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Klebsiella Planticola Endophyte Rhizobacteria and its Interaction with Plants

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Abstract: The *Klebsiella planticola* strain TSHA-91 ability to invade non-leguminous plants was initially studied. During the plant invasion, it was established that the *Klebsiella* cells were persistent and could penetrate into the inside tissues of the plants. The interaction between the *Klebsiella planticola* and non-leguminous plants suggests that the microorganism studied might be of the endophytic bacteria. It was proved that, irrespective of the way of inoculation, bacteria was capable of colonizing the whole plant phytoplane, with the stable level of colonization during the plant vegetation being 10^3-10^4 cell/g dry mass. Different technologies of the "Bioplant-K" bioproduct application for non-leguminous crop inoculation were approbated: traditional pre-seeding treatment of seed, leaves treatment and combination of the two technologies. The study results obtained point to new prospects of utilizing rhizobacteria, particularly of the *Klebsiella planticola* strain TSHA-91, as the basis of the "Bioplant-K" product.

Key words: endophyte rhizobacteria, invasion, persistence, *Klebsiella planticola*, "Bioplant-K".

Introduction

It is known that the study on properties of associative nitrogen fixing bacteria has from the very start been connected with the perspective for their utilization as alternatives to mineral nitrogen fertilizers for non-leguminous crops. A great number of researchers have determined that these bacteria have a complex effect on plants (Bashan, 1997; Reddy et al., 1999). Apart from improving the mineral plant nutrition, they demonstrate their function of stimulating the plant growth and increasing the plant resistance to different phytopathogenic microorganisms. The inoculation technology issue is also of

current interest. While selecting the method of the microbial bioproduct application, apart from the root inoculation, ever greater attention is drawn by a new method - the application of the nitrogen fixing microorganism to the plant phylosphere (Hotjanovic, 1996). The inoculation method efficiency depends on the character of the interaction between the introduced bacteria and the plants.

The research objective was to study the ability of the *Klebsiella planticola* bacteria to colonize plants and invade them while different ways of inoculation were being applied.

Material and Methods

The *Klebsiella planticola* bacteria strain TSHA-91, isolated from the rhizoplane of a cucumber in 1990 and obtained from the Timirjazev Moscow Agricultural Academy Microbiology Department collection, were used as a study material.

The study on the invading and colonizing ability of the bacteria depending on the way of inoculation was conducted by applying the *Klebsiella planticola* TSHA-91 Rif^{200} mutant, rifampicin resistant and capable of growing on the LB medium with the rifampicin antibiotic concentration of 200 mg per 1 ml of nutrient medium, obtained by the method of alternating adaptation to the increased antibiotic concentration (Zvjagincev et al., 1991).

During the cultivation, bacteria were added to the LB (Luria Bertoni) nutrient medium, containing 10 g peptone and 5 g yeast extract (Miller, 1976). The LB and K2 fluid mediums were used for obtaining the accumulating culture. The K2 medium consisted of: peptone - 1.2 g, K_2HPO_4 - 0.5 g, KH_2PO_4 - 0.3 g, $MgSO_4$ - 0.1 g, CaCl - 0.03 g, saccharose - 6g, $(NH_4)_2SO_4$ - 0.14g, yeast extract - 0.1 g per 1000 ml of water supply water.

The "Zaozerskij 85" barley cultivar seed and oat seed were used as a culture-test. The colonizing and acclimatizing abilities of the *Klebsiella planticola* TSHA-91-Rif^{200} bacteria in the barley rhizoplane and phylosphere, depending on the way of the plant inoculation, were estimated in microtrials, set on the Moscow Agricultural Academy Plant Protection Station (1998-2000). The trial scheme consisted of the following inoculation variants: seed treatment, leaf treatment and joint seed and leaf treatment. The seed was inoculated in the 24-hour culture cell suspension with 10^8-10^9 cell/ml titre in the 1:50 dilution within 30 minutes. The phylosphere inoculation was done by hand spraying of shoots using the pulverizer (spray) containing liquid culture of 10^8-10^9 cell/ml titre in 1:50 dilution.

The nitrogenase activity was determined by acetylene method (Zvjagincev et al., 1991) on gas CHROM-4-1 chromatograph furnished with the flame-ionizing detector. The invading properties of the strain were investigated in a sterile model trial on oat seed, cultivated on a sterile soil extract in bacteriological test tubes. Within the 7^{th} day of cultivation the substrate was contaminated with a 10^9 cell/ml suspension of the *Klebsiella planticola* strain TSHA-91-Rif^{200}, concentration 10^9 cell/ml in 1:50 dilution, using a syringe with a long needle at the top, while avoiding contact with the shoots. The identification of strains, separated from the homogenate of the roots and leaves tissue of oat shoots, was performed on the basis of the

phenotype properties (growth on the rifampicin medium) and the plasmid DNA presence. The plasmid DNA was separated by the express Kado method followed by electrophoresis. The DNA electrophoresis was conducted in the agarose gel (0.7%) having the following composition: agarose (type I of the Sigma company), with the application of tri-phosphate buffer according to a classical method (Meyer et al., 1976).

For the purpose of investigating the effects of different ways of inoculation on carbon assimilation in barley plants, as well as of the ability of cells to acclimatize, invade and migrate into plants, the ^{14}C isotope and two-chamber containers, where the upper part of a plant is hermetically separated from the lower one were used in a model trial. The plants were marked once in the $^{14}CO_2$ atmosphere, introduced into the upper compartment. After the trial, the microbe biomass in soil was determined by the extraction-fumigation method (Joergensen, 1995). In order to establish the content of radioactive carbon in the samples of roots, leaves, and soil, the samples were dried to their constant weight at 60^0C, ground and burned in the Oxidazer Model 307 (Canberra Packard) machine, turning carbon, separated in the form of carbon-dioxide, during the process, into the scintillating Permjfluor E^+ (Canberra Packard) cocktail. Samples were, then, analysed on the Liquid Scintillation Counter Tri-Car in 2000CA (Canberra Packard). Data were statistically processed using the "Statistica" computer programme (ANOVA/ MANOVA) and dispersion method (Dospehov, 1979).

Results and Discussion

1. *Klebsiella planticola* in the rhizosphere and phylosphere of barley depending on the way of plant inoculation.

The adaptability of associative root diazotrophic *Klebsiella planticola* strain TSHA-91-Rif^{200} to leaf and stem depending on the way of plant inoculation was investigated in micro-field experiments. In the pre-seeding inoculation of seeds, bacteria moved to the plant surface and their number, growing during the vegetation, reached 10^4 cell/g dry mass, proving that the introduced microorganism had the ability of acclimatizing.

The decrease in the number of living cells of the introduced microorganism population is characteristic of the period of adaptation to the new environment: to food sources and aboriginal epiphyte microorganisms. Further increase of the number of the living cells in the phylosphere is characteristic of the ability of *Klebsiella planticola* to swiftly invade new living environment and the number reached, for epiphyte microorganisms, a rather high, stable level. Therefore, the *Klebsiella planticola* vitality on plants does not depend on the way of inoculation.

Following that, an analogous experiment was set up in order to confirm the obtained data on the ability of the introduced bacteria to acclimate during the non-root inoculation. After the phylosphere inoculation (tab.1), the *Klebsiella planticola* cells were discovered in the plant rhizoplane, without the pre-seeding inoculation of seed with $3.2 \cdot 10^4$ cell/g dry root, which was, as compared to the pre-seeding inoculation variants ($5.3 \cdot 10^7$ - $9.3 \cdot 10^7$), three times lower.

The very fact that *Klebsiella planticola* existed on plant roots in this variant within the first day after the phylosphere inoculation, pointed to the indicated ability of bacteria to migrate towards the roots (hemotaxis) and swiftly colonize their surface. The presence of the *Klebsiella planticola* cells in the plant phylosphere with the pre-seeding treatment of seeds at the level of $1.5 \cdot 10^5$cell/g dry leaves, suggested that *Klebsiella planticola*, having been adsorbed on the seed during the pre-seeding inoculation further colonized the surface of the whole shoot and acclimated in the aboveground plant organs, as well as in the roots.

Table 1. The dynamics of the number of *Klebsiella planticola* strain TSHA-91-Rif^{200} in the rhizosphere, phylosphere and rhizoplane of barley depending on the way of inoculation ($\times 10^3$ cell/g dry mass).

Cases		Inoculation of seeds	Inoculation of seeds and leaves	Inoculation of leaves
Tillering	Roots	93000	52000	32
	Leaves	140	620	620
Booting	Roots	--	--	92
	Leaves	13	73	17
Blooming	Roots	11	7.1	0
	Leaves	1.1	2.8	1.1
Crop	Roots	4.1	8.3	0
	leaves	0	2.1	1.1

-- no data

The obtained data thus confirm the earlier assumption that the *Klebsiella planticola* cells are capable of acclimatizing not only in the rhizosphere but also in the phylosphere of the plants, where they survive during the whole vegetation period, till the harvest, at a rather high level (for epiphytic microorganisms) - 10^3-10^4 cell/g dry leaves. The ability of bacteria, added to phylosphere, to swiftly (during the day) colonize the roots, also in great number - 10^4 cell/g dry root, was also observed.

2. The study on the invasion ability of *Klebsiella planticola* strain TSHA-91.

In order to prove the ability of *Klebsiella* to penetrate into the plant tissue and migrate within the plant, an experiment was set up in sterile conditions using the rifampicin resistant strain of *Klebsiella planticola*. The bacteria cells, isolated from young oat plant organs, were identified not only on the basis of their rifampicin resistance, but also by the express method of the *Klebsiella planticola* plasmid DNA isolation followed by the subsequent electrophoresis.

Table 2. *Klebsiella planticola* strain TSHA-91-Rif^{200} DNA plasmid isolation in oats organs (sterile oat inoculation experiment)

Parts of Plant	*Klebsiella planticola* recombinant plasmids			
	Control dates (days)			
	1	3	5	7
Leaves and stems	Not found --	Not found --	Found +	Found +
Roots	Found +	Found +	Found +	Found +

As seen from Table 2, the invasive ability of *Klebsiella planticola* strain TSHA-91-Rif^{200} as regards young oat plants was shown and the fact that it penetrated into the plant tissue through the root system and migrated into the aboveground plant organs (leaves and stem) was determined. We may suggest that the bacteria studied might be of the endophytic microorganisms. It may be, thus, assumed that interactions between plants and introduced strains are not restricted only to the associative interactions, but that more specific and closer relationships exist between them. In order to confirm the fact that the *Klebsiella planticola* presence in the phylosphere is dependent exactly on the very ability of the strain to colonize all plants, migrating through their tissue, and not on some random contamination, and to provide an answer to the question of the ability of bacteria to migrate through the plant in reverse direction, into the rhizoplane zone, during the inoculation of leaves and stems surfaces, the following investigations were conducted:

In a model experiment, the ability of the inoculant cells to acclimatize and migrate inside the plant was studied. The ways of the cell penetration into the rhizoplane during the inoculation of leaves and into the phylosphere, with inoculation of barley plant roots were determined. In the experiment, the carbon assimilation by plant and carbon translocation into soil was also studied. The ^{14}C isotope and a specially built two-chamber container were used in the study. They allowed hermetical isolation of the upper part of the plant from the lower one and exclusion of the possibility of bacteria to migrate along the plant surface. After the trial, $1.2 \cdot 10^5$cell/g dry mass was determined on the plant roots, and their number was lower on the leaves by an order of multitude and equalled $7.1 \cdot 10^4$cell/g (table 3).

In the variant with the pre-seeding treatment of seed, the number of inoculant cells on the roots almost equalled their number on the plant roots in the variant with the plant surface parts treatment and totalled $4.9 \cdot 10^5$ cell/g. The number of the cell phylosphere was rather lower (by two orders of multitude), being $6.2 \cdot 10^3$ cell/g. In the water by which the washing-out was conducted (in which the roots were washed out to wash soil before adding dilutions for the purpose of analysing rhizosphere), a rather high number of inoculant cells classified into rhizosphere soil inhabitants, was determined.

The experiment showed that the content of carbon, intaken by the plant, grew by 18-11 % during the inoculation.

Table 3. The amount of *Klebsiella planticola* in the plant and soil, depending on the way of inoculation, in the conditions of the isolation of the aboveground plant part from the roots ($\cdot 10^3$ cell/g dry matter) - model experiment

Cases	Amount of *Klebsiella planticola* $\times 10^3$ cell/g					
	Leaves	Roots	Solution	Rhizo-sphere	Soil	Sum
Inoculation of seeds	6.2	490	63	0.42	0.3	560
Inoculation of leaves	71	120	94	0.99	0.8	290

It was thus determined that associative diazotrophic bacteria *Klebsiella planticola* strain TSHA-91 cells have the ability to invade, penetrate into the inner tissue of plants and migrate in two directions - from the roots up to the leaves and vice versa, depending on the way of inoculation. The presence of inoculating bacteria cells in plants indicates their persistence, as confirmed by genetic methods.

3. Nitrogenase activity in rhizosphere, rhizoplane and phylosphere of barley, depending on the way of plant inoculation.

Investigations of the influence of different ways of inoculation on the ability of *Klebsiella planticola* to acclimatize to barley plants, have also determined the character of the emerging properties of the introduced associative nitrogen fixing bacteria. Table 4 shows the results of measuring nitrogenase activity in the barley tillering and booting phases.

It was determined that in the tillering phase, during the pre-seeding inoculation, the nitrogenase activity in the plant rhizoplane was twice that in the control. In the rhizosphere soil, the measured activity was also 1.4 times greater. The next measuring, performed in the stem formation phase, within 14 days after the phylosphere inoculation, resulted in considerable (4 times higher) increase of the nitrogenase activity in the plant rhizoplane with the pre-seeding seed inoculation and leaves inoculation. In the joint inoculation variant, the nitrogenase activity was 7.5 times bigger as compared to the control group.

In the rhizopshere soil of all the inoculated plants (independent of the way of inoculation), nitrogenase activity was 1.8-2 times more intensive than in the control plants. In the plant phylosphere with joint seeds and leaves treatment, nitrogenase activity was twice bigger than in the control, while seed or leaf treatment did not cause essential changes in the intensity of the process.

Therefore, combination of the pre-seeding seed treatment with the phylosphere inoculation had a significant effect on the nitrogenase activity of the plant roots, leaves and soil.

Table 4. Nitrogenase activity in rhizosphere, rhizoplane and phylosphere of barley plants depending on the way of plant inoculation (nmol C_2H_4/g during the day)

Cases	Plant Growth Phases					
	Tillering			Booting		
	Leaves	Roots	Soil	Leaves	Roots	Soil
Without inoculation	--	20.2	13.81	7.41	11.59	9.51
Inoculation of seeds	--	40.82	19.73	9.48	46.99	11.89
Inoculation of leaves and seeds	--	--	--	14.48	86.53	15.51
Inoculation of leaves	--	--	--	10.84	46.22	11.91

Conclusions

On the basis of the study results, the following conclusions may be drawn:

The application of the *Klebsiella planticola* plasmid DNA isolation method has made it possible to establish, above all, the ability of these bacteria to invade non-leguminous plants. Owing to its invading ability, *Klebsiella planticol*a penetrates into the interior plant tissues.

The *Klebsiella planticola* cell presence inside the plants indicates their persistence, where bacteria are capable of migrating to two directions: from the roots up to the leaves and vice versa; *Klebsiella planticola* has therefore been classified into endophyte bacteria.

It has been shown that during the plant inoculation by *Klebsiella planticola* traditional pre-seeding seed treatment, and also by leaf treatment, bacteria colonize actively the whole phytoplane (roots, stems, leaves). Stable number of introduced bacteria cells during the plant vegetation equalled 10^3-10^4 cell/g dry mass; the ability of *Klebsiella planticola* cells to migrate within the plant may result in colonizing the surface of the whole plant phytoplane, independent of the way of inoculation.

Endophyte properties of *Klebsiella planticola* cause its high efficiency while interacting with non-leguminous plants; thus, for instance, nitrogenase activity on roots increases 4-7.5 times, while the accumulation of assimilated carbon in roots and leaves is two times greater.

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ENDOFITALNA RIZOBAKTERIJA *Klebsiella planticola* I NJENA INTERAKCIJA SA BILJKAMA

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Rezime

Proučavanjem invazivnih svojstava *Klebsiella planticola* TSHA-91 u odnosu na neleguminozne biljke utvrđena je visoka perzistentnost ćelija ovog diazotrofa. Ispoljena osobina interakcije *Klebsiella planticola* sa neleguminoznim biljkama omogućava da se ovaj mikroorganizam svrsta u endofitne bakterije. Dokazano je da, nezavisno od načina inokulacije, bakterije koloniziraju čitav fitoplan biljaka, pri čemu nivo stabilizacije brojnosti introducenta u toku vegetacije biljaka iznosi 10^3-10^4 ćelija /g suve mase. Aprobirane su različite tehnologije primene biopreparata "Bioplant-K" za inokulaciju neleguminoznih kultura: tradicionalna predsetvena obrada semena, obrada listova i kombinacija oba načina. Rezultati izvršenih istraživanja ukazuju na nove perspektive iskorišćavanja rizobakterija, a posebno *Klebsiella planticola* soj TSHA-91 kao osnove preparata "Bioplant K".