) increase of 3-indoleacetic acid synthesis compared to control was detected. Addition of nanoparticles to the nutrient medium of *Bacillus subtilis* IMV B-7023 had also promoted synthesis of the abscisic acid in 1.7 times compared to control.

Keywords: IAA, ABA, cytokinins, *Azotobacter vinelandii*, *Bacillus subtilis*, nanomaterials

INTRODUCTION

One of the most perspective ways for sustainable and environmental friendly crop production is the use of microbial preparations. Microorganisms are the essential part of soil organic matter and play the important part in agroecosystems.
About 95% of soil microorganisms can produce different hormonal compounds, regulate all physiological and biochemical reactions in plant, including stress response and play an essential role in plants' growth and development (**Davies, 2004**).
As it was recently established 99% of all soil bacteria function in close association with soil solids (**Costerton *et al.*, 1985**; **Nannipieri *et al.*, 2017**). Bacteria interaction with solid materials of different nature is often followed by the increase of their physiological activity (**Kurdish *et al.*, 2006**), thus influencing the production of plant hormones (**Davies, 2004; Srivastava *et al.*, 2012; Yavorska *et al.*, 2006**). Nevertheless, the bacteria interaction with disperse materials and its impact on the synthesis of biologically active substances is still lacks the detailed explanations.
Thereby, our study was aimed to investigate influence of some types of disperse materials on the biosynthesis of plant hormones by *Azotobacter vinelandii* IMV B-7076 and *Bacillus subtilis* IMV B-7023.

MATERIAL AND METHODS

Microorganisms, nutrient media and culture conditions

In given study two bacterial strains were used from Ukrainian Collection of Microorganisms: *A. vinelandii* IMV B-7076 (**Patent № 72856**) and *B. subtilis* IMV B-7023 (**Patent № 54923A**), which are the active agents of complex bacterial preparation for crops. Bacteria *A. vinelandii* were cultivated for 48 hours on Ashby nitrogen-free sucrose medium having the following composition (g/L): K_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.2, K_2SO_4 0.1, $CaCO_3$ 5.0, sucrose 20.0, pH 7,2 ± 0.2, while *B. subtilis* were grown for 24 hours on glucose-mineral medium having the following composition (g/L): KH_2PO_4 0.5, K_2HPO_4 0.5, $(NH_4)_2SO_4$ 0.5, $MgSO_4 \cdot 7H_2O$ 0.3, NaCl 0.3, KCl 0.3, $CaCO_3$ 5.0, $MnSO_4 \cdot 7H_2O$ trace, $FeSO_4 \cdot 7H_2O$ trace, glucose 10.0, pH 7,2 ± 0.2 at 28 °C.
One-day bacterial culture was used for inoculation. The initial number of bacilli and azotobacter cells was $1 \cdot 10^6$ cells/mL and $1.5 \cdot 10^6$ cells/mL, respectively. The number of cells in cultural media was determined using Kokh's method (**Egorov, 1995**).
Cultural liquid of *A. vinelandii* IMV B-7076 and *B. subtilis* IMV B-7023 after completion of growing were freed from the cells of bacteria and nanoparticles by centrifugation on the centrifuge OPn-8 (Kirgizstan) during 30 min at 6600 g. In the obtained culture medium (CM) of bacteria the indices of produce plant hormonal compounds after influence of disperse materials on the bacteria were determined.

Nanomaterials

The following disperse materials were used in the given study: saponite, vermiculite, titanium dioxide and silica dioxide. *Saponite* is the type of bentonite clays with high magnesium content (up to 12%). The saponite with size up to 200 nm. It was obtained from Tashkivske field in Khmelnitskiy region, Ukraine. *Vermiculite* (Bulgaria origin) in natural mineral of hydromica group with layered structure, has small amounts of Al, Fe, Mg, Si and trace amounts of Ca, Na and K (**Tarasevich *et al.*, 1975**). The vermiculite with size up to 100 nm. *Titanium dioxide* (GOST 9808-84) can be naturally found as rutile, anatase or brukite and obtains 10th place as the natural occurred mineral in the earth's crust (**Samchuk *et al.*, 1982**). The titanium dioxide with size up to 100 nm. *Silica dioxide* was obtained from the O.O. Chuyko Institute of Surface Chemistry NAS of Ukraine. It takes the 2nd place as the natural occurred mineral (around 12%) in the Earth's lithosphere and is also found in bacteria and plants (**Ma *et al.*, 2006**). The silica nanoparticles with size of 5 – 20 nm. All minerals were added to the nutrient mediums before sterilization in optimal for bacteria growth concentration – 5.0 g/L (**Chobotarov *et al.*, 2010**).

Assay of Phytohormones Activity

Phytohormones activity in CM was detected using bioassay methods (**Volkogon *et al.*, 2010)**. Quantitative analysis of plant hormones in the cultural medium was detected using high performance liquid chromatography (***Methods of plant hormones identification*, 1988**) on liquid chromatograph Agilent 1200 LC (USA) with diode-array detector G 1315 B (column Eclipse XDB-C 18, 4.6 x 150 mm, particle size 5 microns). Hormonal compounds were eluted in the methanol:water (37:63) solvent system. Samples were analyzed in online mode. Chromatogram calculation was performed using the Chem Station software.

Statistical analysis

Microsoft excel (Microsoft corporation, USA) was used to analyze data on the average of three replicates (±SE) obtained from three independent experiments.

RESULTS AND DISCUSSION

Using bioassay methods the ability of *A. vinelandii* bacteria to produce plant hormones and especially compounds of cytokinin nature was revealed. In variants with 1:400 CM dilution ratio the 7.3% increase of cytokinin activity as compared with the control was observed, while variants with 1:1000 dilution had demonstrated higher by 43.3% cytokinin activity. The highest cytokinin activity was observed in variants with CM dilution 1:1500, exceeding the control values by 71.4% (Tab 1).
The addition of disperse materials into the nutrient medium of *A. vinelandii* had minor effect to the accumulation of cytokinins in CM this bacteria. Thus, upon the addition of titanium dioxide to the nutrient medium, its activity at 1:400 dilution ratio was at the control level. However, further dilution of this strain CM had demonstrated the increase of cytokinin activity by 61.1% (1:1000 dilution ratio) and 75.0% (1:1500 dilution ratio) as compared to control and had exceeded the performance by 17.8% and 3.6%, respectively the CM activity in variants without disperse materials (Tab 1).
Both vermiculite and saponite particles had no effect on the production of cytokinins by *A. vinelandii*. Thus, it was shown that undiluted CM had the same as control level of cytokinin activity. Variants with further dilutions, as it was observed with the addition of titanium dioxide to the nutrient medium, had higher level of cytokine activity with their maximum at 1:1500 dilution ratio – 42.9% and 50.0%, respectively (Tab 1), which, nevertheless, were lower than the cytokinin activity of *A. vinelandii* CM without dispersed particles (Tab 1).

Table 1 Cytokinin activity of *Azotobacter vinelandii* IMV B-7076 culture media (% to control) separately and upon addition of disperse materials

Variant	Control w/o DM	Titanium dioxide	Saponite	Vermiculite
Control (water)	100.0			
Standard (BAP, 10^{-5} M)	334.9 ± 1.5			
Dilution ratio				
CM:water 1:100	n	n	n	n
CM:water 1:200	97.6 ± 7.5	109.8 ± 2.9	112.2 ± 4.3	82.9 ± 3.6
CM:water 1:400	107.3 ± 9.1	100.0 ± 2.8	92.7 ± 4.2	100.0 ± 3.5
CM:water 1:600	117.8 ± 9.0	n	117.8 ± 9.5	104.3 ± 8.2
CM:water 1:1000	143.3 ± 5.4	161.1 ± 9.3	96.7 ± 6.0	110.2 ± 8.2
CM:water 1:1500	171.4 ± 6.1	175.0 ± 5.9	142.9 ± 7.5	150.0 ± 6.2
CM:water 1:2000	160.7 ± 6.7	160.7 ± 5.7	132.1 ± 8.9	132.1 ± 6.5

Legend: CM – culture media, DM - disperse materials, BAP – 6-benzylaminopurine, n – was not determined

Besides cytokinins *A. vinelandii* bacteria can accumulate in the culture medium compounds of auxin nature (Tab 2). However, in pure medium studied *A. vinelandii* had accumulated minor amounts of auxin. Maximum auxin activity was observed in variants with CM dilution with water environment at 1:200 ratio which was only 5% higher as compared with control. At further dilution the auxin activity had decreased (Tab 2).
Addition of titanium dioxide particles into the nutrient medium of *A. vinelandii* bacteria had led to a minor increase in accumulation of auxin compounds in CM this bacteria. CM of bacteria which cultivated with vermiculite and saponite particles had higher auxin activity with its maximum at 1:400 dilution ratio. It was shown the auxin activity of CM was higher by 21.3% and 28.8%, when compared to the control and had exceeded the values obtained in variants without saponite and verimuculite by 17.5% and 25.0%, respectively (Tab 2). Moreover the CM with these disperse materials was exceeding positive control (IAA, 10^{-5} M) by 2.7% and 10.2%, respectively (Tab 2). The auxin activity of CM had gradually decreased at further dilution, unlike in bioassays for determining of cytokinin activity (Tab 1). However, higher auxin activity of CM with disperse materials add-ons when compared to controls was observed in all studied variants (Tab 2).

Table 2 Auxin activity of *Azotobacter vinelandii* IMV B-7076 culture media (% to control) separately and upon addition of disperse materials

Variant	Control w/o DM	Titanium dioxide	Saponite	Vermiculite
Control (water)	100.0			
Standard (IAA, 10^{-5} M)	118.6 ± 1.2			
Dilution ratio				
CM:water 1:100	n	n	n	n
CM:water 1:200	105.0 ± 5.5	108.8 ± 1.9	113.8 ± 3.4	115.0 ± 2.6
CM:water 1:400	103.7 ± 7.1	105.0 ± 2.8	121.3 ± 4.2	128.8 ± 3.5
CM:water 1:600	103.3 ± 4.3	n	110.0 ± 8.5	110.0 ± 7.2
CM:water 1:1000	103.3 ± 8.1	104.4 ± 8.3	103.3 ± 5.9	95.6 ± 6.2
CM:water 1:1500	94.1 ± 4.1	101.0 ± 4.9	95.1 ± 7.5	99.0 ± 3.2
CM:water 1:2000	98.0 ± 6.7	101.0 ± 5.7	97.1 ± 7.9	100.0 ± 6.5

Legend: CM – culture media, DM - disperse materials, IAA – 3-indoleacetic acid, n – was not determined

Thus the addition of studied disperse materials to the nutrient medium of *A. vinelandii* had promoted accumulation of plant hormonal compounds with cytokinin and auxin activities in CM of this strain.
It is known that about 80% growth promoting rhizosphere bacteria are capable of biosynthesis of IAA (**Yavorska *et al.*, 2006**), which precursors – tryptophan or indole, can be found in root exudates mostly composed of carbohydrates (about 60%) (**Lunsdorf *et al.*, 2000**). By our previous research it was found (**Tserkovnyak *et al*., 2009**) that rhizosphere bacteria *Azotobacter vinelandii* IMV B-7076 can accumulate in the Ashby medium with L-tryptophan about 140 ng/mL of free IAA, not associated with other organic substances and about 160 ng/mL of bound auxin.
Cultivation of *A. vinelandii* bacteria with dispersed silica or vermiculite particles without L-tryptophan had resulted in accumulation of minor amounts of both free and bound forms of IAA (Tab 3). Thus, in variants with silica dioxide nanoparticles to the nutrient medium of *A. vinelandii* bacteria the amount of synthesized IAA was lower if compared to the control. Cultivation of *A. vinelandii* in the nutrient with 5.0 g/L of vermiculite had resulted in accumulation of bounded forms of IAA, although its amount was lower than in control (Tab 3), which might be due to the compounds sorption with nanoparticles.

Table 3 Disperse materials impact on the ability of *Azotobacter vinelandii* IMV B-7076 and *Bacillus subtilis* IMV B-7023 to accumulate IAA in culture media

Disperse materials	**IAA form**	**IAA accumulation, ng/mL**	
		A. vinelandii	*B. subtilis*
Control (without DM)	Free	26.3 ± 1.3	46.0 ± 0.7
	Bound	40.8± 1.1	n
Nano-SiO_2	Free	2.7 ± 0.9	54.0 ± 0.8
	Bound	6.2 ± 0.8	20.9 ± 0.6
Vermiculite	Free	10.6 ± 0.5	77.8 ± 0.2
	Bound	21.6 ± 1.0	127.8 ± 0.2

Legend: DM - disperse materials, IAA – 3-indoleacetic acid, n – was not determined

Analysis of the CM of *B. subtilis* IMV B-7023 had revealed the bacterial ability to synthesize IAA (Tab 3). As it was showed the amount of free form of IAA in CM was about 46.0 ng/mL while its bound form was found in trace quantities (Tab 3).
The addition of nano-SiO_2 or vermiculite particles has promoted accumulation of IAA in the culture medium of the studied bacteria. Thus, in variants with nano-SiO_2 the amount of free IAA forms in CM was 1.2 times higher if compared to the control, while the amount of bound IAA forms had reached 20.9 ng/mL. The concentration of free IAA form in CM of bacilli had increased cultivation of studied bacteria with vermiculite particles in 1.7 times while the content of bound IAA forms was 127.8 ng/mL compared with control (Tab 3). Thus, the accumulation of IAA in the CM of *B. subtilis* was observed at cultivation of given bacteria with disperse materials.
Among phytohormones abscisic acid (ABA) plays an important role in plants. ABA, the "stress hormone", is accumulating in plants upon their exposure to stress factors (**Kulaeva, 1994**) and is involved into the buds differentiation, fruit development and formation of additional roots (**Bleecker, 1999**).
The data obtained have revealed that *A. vinelandii* accumulates abscisic acid into the cultural medium (Tab 4). ABA content not associated with other organic compounds was 8.1 ng/mL while the content of its bounded form was 21.5 ng/mL.

Table 4 Disperse materials impact on the ability of *Azotobacter vinelandii* IMV B-7076 and *Bacillus subtilis* IMV B-7023 to accumulate abscisic acid in culture media

Disperse materials	**ABA form**	**ABA accumulation, ng/mL**	
		A. vinelandii	*B. subtilis*
Control (without DM)	Free	8.1 ± 0.4	41.4 ± 1.1
	Bound	21.5 ± 1.0	n
Nano-SiO_2	Free	10. 8 ± 0.5	10.5 ± 0.5
	Bound	9.8 ± 0.5	14.7 ± 0.7
Vermiculite	Free	48.6 ± 1.4	65.0 ± 1.8
	Bound	20.3 ± 1.0	4.2 ± 0.2

Legend: DM - disperse materials, ABA - abscisic acid, n – was not determined

Addition to the nutrient medium of 5.0 g/L of nano-SiO_2 had stimulated biosynthesis of free form of ABA in CM *A. vinelandii* IMV B-7076 in 1.3 times, and in 2.2 times the amount of bounded ABA. In variants with 5.0 g/L of vermiculite particles into the nutrient medium of *A. vinelandii* the substantial increase in ABA synthesis was observed. The total ABA content in CM of these bacteria was 2.3 times higher than in the control variant. The most noticeable increase of was observed for free ABA form not associated with other organic compounds (6 times compared to control). The number of bounded ABA forms was 2.5 times higher than in the control (Tab 4).
B. subtilis is also capable to the ABA accumulation in the culture medium (41.4 ng/mL). The total number of the ABA in CM of *B. subtilis* IMV B-7023 was lower at addition of SiO_2 nanoparticles into nutrient medium, that in control. Thus, the content of free and bound ABA forms was 10.5 ng/mL and 14.7 ng/mL, respectively (Tab 4).
Vermiculite addition to the nutrient medium of *B. subtilis* IMV B-7023 had increased synthesis of free ABA form in 1.4 times compared with control (to 65.0 ng/mL), whereas the concentration of its bound forms was lower in 3.5 times than in the variants with nano-SiO_2 (Tab 4).
Cytokinins play an important role in the regulation of plants growth and development (**Giron *et al.*, 2013**). It was established that *A. vinelandii* bacteria can accumulate in the culture media different compounds of cytokinin nature (Tab 5). The amount of zeatin, zeatin-riboside, zeatin-glycoside and isopentyl adenine was, respectively, 53.9; 49.2; 104.3 and 61.8 ng/mL. The content of isopentyl adenosine in CM of *A. vinelandii* bacteria was 4.6 ng/mL. Cultivation of these bacteria in a medium with SiO_2 nanoparticles had promoted accumulation of zeatin in 2.4 times, zeatin-riboside – in 3.6 times, zeatin-glycoside – by 3% and reduced the amount of izopentyl adenine in 1.3 times compared to control (Tab 5).

Table 5 Disperse materials impact on the ability of *Azotobacter vinelandii* IMV B-7076 to accumulate cytokinins in culture media

Disperse materials	**Accumulation of cytokinins, ng/mL**				
	Z	ZR	ZG	iP	iPA
Control (without DM)	53.9 ± 1.7	49.2 ± 1.5	104.3 ± 1.2	61.8 ± 1.1	4.6 ± 0.2
Nano-SiO_2	129.2 ± 1.5	176.8 ± 1.3	107.4 ± 1.3	48.9 ± 1.3	n
Vermiculite	53.6 ± 1.7	113.6 ± 1.7	83.3 ± 1.2	39.5 ± 1.0	4.4 ± 0.2

Legend: DM - disperse materials, Z – zeatin, ZR – zeatin-riboside, ZG – zeatin-glycoside, iP –izopentyl adenine, iPA –izopentyl adenosine, n – was not determined

Addition of vermiculite particles to the nutrient medium of *A. vinelandii* had stimulated the synthesis of the zeatin-riboside which content in CM of studied bacteria was 2.3 times higher compared to the control. The amount of zeatin was at control level, while the number of zeatin-glycoside and izopentyl adenine was 20 % and 36 % lower than in the control variant, respectively (Tab 5).
B. subtilis IMV B-7023 bacteria are also capable of cytokinins production. Thus, upon the cultivation of bacteria in the absence of dispersed materials in nutrient medium the 21.5 ng/mL of izopentyl adenine was detected (Tab 5).
Vermiculite and silica dioxide particles addition to the nutrient medium of *B. subtilis* had resulted in a significant increase in cytokinin production (date was present early) (**Kurdish *et al.*, 2015**). Besides, the addition of vermiculite to the nutrient medium of *B. subtilis* had stimulated the production of izopentyl adenine in 4.0 times (Tab 5).
Thus, the *A. vinelandii* IMV B-7076 and *B. subtilis* IMV B-7023 bacteria are capable of plant hormone synthesis. Cultivation of bacteria in the nutrient mediums with silica or vermiculite nanomaterials had promoted accumulation of phytohormones.

CONCLUSION

The results indicate that the interaction of *Azotobacter vinelandii* IMV B-7076 and *Bacillus subtilis* IMV B-7023 with nanomaterials differently, causing the interference phytohormone synthesis of these strains.
It is known that nanoparticles natural materials are important components of the soil (**Maurice *et al.*, 2008; Vijendra Kumar Mishra *et al.*, 2009**). They cause a significant impact on his fertility, abiotic and biotic components of the soil (**Gilbert *et al.*, 2005**). For interaction of a number of species of bacteria nanoparticles different nature there are significant changes in physiological and biochemical activity of the studied population (**Skorochod *et al.*, 2016; Yanbo Wang *et al.*, 2010**). The result of this interaction is largely affected by a number

of physical and chemical factors (**Yanbo Wang *et al.*, 2010**), which are determined to this time is not enough, indicating the need for further research in this urgent scientific direction.

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