ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 5, pp. 531–538. © Pleiades Publishing, Ltd., 2014.
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EXPERIMENTAL ARTICLES

Auxin Production by the *Klebsiella planticola* Strain TSKhA-91 and Its Effect on Development of Cucumber (*Cucumis sativus* L.) Seeds

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Received December 3, 2013

Abstract—Capacity of *Klebsiella planticola* strain TSKhA-91 for synthesis of indole-3-acetic acid (IAA) and other auxins was studied. The qualitative and quantitative composition of these compounds depends on the presence of exogenous tryptophan and on the nitrogen source. The highest IAA yield was obtained at the stationary phase of growth. Addition of L-tryptophan to the medium resulted in a significant increase (up to 85.5 μg/mL) of auxin biosynthesis, especially in the presence of nitrates. Thin-layer chromatography revealed that the indole-3-acetamide pathway was not active in this strain. The biological activity of auxins was confirmed by assay with kidney bean cuttings; the height of root formation and root number increased 16- and 6-fold, respectively. Under conditions of low-temperature stress, protective effect of *K. planticola* TSKhA-91 on development of cucumber (*Cucumis sativus* L.) seeds and stimulation of germination and root formation by its seeds were shown.

Keywords: *Klebsiella planticola*, auxins, indole-3-acetic acid, plant growth-promoting rhizobacteria (PGPR), *Cucumis sativus* L.

DOI: 10.1134/S0026261714050063

One of the properties of plant growth-promoting rhizobacteria (PGPR) is the biosynthesis of phytohormones, along with dinitrogen fixation, formation of readily available forms of iron and phosphorus, increased accessibility of nutrients for plants, and suppression of growth and development of plant pathogens [1–3]. Auxins are the main regulators of plant growth and development, and indole-3-acetic acid (IAA) is the most active indolic compound in this group. The capacity for auxin biosynthesis is widespread among heterotrophic and phototrophic bacteria, symbiotic and pathogenic microorganisms. The most complete information about IAA producers—different pathways of its biosynthesis, molecular and genetic mechanisms of regulation of the metabolic pathways resulting in formation of various indolic compounds of microbial origin—can be derived from the reviews published earlier [3–5].

One of the most important applied aspects of the capacity for IAA formation by the PGPR strains is their promising use in agrotechnology for inoculation of seeds and seedlings and for sapling treatment [3, 6–8]. The effectiveness of such treatment shows up in the stimulation of root formation, facilitation of seed germination and biomass build-up, and greater resistance to environmental unfavorable abiotic factors and to plant pathogens, which, in turn, increases the germinative capacity and the productivity of agricultural and industrial crops, as well as of ornamental plants. However, the choice of effective PGPR strains depends not only on the biosynthesis of, for example, IAA but also on their capacity for active root colonization [2]. This is of great importance in the agricultural technology of crops in temperate and cold climates, which necessitate the cultivation of early-ripening crops with a short vegetation period, high yields, and resistance to plant pathogens and various types of stress (temperature, water–salt balance) [9, 10].

Earlier, the strain *Klebsiella planticola* TSKhA-91, which proved to be an active representative of growth-promoting rhizobacteria, was isolated from the cucumber rhizoplane [11]. The strain was tested in the geographic network of the RAAS experiments in Russia and abroad (the United States, Vietnam, China, and India) on a wide spectrum of various vegetable, technical, and grain crops [11]. Its high efficiency in increasing the yield of these agricultural crops by 20–25% due to, among other things, offsetting the negative consequences of moisture limitation, was established [12]. The representatives of the genus *Klebsiella* are known to have a favorable effect on plant development, not only due to their nitrogen-fixing activity but also by auxin formation [13–16].

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The goal of the present work was to study the capacity of *K. planticola* TSKhA-91 for the synthesis of indole-3-acetic acid and other auxins under various cultivation conditions, as well as to determine the influence of these substances on root formation of the plants and stimulation of germination and development of *C. sativus* seeds including the conditions of low-temperature stress.

MATERIALS AND METHODS

The *subject of study* was the strain *K. planticola* TSKhA-91 deposited in the collection of the Department of Microbiology and Immunology, Timiryazev Agricultural Academy, Moscow. The strain was cultivated in the dark on a shaker (180 rpm) at 32°C under aeration conditions in 100-mL conical flasks containing 20 mL of one of the following nutrient media (g/L): (1) LB: tryptone, 10,0; yeast extract, 5.0; NaCl, 10.0; (2) K2: peptone, 1.25; K_2HPO_4, 0.5; KH_2PO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 0.75; $CaCl_2 \cdot 6H_2O$, 0.03; sucrose, 6.0; $(NH_4)_2SO_4$, 0.14; yeast extract, 0.1; trace element solution according to Fedorov, 1 mL; (3) nitrogen-free modification of K2 ($K2/N_0$): the same as K2, but without peptone, $(NH_4)_2SO_4$, or yeast extract; and (4) modification of K2 with nitrate nitrogen ($K2/NO_3$): the same as K2, but without peptone or $(NH_4)_2SO_4$; $NaNO_3$ (1.25 g/L) was used a source of nitrogen. In order to study the effect of various precursors on auxin biosynthesis, 200 μg/mL of L-tryptophan (Trp), indole-3-acetamide (IAM), and indole-3-pyruvic acid (IPA) (ICN, Germany), were introduced into medium 4 at 0.5 mM concentrations.

Assessment of culture growth was carried out by the optical density (OD) in the dynamics of growth (after 24 h), determined nephelometrically on a KFK-3-01 photometer (ZOMZ, Russian Federation), λ = 590 nm.

Colorimetric determination of the amount of indole-3-acetic acid in the culture liquid (CL) was performed using Salkowski reagent according to the method described earlier [17, 18] on an Ultrospec II spectrophotometer (LKB Biochrom, United Kingdom). Uninoculated medium served as the control. The standard curve was constructed based on serial IAA dilutions (ICN, Germany).

For analysis of indolic substances, thin-layer chromatography was used. For this purpose, an aliquot of the supernatant (800 μL) was transferred to an Eppendorf tube; pH was adjusted to 2.8; 1 mL of ethyl acetate was added; and the tube was vigorously shaken for 10 min. After separating the phases, the upper fraction was collected into light-proof test tubes; ethyl acetate was evaporated at room temperature; and the precipitate formed was dissolved in 30 μL of methanol. The samples were applied onto silica gel plates on aluminum support with an UF 254 indicator (Macherey-Nagel GmbH&Co. KG, Germany). Chromatography was carried out in the chloroform : ethyl acetate : formate system at a 50 : 40 : 10 ratio (vol/vol); the plates were examined under UV illumination. The standards of indolic compounds IAA (indole-3-acetic acid), IAM (indolyl-3-acetamide), and IPA (indolyl-3-pyruvic acid) applied to the plates as 1 mM solutions were used as markers. The marker R_f values were compared to the experimental samples.

To study the biological activity of IAA, the previously described biological test, which determined the influence of the growth stimulators in the CL on rooting bean (*Phaseolus vulgaris*) cuttings, was used [18]. Sterile tap water and nutrient media were used as the controls. The height of root formation and the root number served as the criteria for assessing the effect of IAA on root formation by the cuttings. For the variants with high auxin content, the CL was diluted with distilled water (2-, 5-, 10-, 50-, and 100-fold).

For seed germination, the hybrid F1 of the cucumber (*Cucumis sativus* L.), Maryina roshcha variety, was used together with the bacterial culture. The seeds were washed in soap solution for 15 min and then washed off thrice for 5 min in sterile distilled water. The seeds were treated under sterile conditions in a laminar hood. The seeds were introduced into flasks with 20–30 mL of distilled water or $K2/NO_3$ nutrient medium as the control. The seeds of the experimental samples were soaked in (1) undiluted 48-h *K. planticola* TSKhA-91 culture grown in medium $K2/NO_3$ and in (2) 100-fold diluted culture. The experiment was carried out under different temperature conditions: at the temperature optimal for seed germination (28°C) and under the cold stress-modeling conditions, for which purpose the experimental samples were incubated for a week at 12°C and then placed in a thermostat at 28°C. The efficiency of seed bacterization was determined by enumerating their germination, the number of lateral roots, the length of the tap root and the seedlings, and by quantifying the root and seedling biomass [19].

All the experiments were made in 3–5 replicates. The data obtained were processed using variational statistics analysis taking the criterion of probability to be $p < 0.05$.

RESULTS AND DISCUSSION

The microbe–plant relationships mediated by auxin formation may be symbiotic (e.g., with the involvement of rhizobia) or, on the contrary, parasitic (the phytopathogenic strains of agrobacteria and rhodococci are active producers of IAA) [4, 5]. But the main mass of microorganisms (by both their abundance and the diversity of species) colonizing the rhizosphere, the rhizoplane, the phylloplane, and the internal plant tissues enters into associative interactions with the host plant without forming special compartments (tubercles, galls). Associative microorgan-

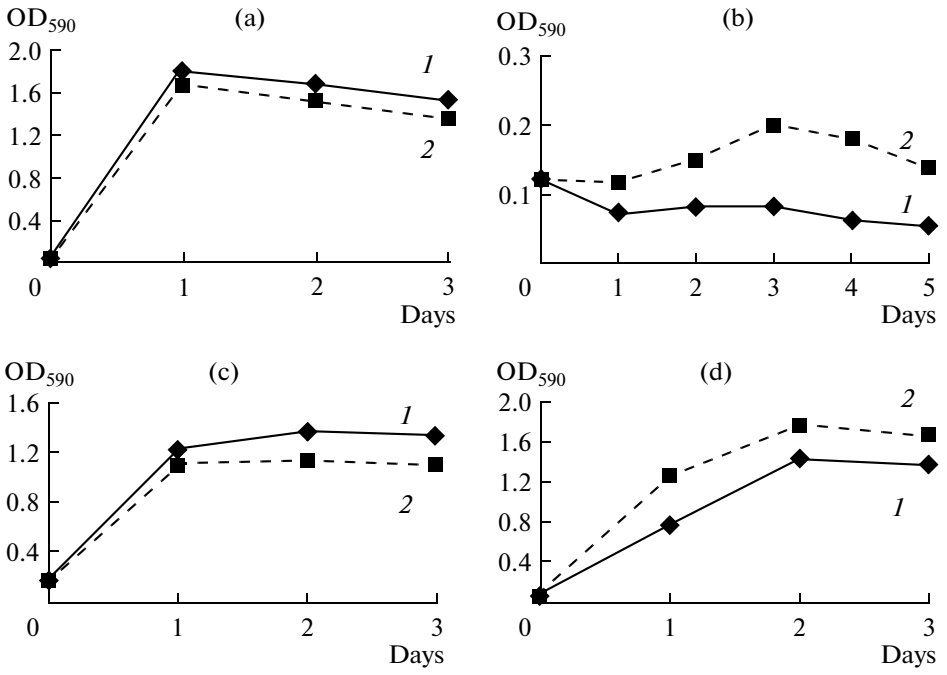

Fig. 1. Growth dynamics of *K. planticola* TSKhA-91 in different media. Medium LB (a); nitrogen-free medium K2/N_0 (b); ammonium medium K2 (c); and nitrate medium K2/NO_3 (d) without added tryptophan (*1*) and with addition of 200 μg/mL of L-tryptophan (*2*). The value discrepancies among the experimental replicates were less than 5%.

isms appear to be more competitive and gain adaptive advantages due to the formation of a consortium with a plant obtaining nutrients with plant exudates [2] and the ecological niche, which, due to growth of plant tissues, constantly provides new territories for colonization [5].

The capacity for IAA biosynthesis was found in many associative bacteria, for example, *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Serratia*, and *Sphingomonas* species, as well as streptomycetes and methylobacteria [3, 8, 13, 18, 20, 21]. Of special interest are the PGSR strains favorably influencing the growth and development of agricultural plants [3, 6–8, 22], and in this respect, enterobacteria are among the most efficient groups [13, 15].

In order to study the effect of the composition of the nutrient medium on auxin formation by *K. planticola* TSKhA-91, the strain (which is known for its nitrogen-fixing activity) was cultivated in nitrogen-free media and in media with bound nitrogen in the nitrate, ammonium, and organic forms. The culture attained the maximal biomass yield (OD = 1.4–1.8) after 24 and 48 h of growth in a rich LB medium and in nitrogen-containing mineral media, respectively (Fig. 1). Addition of tryptophan had no significant influence on growth in LB medium, which is rich in amino acids and oligopeptides, whereas in nitrogen-free medium, it resulted in a more than twofold biomass increase, although the maximal yield did not exceed 0.2 units.

Colorimetric assessment of the IAA content in the culture liquid showed that *K. planticola* TSKhA-91 formed it in considerable amounts (Fig. 2). Due to the high content of amino acids (including tryptophan) in LB medium, the basal level of IAA biosynthesis under these conditions (without tryptophan) was high (about 7 (μg/mL). On mineral media, IAA formation without exogenous tryptophan was 1.0–5.0 μg/mL, and on nitrogen-free medium, IAA was not detected at all. Addition of tryptophan to the media significantly stimulated IAA biosynthesis, since this amino acid is frequently precursor in the biosynthesis of microbial auxins [5]. A source of tryptophan for associative bacteria is the root exudates of higher plants, and although the content of this amino acid may vary considerably depending on the plant species [2], microorganisms consume it actively and convert it to IAA. On the whole, addition of 200–500 μg/mL of tryptophan results in an increase in the level of IAA biosynthesis by rhizobacteria (including *Klebsiella* species) from 5–20 to 100–150 μg/mL [6, 8, 15, 16, 18, 20, 21]. In LB (with tryptophan), the strain *K. planticola* TSKhA-91 formed the least amount (14.5 μg/mL) of indolic compounds; the greatest amount (85.5 μg/mL) was produced in the nitrogen-free medium, indicating efficient use of tryptophan and its conversion to auxin. A high level of IAA biosynthesis (80.6 μg/mL) was also noted when tryptophan was introduced into the medium with nitrate nitrogen, in contrast to the medium with ammonium nitrogen where the level of biosynthesis did not exceed 5 μg/mL. The maximal

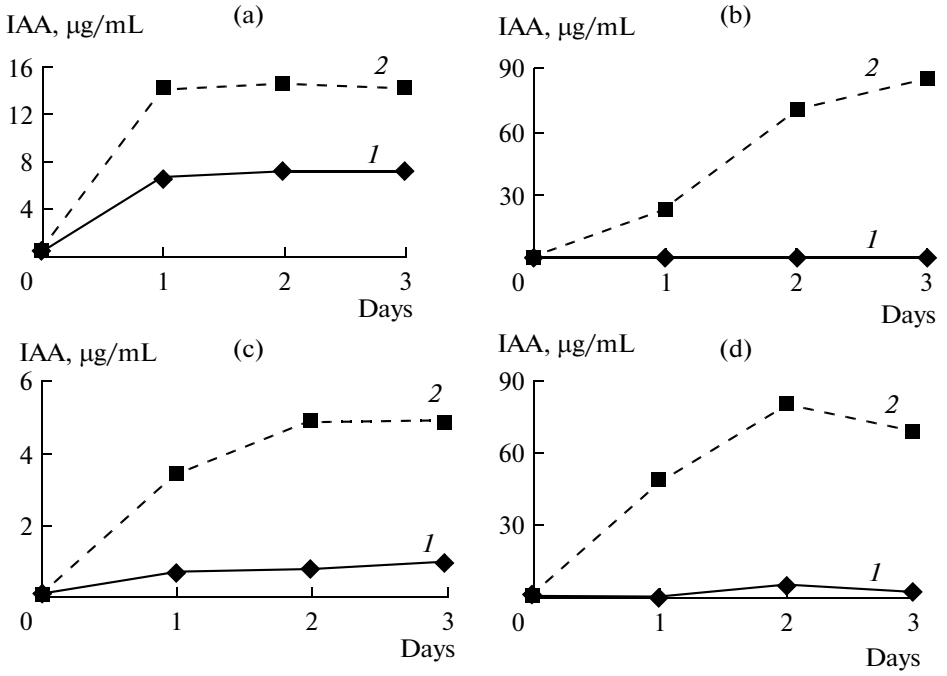

Fig. 2. Influence of exogenous tryptophan on IAA formation by *K. planticola* TSKhA-91 in different media. Medium LB (a); nitrogen-free medium K2/N_0 (b); ammonium medium K2 (c); and nitrate medium K2/NO_3 (d) without added tryptophan (*1*) and with addition of 200 μg/mL of L-tryptophan (*2*). The value discrepancies among the experimental replicates constituted less than 5%.

IAA yield corresponded to the stationary phase of growth, which agrees with the data of other authors [23, 24] (including those obtained for the representatives of the genus *Klebsiella* [13]) and is consistent with the consensus that the release of auxins under conditions unfavorable for the optimal existence of the bacteria is of great functional significance, increasing the probability of formation of a consortium with plants [23]. This is also confirmed by our data on the influence of the composition of nutrient media on IAA biosynthesis: under nitrogen limitation, the amount of the IAA formed was the maximal even at a very low population density (Fig. 1), whereas in the rich LB medium, the yield of auxins was low even with additional tryptophan. It was noted that in the media containing the nitrate forms of nitrogen, considerable amounts of auxins were formed [21], while, on the contrary, ammonium ions inhibited IAA biosynthesis, probably at the stage of removal of the tryptophan amino group, especially in the bacteria possessing the IPA biosynthetic pathway (saprophytic strains of *Pseudomonas, Agrobacterium, Azospirillum*, and *Azotobacter*) [20, 24]. Investigation of some of the factors influencing the IAA yield (temperature, carbon source, and tryptophan concentration) showed the source of nitrogen to be the most significant of them [25].

Application of the colorimetric method of quantification of auxins may result in overestimated IAA content, since Salkowski reagent reacts not only with IAA, but also with indole, indolyl-3-pyruvate, indolyl-3-acetamide, and some other auxin derivatives [20, 21, 26]. At the same time, IAA is quantitatively the main auxin contained in the medium, while, for example, IPA is characterized by extreme instability; therefore, the use of this method is justified.

For IAA identification in the composition of the auxins formed by *K. planticola* TSKhA-91, thin-layer chromatography was performed, and the following results were obtained (Fig. 3). No indolic compounds were detected on mineral media without addition of tryptophan. On LB without tryptophan, formation of indolyl-3-acetic acid corresponding to the marker by R_f is explained by the presence of amino acids, including tryptophan, in the medium. The fact that the content of indolic compounds increased significantly on addition of tryptophan to synthetic cultivation media was also supported by the TLC data. This effect was particularly noticeable when the strain was cultivated in mineral media. Different indolic compounds were revealed in the culture liquid, and their concentration and diversity depended on the nitrogen source used in the medium (Fig. 3). For example, addition of tryptophan to the K2 ammonium medium resulted in formation of IAA and another compound close to IAA by R_f, although the formation rate of these substances was extremely low. Under nitrogen-free conditions, when the culture developed at the expense of nitrogen fixation, maximum diversity of auxins was observed. Under these conditions, IAA was not the main com-

ponent; a substance with R_f not corresponding to any of the markers used in this work was released in considerable amounts (according to the intensity of luminescence under UV illumination). It was noted that in the culture developing in nitrate medium, the quantitative and qualitative auxin composition changed in the process of growth: IAA became the dominant indolic compound. Having attained its maximal concentration at 48 h, IAA broke down at 72 h to form other indolic components; new bands, which had not previously been detected, were visualized.

Apart from indolyl-3-acetic acid, we also identified some of its precursors: indolyl-3-acetamide (IAM) and indolyl-3-pyruvate (IPA), which are the key intermediates in two different pathways of IAA biosynthesis. We did not succeed in reliably visualizing any band corresponding to these markers in the nitrogen-free medium and LB, whereas in other mineral media, compounds with the R_f values close to them were detected. Many microorganisms are known to be capable of forming IAA simultaneously along several biosynthetic pathways [5]. In order to specify the pathway of IAA biosynthesis by the strain studied, the intermediates IAM and IPA were exogenously added to the cultivation medium and detected chromatographically, whether they remained in the medium or were involved in IAA biosynthesis. It was found that IAM was almost not consumed althoug a certain amount of a compound not not corresponding to any of the standards was formed in the medium. Moreover, on addition of IAM, several new UV-fluorescent compounds were formed; their concentration, however, changed little with time. Thus, the indole acetamide pathway of IAA biosynthesis was not active in the studied *K. planticola* strain. At the same time, exogenous indolyl-3-pyruvate (IPA) was metabolized to a significant degree, and at 48 h of cultivation, we revealed in the cultivation medium a compound with R_f similar to that of indolyl-3-acetaldehyde (IAld), from which IAA was then formed along this pathway of biosynthesis. However, both of these compounds are known for their instability [13]. In the case of *Klebsiella pneumoniae* it was shown in [13] that tryptophol formed from IAld was present at high concentrations in its culture liquid. Importantly, both IAld and IPA were not undetected or were present in small amounts due to their extreme instability. Thus, the authors suggested the presence of the IPA pathway of IAA biosynthesis in this bacterium. Other investigators [14] revealed a number of intermediates of auxin biosynthesis in the culture liquid of *Klebsiella oxytoca*—tryptamine, IAM, tryptophol, and indolyl-3-acetonitryl, but they also did not detect IPA and IAld due to their instability. The presence of a considerable amount of tryptophan also gave evidence of the highest activity of the IPA pathway of IAA biosynthesis in the studied *Klebsiella* strain. Biosynthesis along the IPA pathway is known to be the most common among microorganisms, especially the PGPR strains [3, 5]. IPA, which is converted to indole-3-aceticaldehyde and then to IAA, is formed from tryptophan along this pathway. The functioning of this pathway in the studied strain seems to be highly probable. Since IAM is the only intermediate in this pathway of IAA biosynthesis, the fact that exogenous IAM remained unutilized indicates that this pathway cannot be the main pathway of IAA biosynthesis.

For symbiotic *Klebsiella* strains, their influence on the growth of a number of agricultural crops is known. We showed earlier [11] that upon bacterization of the cucumber seeds, *K. planticola* TSKhA-91 increased the productivity of bee-pollinated varieties and hybrids of the cucumber plants by an average of 24%, as well as decreased the nitrate content in the fruits. Our experiments also demonstrated the growth-promoting effect of IAA formed by the *Klebsiella* strain studied: root formation considerably increased in the bean cuttings treated with CL (table; Fig. 4). The treatment of the cuttings contributed to formation of the root brush, with the root formation height exceeding the control sample values 5–16-fold.

The substances contained in the supernatant of the bacterial culture grown in K2/NO_3 medium with tryptophan actively stimulated the process of rooting. However, the content of auxin (over 80 μg/mL) and other biologically active substances in the initial CL was probably extremely high: along the cutting height, a multitude of tubercles corresponding to rooting was revealed, but the roots failed to develop further. Moreover, in some places, the stem was stratified. When the auxin concentration was decreased by diluting the bacterial CL, the number of germinated roots and the height of the stem on which they formed increased (table). For example, when the bean cuttings were treated with the CL from the K2 + tryptophan medium diluted 10-fold with distilled water, the roots were formed 1.3 cm higher compared to the undiluted variant. When the CL of the bacteria grown in nitrate medium with tryptophan was diluted two- and fivefold times, the number of germinated roots, compared to the control, increased 1.9- and 4.5-fold, respectively. Both dilutions (2- and 5-fold) of the CL of the K2 + tryptophan-grown culture proved to be the most efficient for stimulating root formation by bean cuttings. The authors of the above-cited work [13] also noted that it was the diluted (up to 6–8%) supernatant fluid of *K. pneumoniae* that exerted a stimulating effect on root formation of rice in hydroponic culture: it facilitated root formation and increased the root surface and the mass of dry roots. High concentrations, on the contrary, inhibited the processes of root growth and development.

In the experiments with the Maryina roshcha cucumber hybrid, in the seeds treated with the strain culture diluted 100-fold and incubated for seven days at 12°C, the length of the tap root and the height of the seedlings were greater (by 18 and 20%, respectively) five days after germination than in cold-treated seeds

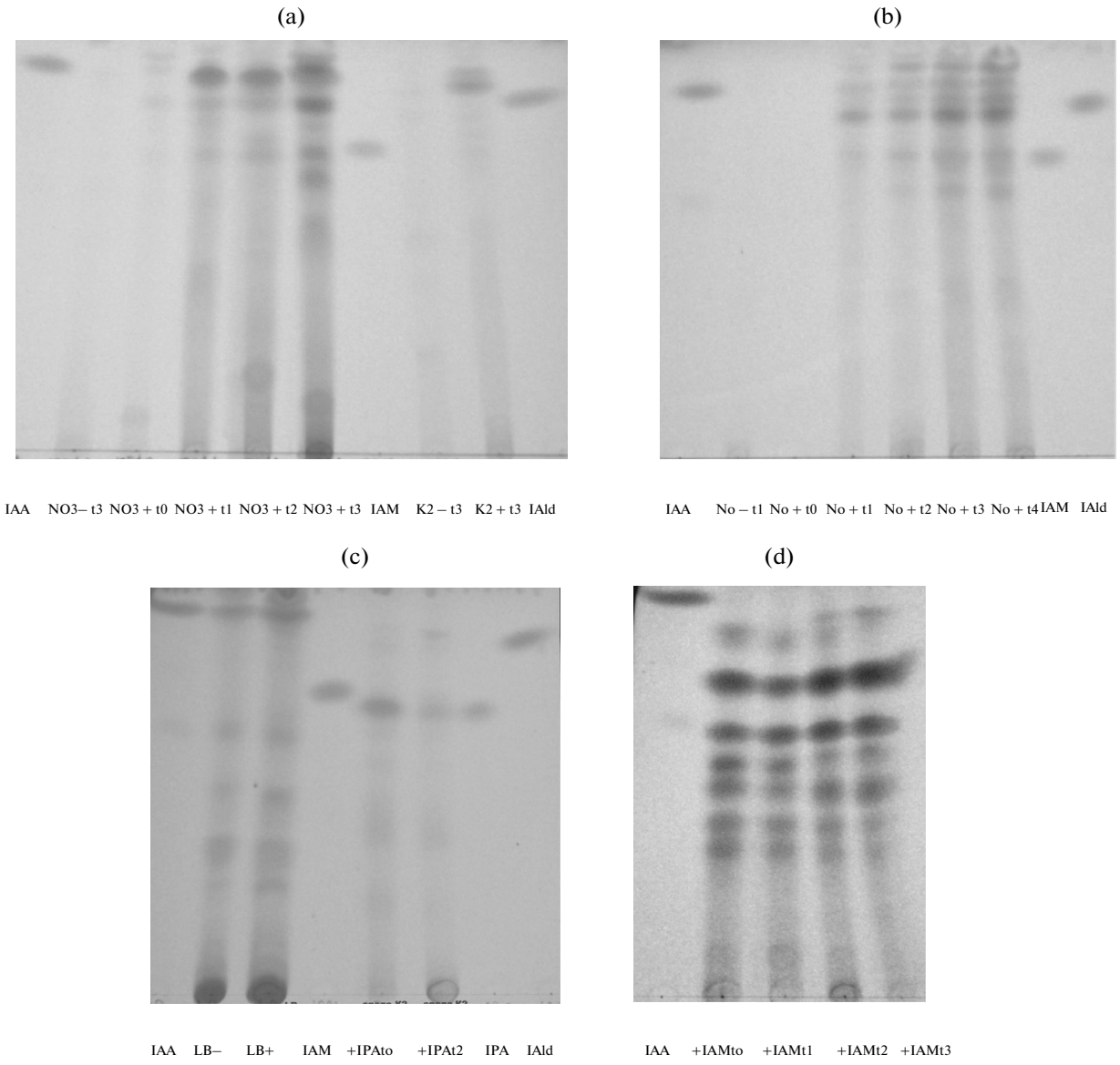

Fig. 3. Thin-layer chromatography of the indolic substances formed by *K. planticola* TSKhA-91 in different media. K2/NO_3 and K2 (a); K2/N_0 (b); LB and K2 with the addition (+) of IPA (c); and K2 with the addition (+) of IAM (d). Cultivation was carried out without tryptophan (–) and with tryptophan (+) at the zero point (t0), on the first (t1), second (t2), third (t3) or fourth (t4) day. In the samples where 't' is not indicated, sampling was made on the third day. Markers (ICN): IAA, indole-3-acetic acid; IAld, indolyl-3acetaledhyde; IAM, indolyl-3-acetamide; IPA, indolyl-3-pyruvate. The auxins were extracted with ethyl acetate. Mobile phase: chloroform : ethyl acetate : formate (50 : 40 : 10, vol/vol); the plates were examined under UV illumuination.

soaked in water. Under normal conditions (28°C), all the seeds showed similar values in terms of all the biometric parameters (the length of the tap root, the number of branch roots, and the height of the seedlings). The dry root mass of the cucumber from the experimental variant (treated with 100-fold diluted bacterial culture) was also greater compared to the control variant (the seeds soaked in water) by 42%, which confirms the stimulating effect of auxins on the root formation process, whereas in terms of the biomass of the green part of the plant, both variants had similar values (13.4 and 13.5 mg from one plant, respectively). In all the experimental variants, the number of lateral roots in the plants subjected to hardening was more by 10–30% than in the seeds germinating at 28°C. It is necessary to note that the biometric values of the seeds soaked in undiluted culture of the strain under both temperature modes were worse by an average of 30% than the control values (data not shown). However, treatment of the cucumber seeds with 1 : 100 diluted culture of *K. planticola* TSKhA-91 grown in the medium with nitrate dilution resulted in a considerable enhancement of the ecological toler-

Effect of the culture liquid of *K. planticola* TSKhA-91 on root formation of bean *Phaseolus vulgaris*) cuttings

Experimental variant	IAA content in CL (μg/mL)	Height of the stem at which roots are formed		Root number per one cutting
		cm	increased (-fold)	
Distilled water	–	0.5 ± 0.1	0	6.2 ± 4.3
CL (LB)	7.1	4.3 ± 0.5	8.5	7.5 ± 5.6
CL (LB, 1 : 50)	–	3.4 ± 1.5	6.8	10.4 ± 2.8
CL (LB + Trp)	**14.4**	**4.8 ± 1.1**	**9.6**	**20.5 ± 6.2**
CL (LB + Trp, 1 : 50)	–	**4.6 ± 0.3**	**9.2**	**7.3 ± 0.8**
CL (K2/N_o)	0.2	2.7 ± 1.5	5.4	6.75 ± 1.3
CL (K2/N_o + Trp)	**85.5**	**5.6 ± 1.9**	**11.2**	**22.2 ± 11.6**
CL (K2)	1.0	3.3 ± 0.9	12.3	20.8 ± 6.6
CL (K2 + Trp)	**4.9**	**5.6 ± 1.3**	**11.2**	**29.2 ± 9.3**
CL (K2/NO_3)	4.9	3.4 ± 1.5	6.8	21.4 ± 13.1
CL (K2/NO_3 + Trp)	**80.6**	**7.8 ± 0.6**	**15.6**	*
CL (K2/NO_3 + Trp, 1 : 2)	–	**6.8 ± 1.9**	**13.6**	**16.8 ± 7.3**
CL (K2/NO_3 + Trp, 1 : 5)	–	**7.8 ± 1.4**	**15.6**	**39.6 ± 14.1**

Experimental variants with addition of exogenous tryptophan to the medium are in bold. A dash in the "IAA content" column indicates that the IAA concentration was not measured or was equal to zero (control).

* Graft stratification due to excessive concentration of biologically active substances in the CL occurred. The confidence interval is indicated at $p = 0.95$.

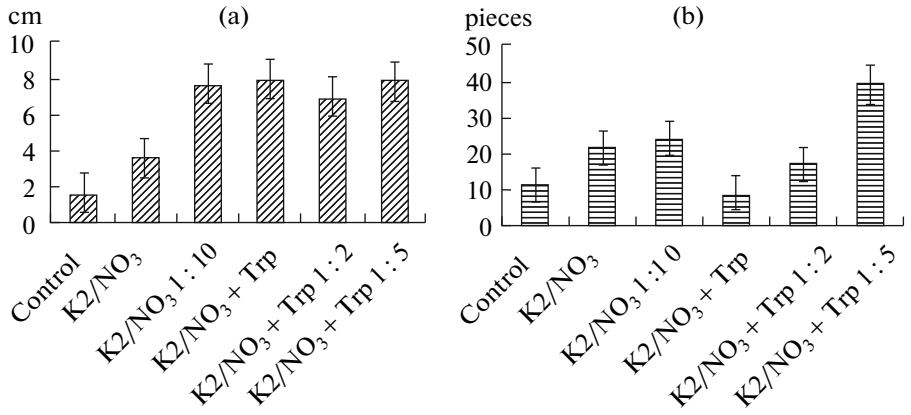

Fig. 4. Effect of the auxins contained in the culture liquid of *K. planticola* TSKhA-91 on the height of root formation (a) and the number of the roots (b) formed by the kidney bean (*Phaseolus vulgaris*) cuttings. Designations: control, water; K2/NO_3, modification of K2 with nitrate nitrogen; +Trp, L-tryptophan (200 μg/mL) was added to the cultivation medium. 1 : 2, 1 : 5, 1 : 10 are the 2-, 5-, and 10-fold dilutions of the initial culture fluid with water, respectively.

ance of the plant to the temperature (cold) stress conditions.

Our investigations confirm that the use of the associative diazotrophic bacterial strain *K. planticola* TSKhA-91 shows much promise in the cultivation of agricultural crops. It is expedient to obtain an efficacious strain-based biopreparation for use as an ecologically safe protector of heat-loving plants against low-temperature stress.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project no. 12-04-31446 mol_a.

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Translated by E. Babchenko