



Influence of Enhanced Synthesis of Exopolysaccharides in *Rhizobium ruizarguesonis* and Overproduction of Plant Receptor to these Compounds on Colonizing Activity of Rhizobia in Legume and Non-Legume Plants and Plant Resistance to Phytopathogenic Fungi

Elizaveta S. Kantsurova¹ · Andrey D. Bovin¹ · Alina M. Dymo¹ · Natalya A. Komolkina¹ · Alexandra A. Shalyakina¹ · Elizaveta A. Salnikova¹ · Olga A. Pavlova¹ · Oleg S. Yuzikhin¹ · Nadezhda A. Vishnevskaya¹ · Elena A. Dolgikh¹

Received: 20 August 2024 / Accepted: 29 September 2024

© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2024

Abstract

Rhizobial exopolysaccharides (EPS) may provide stabilization of membranes against external factors, as well as improved surface adhesion, but their role in interaction with legume and non-legume plants is still far from understanding. In this work, the transcriptional regulator RosR of *Rhizobium ruizarguesonis*, which regulates the synthesis of EPS, was overproduced in a pHC60 plasmid and expressed in the RCAM 1026 strain. This resulted in an improved production of EPS by this recombinant strain. Comparative analysis of the inoculation of pea *Pisum sativum* plants with *R. ruizarguesonis* pHC60-rosR and strain carrying the empty plasmid revealed an essential increase in the number of nodules, root length and biomass in plants inoculated with this EPS-overproducing strain. It demonstrates that the enhanced EPS synthesis by rhizobia may stimulate plant root colonization and subsequent nodule formation in pea plants. The influence of enhanced EPS synthesis in rhizobia on colonizing activity was also estimated in non-legume plant tomato *Solanum lycopersicum*. Our findings shown the increased colonization of the root surface and stimulation of the shoot biomass of inoculated plants. Inoculation of pea and tomato with EPS-overproducing rhizobial strain essentially increased plant resistance to phytopathogenic fungi *Fusarium culmorum* and *F. oxysporum* in both legume and non-legume plants, demonstrating a significant biocontrol effect of this recombinant strain. Furthermore, we have identified the *PsLYK10* gene that encodes a putative EPS receptor in *P. sativum*, although no significant effect of *PsLYK10* overexpression on nodulation in legume (pea *P. sativum*) and colonization of roots of non-legume plants by rhizobia was found compared to enhanced production of EPS by rhizobia.

Abbreviations

EPS	Exopolysaccharides
CG	Cyclic glucans
GM	Glucomannans
LPS	Lipopolysaccharides
CPS	Capsular polysaccharides

Introduction

Soil bacteria collectively called “rhizobia” are able to form symbiosis with legume plants that results in colonization of host plant roots followed by formation of specialized structures—the nitrogen-fixing root nodules. Rhizobia are also able to colonize the surface of the roots of non-legume plants as it was shown for strawberry [1], lettuce, carrot [2], spinach [3], rice [4], pepper, and tomato [5]. Such colonization may stimulate the growth and productivity of crop plants. Therefore, development of approaches to improve and stabilize the process of colonization of the roots of legume and non-legume plants by various rhizobial strains is significant for the development of sustainable agriculture.

In legume plants, mutual recognition and subsequent attachment of rhizobia to the root surface of plants depend on signal exchange between partners, including flavonoids

Elizaveta S. Kantsurova and Andrey D. Bovin have contributed equally to this work.

✉ Elena A. Dolgikh
ea.dolgikh@arriam.ru; dol2helen@yahoo.com

¹ All-Russia Research Institute for Agricultural Microbiology, Podbelsky Chausse 3, 196608 St.-Petersburg, Russia

and Nod factors, as well as specific proteins and surface compounds of rhizobia. Perception of rhizobial signals, Nod factors, by plant receptors provides the initial recognition of rhizobia followed by their attachment to plant roots. The attachment of rhizobia to plant cells is a complex process that includes primary and secondary stages, in which multiple factors are involved. Rhizobia may produce some proteins, providing their improved adhesion to the roots [6]. Primary attachment is mediated by bacterial proteins with adhesive properties collectively called adhesins [7]. One of these well-known adhesins is bacterial agglutinin, the *Rhizobium*-adhering protein A (RapA). RapA adhesin was first described in *Rhizobium leguminosarum* bv. *trifolii* strains and belongs to extracellular calcium-binding proteins, which are directly involved in the colonization of plant roots [8, 9]. The increase in RapA production by rhizobial strains stimulated rhizobial competitiveness, root colonization, and number of nodules on roots in inoculated legume plants [9].

In addition, plant lectins secreted at the root hair tip are involved in primary attachment [10–12]. Legume lectins bind with high specificity to a polar-localized rhizobial neutral polysaccharide called glucomannan and therefore promote the development of legume-rhizobia symbiosis and improve rhizobial adhesion to the root surface [13]. As an example, plant lectin PSL expressed on root hair tips promotes attachment of *Rhizobium leguminosarum* bv. *viciae* to pea root hairs by binding to a rhizobial glucomannan [13]. Root hair lectin and seed lectin were shown to represent the same protein encoded by one functional gene *psl* in pea [14]. The importance of lectins in the regulation of interactions rhizobia with non-legumes was also shown by the introduction of the pea seed lectin *PSL* gene into non-legume plants, which increased the specificity and stability of root colonization of these plants by rhizobia [15]. A secondary attachment step suggests the anchoring by bacterial cellulose fibrils, but it plays a not so important role as the primary one [10].

The significant role in colonization may play rhizobial surface polysaccharides, such as cyclic glucans (CG), high-molecular weight neutral polysaccharides (glucomannans, GM), lipopolysaccharides (LPS), capsular polysaccharides (CPS), beta-glucans, and exopolysaccharides (EPS) [16]. Among rhizobial surface polysaccharides, EPS are of special interest because of their important biological functions. In bacteria, these compounds provide stabilization of membranes against external factors, as well as improvement of surface adhesion [17]. EPS are acidic polysaccharides released out of the cell or weakly associated with the cell surface. They consist of repeating units of sugar monomers and some non-carbohydrate substituents, such as succinate, glycerol, acetyl, or pyruvate. The most well-known EPS from *Sinorhizobium meliloti* is a polymer of repeating units such as the octasaccharide composed of seven glucose residues and one galactose residue with

acetyl, succinyl, and pyruvyl modifications. In the case of *R. leguminosarum*, a repeating unit is composed of five glucose, one galactose, and two glucuronic acid residues, decorated by acetyl and pyruvyl groups. EPS may exist in a high-molecular weight form, as a polymer containing many repeating units, and a low-molecular weight form, composed of one or several repeating units. Previous studies showed that EPS are necessary for rhizobia attachment and colonization of root hairs, infection thread initiation and extension, as well as intracellular accommodation of rhizobia after release into the cell [18–21]. However, the role of rhizobial EPS was predominantly studied in interaction with model legume plants such as *Lotus japonicus*, *Medicago truncatula*, and *Trifolium repens* [22–24], but not with crop legumes.

Lysin-motif (LysM) receptor-like kinase LjEPR3 in *L. japonicus* and MtLYK10 in *M. truncatula* are involved in the recognition of EPS and distinguishing its structure in compatible rhizobia [24–26]. This type of receptor may be present in legume as in non-legume plants. Since it was identified in a wide range of dicotyledon and monocotyledon plants [27], the recognition of microbial EPS can be considered a conservative feature of plants [28]. The main function of the LjEPR3 and MtLYK10 receptors in legume plants was related to the regulation of intracellular accommodation of rhizobia during the infection process and suppression of defense responses [24–26]. Expression of these receptors is inducible by Nod factors and sufficient to select compatible bacteria through recognition of their EPS.

In this article, we studied the effect of enhanced EPS synthesis in rhizobia and stimulated EPS perception by plants on nodulation in pea *P. sativum* L. Previously, the *rosR* rhizobial gene encoding a positive transcriptional regulator was shown to be involved in the biosynthesis and regulation of EPS production [23, 29–31]. In this work, the transcriptional regulator RosR of *Rhizobium ruizarguesonis* was overproduced in a pHC60 plasmid under the *lac* promoter and expressed in the RCAM 1026 rhizobial strain. In our experiments, the resistance of pea plants to the phytopathogenic fungus *Fusarium culmorum* was studied in response to inoculation with EPS-overproducing and non-modified strains. The influence of enhanced EPS synthesis in rhizobia on colonizing activity was also estimated in non-legume plant tomato *Solanum lycopersicum* as well as tomato resistance to *Fusarium oxysporum* fungal infection. Furthermore, we have identified the *PsLYK10* gene that encodes a putative receptor for EPS in *P. sativum* (as demonstrating a high level of similarity to MtLYK10 and LjEPR3) and studied the effect of *PsLYK10* overexpression on nodulation in legume (pea *Pisum sativum* L.) and colonization of roots of non-legume (tomato *S. lycopersicum*) plant by rhizobia to estimate its specificity and effect on the colonization.

Materials and Methods

Plant Material and Growth Conditions

The Frisson cultivar pea seeds were cultured in concentrated sulfuric acid for 5 min and washed with sterile water at least 5 times. Seeds were transferred to 1% water agar and placed for 4–5 days in darkness at 25 °C.

Tomato *Solanum lycopersicum* seeds of cultivar Carmello were sterilized with 15% sodium hypochlorite NaOCl (0.1 M) for 5 min, then washed 6 times with distilled sterile water, and kept in 10% H₂O₂ for 2 min. After that, the seeds were washed 3 times with large volumes of sterile water. The sterilized seeds were placed in Petri dishes on 1% water agar and left for 1 day at 4 °C and then for 3 days at room temperature in darkness. Young seedlings were placed in Petri plates on Murashige–Skoog (MS) agar medium without sucrose [32] and then cultured in the phytotron (MLR-352H, Panasonic, Japan) at 21 °C, 60% humidity, and a photoperiod of 16 h/ 8 h for 5 days in light conditions.

Fungal Strains

Infection with phytopathogenic fungi *Fusarium culmorum* Schldl. strain. 4–5-day pea *Pisum sativum* L. seedlings cv. Frissons were transferred to 250-ml plastic vessels with 8–10-mm sterilized vermiculite saturated with Jensen's medium and containing 3×10^5 conidia of the pathogenic fungus. To infect the plants, an inoculum of highly aggressive phytopathogenic fungi *Fusarium culmorum* strain 334 was used along or together with *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry* or EPS-overproducing *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* strains and incubated for 20 days. To obtain the inoculum, the *F. culmorum* 334 strain was grown on Chapek agar for 10–14 days and then washed off the plates with sterile water. The plants were grown in a growth chamber at 21 °C in 16-h light/ 8-h dark cycles, with 60% humidity. The intensity of plant infection with phytopathogenic fungi was carried out according to the formula proposed earlier [33]. Visual disease symptoms were assessed 21-day post-inoculation (dpi) using a four-point scale (0—symptomless, 1—slightly necrotic, 2—moderately necrotic, 3—severely necrotic, 4—completely necrotic) [34]. To collect the material, the roots were washed 3–4 times with tap water and used for estimation of infection development. In addition, roots were frozen for DNA isolation.

The single-spore *Fusarium oxysporum* Schldl. strain, MFG 58284 was taken from the culture collection (MF) of the All Russian Institute of Plant Protection (VIZR, St.

Petersburg, Russia) [35]. Grown in potato dextrose agar (HiMedia Laboratories Pvt. Limited, India), in a thermostat at +28 °C in the dark for 2 weeks. The washing was done with sterile distilled water. Conidia were examined in Goryaev's chamber, in large cages at a wash dilution of 1:1000. The number of conidia was calculated according to the formula: mean (2.25) * 250 * 10³ = 5.6 * 10⁶—in one ml 1:1000 washout. The density of the inoculum is 3.2 * 10⁶ in one ml. An inoculum with the addition of 50 µl of Tween-20 was mixed with coarse vermiculite (8 mm), moisture absorption of vermiculite.

Bacterial Strains

Escherichia coli XLBlue MRF' and TOP10 strains (Thermo Fisher Scientific, USA) were used for standard cloning procedures. *Rhizobium ruizarguesonis* sp. nov. RCAM 1026 strains carrying pHC60-*mCherry* or pHC60-*rosR* were cultured at 28 °C in YEM agar medium [36] supplemented with 0.5-mg/ml streptomycin. The fresh bacterial inoculum was introduced into 100 ml of B⁻ medium [37] and 0.5-mg/ml streptomycin and cultivated with stirring at 220 rpm at 28 °C. *The Agrobacterium rhizogenes* Arqua 1 strain was used for plant transformation. *A. rhizogenes* Arqua 1 was grown at 28 °C on TY (tryptone yeast) agar medium supplemented with 0.05-mg/mL spectinomycin [38].

Generation of Constructs for Plant and Rhizobia Transformation

Cloning of the rhizobial rosR gene. To generate the pHC60-*rosR* vector for rhizobial transformation, carrying the gene of interest, the coding sequence of *rosR* gene (700 bp) was amplified using rhizobial RCAM 1026 DNA as a matrix with corresponding primers flanked with the sequences for *Clal* restriction enzyme. Amplification was done using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA). The amplified DNA product was subcloned into the pBlueScript SK(+) vector restricted by the *SmaI* enzyme. The ligation mixture was introduced in the DNA methylation deficient *E. coli* GM2163 strain (*dam*⁻, *dcm*⁻). After purification of the plasmid, the fragment of *rosR* DNA was restricted by *Clal* from pBlueScript SK (±) and cloned under the *lac* promoter in the pHC60 vector, also carrying *GFP* under the T7 promoter (Fig. S1). The final construct was purified from *E. coli* TOP10 (Thermo Fisher Scientific, USA) cultured in the presence of 0.01-mg / ml tetracycline and verified by sequencing. Subsequently, it was transferred to *R. ruizarguesonis* RCAM 1026 for inoculation of the plant.

Cloning of the pea LYK10 gene. To generate pKGWD,0 vectors for plant transformation, the coding sequence of the *PsLYK10* gene (1860 b.p.) gene without stop codon

was amplified using cDNA as a matrix (total RNA was isolated from 21 dai pea nodules of cv. Finale) with the corresponding primers. Amplification was done using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA). The amplified product was restricted with *XbaI/EcoRI* and subcloned into the pMON vector under the 35S promoter in the frame with the sequences encoding YFP and nopaline synthase terminator (Tnos). The p35S::*PsLYK10*::YFP fragment was amplified with primers carrying *attB1* and *attB2* sites and cloned into pDONR221 using BP clonase (Thermo Fisher Scientific, USA). In the next step, the p35S::*PsLYK10*::YFP from the pDONR221 vector was transformed into the destination vector pKGWD,0 using LR clonase (Thermo Fisher Scientific, USA). The final construction was introduced into the *Agrobacterium rhizogenes* strain Arqua1.

Analysis of Root Surface Colonization by Rhizobia and Fungi Using PCR

To build the calibration graphs, fragments of the genes encoding hypervariable regions V4–V6 in *Fusarium oxysporum* 18S rRNA (*FOX*) and *R. ruizarguesonis* NodA acyltransferase (*nodA*) were amplified using fungal and *R. ruizarguesonis* RCAM 1026 DNA as matrix. The fragment of the housekeeping *GAPDH* gene of tomato was amplified using complementary DNA (cDNA) as a matrix (total RNA was isolated from tomato roots). The amplification was performed using Taq DNA polymerase (Evrogen, Russia). The amplified products were cloned in the vector pAL2-T (Evrogen, Russia), verified by sequencing and used for the construction of calibration graphs with a range of concentrations for the cloned genes (*R. ruizarguesonis nodA* gene, *Fusarium oxysporum FOX* gene encoding 18S rRNA, pea housekeeping *GAPDH* gene) (Fig. S2). It allowed us to calculate the amount of rhizobial and fungal DNA in the probes. Rhizobial colonization was determined by quantifying the expression of the *R. ruizarguesonis nodA* gene by qRT-PCR and calculating the number of gene copies (Fig. S2), then normalizing these values to tomato *GAPDH* normalization factor was done. Fungal colonization was estimated by quantifying the expression of fungal *F. oxysporum 18SrRNA* gene by qRT-PCR and calculating the number of gene copies (Fig. S2), then normalizing these values to tomato *GAPDH* normalization factor was done. All primers used for cloning are presented in Table S1.

EPS Isolation

For EPS isolation, 10-ml cultures of rhizobia were grown in YEM medium for 2 days at 28 °C in a rotary shaker. EPS was precipitated from culture supernatants with 30 ml of 96% ethanol, the precipitates were dissolved in water

and then analyzed for carbohydrates as described earlier [39]. Total sugars content was calculated in glucose equivalents.

Plant Transformation

Sterile young tomato seedlings (4–5 days after transfer to the light) were cut in the hypocotyl region and transformed with *Agrobacterium rhizogenes* Arqua 1 by applying bacterial mass to the wounding site. Transformed seedlings (6–8 per plate) were placed on MS agar medium without sucrose in a Petri dish between two sheets of filter paper soaked in sterile distilled water. The root area of the plants located on the plates was covered with foil. The dishes were placed in a phytotron and cultivated in a vertical position at 21 °C, 60% humidity, and with a photoperiod of 16-h light/ 8-h dark for 10–14 days until callus appeared. Subsequently, the plants were transferred to MS medium with sucrose (3%) containing 0.3 mg/ml of cefotaxime antibiotic and further incubated under the same conditions for 10–14 days until transgenic roots appeared. Composite plants were selected for GFP fluorescent roots and then transferred into aeroponics with LIPM medium [40] and incubated for 5 days at room temperature with a photoperiod of 16 h/ 8 h. After that the plants were transferred to vermiculite moistened with Fahraeus medium. Inoculation with *R. ruizarguesonis*, strain RCAM 1026 pHC60-*mCherry* or RCAM 1026 pHC60-*rosR* strain with the introduced *rosR* gene was performed 2 days later (2 ml of bacterial inoculum per plant with OD₆₀₀ = 0.5) and plants were incubated for 14 days after inoculation at room temperature with 16-h/ 8-h photoperiod. Plants transformed with the *GUS* gene that encodes beta-glucuronidase under constitutive promoter p35S were used as controls (*GUS* control) at the same stage after inoculation.

DNA Isolation and Quantitative Reverse Transcription PCR

DNA from plant roots was isolated using an extraction buffer (100-mM Tris-HCl (pH 8.0), 20-mM EDTA (pH 8.0), 20-mM 2-β-mercaptoethanol, 1,4-M NaCl, 2% CTAB) [41]. Quantitative reverse transcription PCR (qRT-PCR) analysis was performed using the CFX96 real-time system and iQ SYBR Green SuperMix (Bio-Rad Laboratories, USA). All primers (Table S1) were designed using the Vector NTI program and produced by the Evrogen company (www.evrogen.com). The specificity of the PCR amplification was verified using a dissociation curve (55–95 °C). The relative quantities of DNA were calculated as ratios relative to non-inoculated root expression levels. Data from two to three independent biological experiments were analyzed.

Measuring the Nitrogenase Activity

Pea roots with nodules were placed into the Falcon tubes with hermetically sealed lids at room temperature (approximately 50 mL of headspace atmosphere in the Falcon tube). 10% of the headspace atmosphere were replaced with 5-ml C_2H_2 using a 5-ml syringe with needle. Using a 1-mL syringe, aliquots of the headspace atmosphere were extracted in 24-h interval and the C_2H_4 production was measured by gas chromatography. A Shimadzu GC-2014 gas chromatograph equipped with GC SUS 2.0 m \times 5.0 mm column was used. Oven temperature was 70 °C with flame ionization detector (FID) temperature set to 70 °C. Flow rate of the nitrogen carrier gas was 75 mL/min. Total C_2H_4 production in the probe is calculated by evaluation the C_2H_4 peak area compared to calibration graphs for C_2H_4 .

Phylogenetic Analysis

To build a phylogenetic tree, amino acid sequences were used, which were aligned using the ClustalW method in the MEGA11 program [42]. Evolutionary relationships were reconstructed using the Maximum likelihood method and the JTT matrix model [43]. The publication shows the tree with the highest logarithmic probability (− 9499,06). The source trees for the heuristic search were generated automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then choosing a topology with a higher log-likelihood value. A discrete gamma distribution was used to model evolutionary rate differences between sites (5 categories (+ G, parameter = 0.7851)). This analysis involved 12 amino acid sequences. All positions containing spaces and missing data have been eliminated (complete removal option). In total, there were 678 positions in the final dataset. Evolutionary analysis was carried out in MEGA11 [42].

Statistical Analysis

One-way analysis of variance (ANOVA) was used to check differences in root length, fresh weight, and number of nodules. Data from two–three independent biological repeats were used for analysis. The Tukey's post hoc test was performed between all groups to determine the differences within the groups.

The analysis of changes in gene expression was carried out on the basis of 2–3 biological repeats and included 5 to 6 plants for each control and treated variants in one repeat. The threshold cycle (Ct) values were calculated using the Bio-Rad CFX Manager 1.6 program and analyzed using

the $2^{-\Delta\Delta Ct}$ method. Standard one-way analysis of variance (ANOVA) and Tukey post hoc test were performed.

Results

Effect of Enhanced EPS Synthesis in *Rhizobium ruizarguesonis* on the Nodulation in pea *P. sativum* L.

The *rosR* rhizobial gene encoding a transcriptional regulator was shown to be involved in the biosynthesis and regulation of EPS production in various rhizobial species [23, 29–31]. In this work, the *R. ruizarguesonis* gene that encodes the transcriptional regulator RosR was overproduced in a pHC60 plasmid and expressed in the *R. ruizarguesonis* RCAM 1026 rhizobial strain (Fig. S1). To estimate the production of EPS by rhizobia, the previously described method [23, 39] was applied in our experimental work. The EPS content in the rhizobial strain carrying the pHC60-*rosR* vector with an additional copy of the *rosR* gene was shown to be approximately two times higher than in the strain containing the empty vector pHC60-*mCherry* only (Table 1).

Therefore, it allowed us to estimate the effect of improved EPS synthesis in rhizobia on the development of symbiosis in pea plants. At 28 days after inoculation with rhizobial strains, pea plants were collected and the number of nodules on the roots, as well as the morphometric parameters of the plants were evaluated. The number of nodules was shown to increase significantly in plants inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* (Fig. 1). Additionally, we found an increase in root length and plant biomass in plants inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* (Fig. 2). It suggests that the enhanced EPS synthesis and secretion by rhizobia may stimulate plant root colonization and subsequent nodule formation in pea plants.

The influence of the RosR transcription regulator was also estimated for strains that form ineffective symbiosis with pea. As previously shown, the strain *R. ruizarguesonis* RCAM 1064 had decreased colonization of pea roots, which seems to be related to impaired EPS or LPS synthesis [44]. On pea roots infected with the *R. ruizarguesonis* RCAM 1064 strain, a large number of ineffective white nodules were observed (Fig. 1). However, when plants were inoculated

Table 1 The EPS content in the rhizobial strains

Strain	Concentration, mg/ml \pm SEM
<i>Rhizobium ruizarguesonis</i> RCAM 1026 pHC60- <i>mCherry</i>	0.258 \pm 0.031
<i>Rhizobium ruizarguesonis</i> RCAM 1026 pHC60- <i>rosR</i>	0.400 \pm 0.024

SEM standard error of the mean

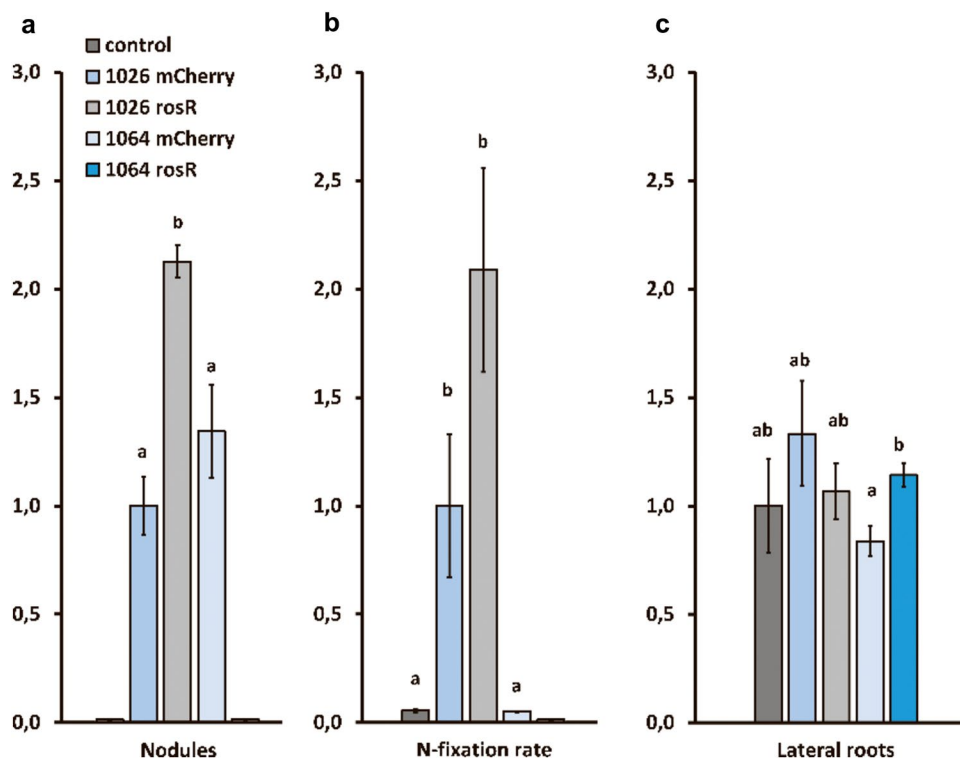


Fig. 1 Ratio of the number of nodules (a), nitrogenase activity (μl of C_2H_4 per plant) (b), and number of lateral roots (c) in plants inoculated with different effective *R. ruizarguesonis* RCAM 1026 and ineffective *R. ruizarguesonis* RCAM 1064 rhizobial strains. Effect of EPS overproduction was estimated in strains carrying the pHC60-*rosR* plasmid compared to pHC60-*mCherry* plasmid (without *rosR*). A, B—the values in different variants were compared with control RCAM 1026 strain carrying pHC60-*mCherry* plasmid (that was taken

as 1). The number of nodules were scored only in nodule-forming variants. C—the numbers of lateral roots in various variants were compared with control plants without inoculation (that was taken as 1). One-way analysis of variance (ANOVA) and Tukey post hoc test were performed. Different lower-case letters indicate significant difference among the various variants. Error bars indicate standard error of the mean of four-five replicates

with *R. ruizarguesonis* RCAM 1064 pHC60-*rosR*, practically no nodules were observed on the roots (Fig. 1). It demonstrates that overproduction of the RosR transcription regulator may prevent nodulation by this ineffective strain.

Resistance of the Pea Plant to the Phytopathogenic Fungus *Fusarium culmorum* in Response to Inoculation with the EPS-Overproducing Rhizobial Strain

In our experiments, the resistance of the pea plants to the phytopathogenic *Fusarium culmorum* fungus was also studied in response to inoculation with the EPS-overproducing strain (Fig. 3). Infection of pea plants with highly aggressive strain 334 of *F. culmorum* fungus [45] resulted in the development of root rot 21 days after incubation, when the root lesions became visible and resulted in their necrosis (Fig. 3a). However, co-inoculation of pea plants with the *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* strain significantly reduced disease symptoms, probably due to competition for available niches on the root surface or increasing

plant resistance to phytopathogen (Fig. 3a). Such plants had a higher fresh shoot weight compared to plants inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry* (Fig. 3b). The reduction in disease symptoms was less pronounced in plants inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry* (Fig. 3). Finally, it shows that enhanced EPS production by rhizobial strains may reduce plant susceptibility to *F. culmorum* infection.

Influence of Enhanced EPS Synthesis in Rhizobia on Colonizing Activity in Non-Legume Plant Tomato *S. lycopersicum*

As mentioned before, rhizobia are also able to colonize the surface of the roots of non-legume plants. Since such colonization may stimulate the growth and productivity of inoculated plants, the effect of enhanced EPS synthesis in rhizobia on colonizing activity in non-legume plants was also estimated. To test this influence, inoculation of tomato *S. lycopersicum* plants with rhizobial strains that differed in EPS production was performed in our experiments (Fig. 4).

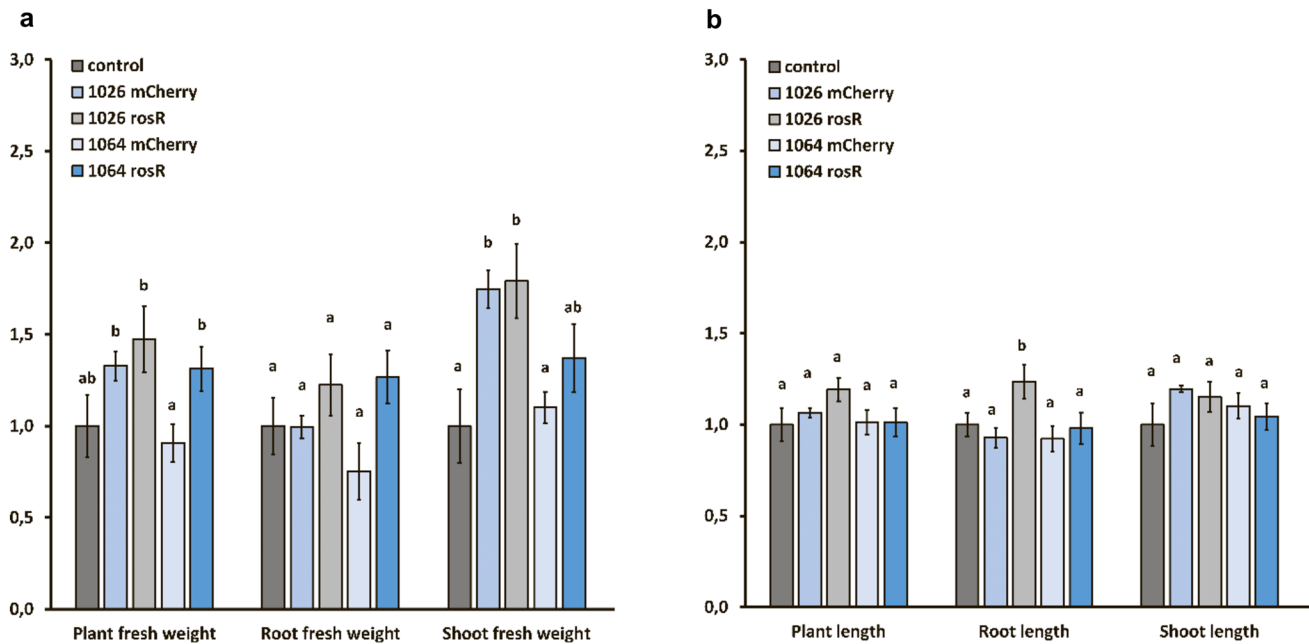


Fig. 2 Different growth parameters of pea plants after inoculation with effective *R. ruizarguesonis* RCAM 1026 and ineffective *R. ruizarguesonis* RCAM 1064 rhizobial strains. Effect of EPS overproduction was estimated in strains carrying the pHC60-*rosR* plasmids compared to pHC60-*mCherry* (without *rosR*) on plant fresh weight (a) and plant length (b). The values in different variants were com-

pared with control plants without inoculation (that was taken as 1). One-way analysis of variance (ANOVA) and Tukey post hoc test were performed. Different lower-case letters indicate significant difference among the various variants. Error bars indicate standard error of the mean of four-five replicates

To estimate the level of colonization of the root surface by rhizobia, the amount of rhizobial DNA in the root probes related to the amount of plant DNA was measured using qPCR (Fig. 4a). The significantly enhanced content of rhizobia was found on the root surface of tomato plants inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* compared to *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry*, using qPCR (Fig. 4a) and fluorescent microscopy (Fig. 4b,c). Therefore, the results of these experiments showed the positive influence of enhanced EPS production by rhizobia on colonization of the root surface of tomato *S. lycopersicum*.

Resistance of the Tomato Plants to the Phytopathogenic Fungus *Fusarium oxysporum* Schltdl. MFG 58284 Strain in Response to Inoculation with the EPS-Overproducing strain

The resistance of the tomato plants to the phytopathogenic *Fusarium oxysporum* Schltdl. MFG 58284 fungus was also studied in response to inoculation with the EPS-overproducing strain (Fig. 5a–c). Infection of tomato plants with *F. oxysporum* MFG 58284 fungus resulted in the leaf chlorosis and necrosis of leaf tips 14 days after infection. At the same time, co-inoculation of tomato plants with the *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* strain significantly reduced disease symptoms. Such plants had a higher number of

leaves and their weight compared to plants inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry* (Fig. 5a,b). The reduction in disease symptoms was less pronounced in plants inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry* (Fig. 5a–c). Finally, it shows that enhanced EPS production by rhizobial strains may reduce plant susceptibility to *F. oxysporum* infection.

Influence of Overproduction of Plant Receptor to EPS on rhizobia Colonizing Activity on the Root Surface of Legume and Non-Legume Plants

At the next stage of our research, we have tried to estimate the effect of increased production of putative receptor to EPS upon rhizobial colonization in legume and non-legume plants. The search for homologues of the genes encoding putative receptors to EPS, the LysM receptor-like kinases MtLYK10 and LjEPR3 in *M. truncatula* and *L. japonicus*, was carried out in the genomes of pea and other legume and non-legume plants using the coding sequences (CDSs) of *MtLYK10* and *LjEPR3* for analysis based on the BLASTN method (Table S2) [46].

Using these newly identified sequences, we were able to reconstruct the phylogenetic relationships between the homologues of MtLYK10 and LjEPR3 found in various agriculturally important crops (Fig. S3). The list of genes

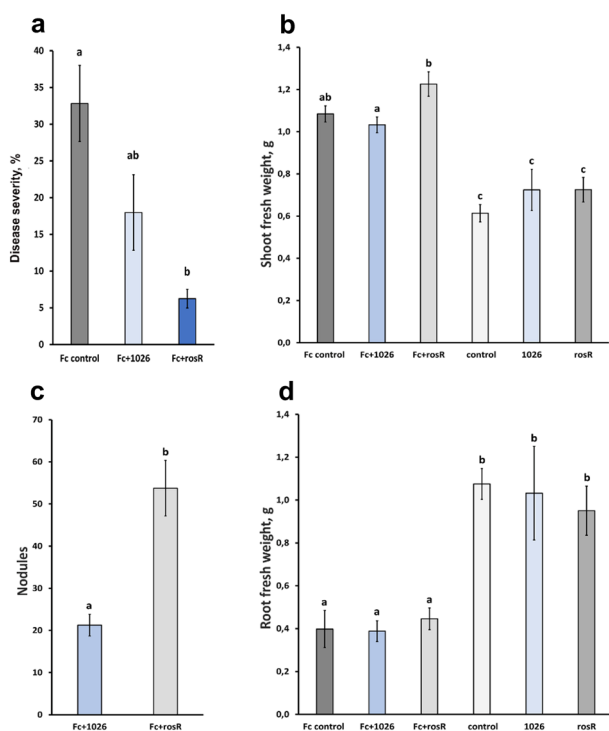


Fig. 3 Pea plant susceptibility to *Fusarium culmorum* infection. Disease severity (%) in plants inoculated with the *R. ruizarguesonis* RCAM 1026 pH60-*rosR* strain and *R. ruizarguesonis* RCAM 1026 pH60-mCherry along with the phytopathogenic fungus *Fusarium culmorum* 334 (a). Different lower-case letters indicate significant difference among the various variants. Error bars indicate standard error of the mean for 15–16 plants. Shoot fresh weight (b), number of nodules (c), and root fresh weight (d) in plants inoculated with the *R. ruizarguesonis* RCAM 1026 pH60-*rosR* strain and *R. ruizarguesonis* RCAM 1026 pH60-mCherry (without *rosR*) along with the phytopathogenic fungus *Fusarium culmorum* 334. One-way analysis of variance (ANOVA) and Tukey post hoc test were performed. Different lower-case letters indicate significant difference among the various variants. Error bars indicate standard error of the mean for 15–16 plants

and organisms is presented in Table S2. The LYK10/EPR3 homologues in *Pisum sativum*, *Cicer arietinum*, and *Medicago truncatula* form a single group with a common ancestor, such as LjEPR3. Therefore, *P. sativum* PsLYK10 is a homologue of MtLYK10 from *M. truncatula* and LjEPR3 from *Lotus japonicus*. The phylogenetic tree also showed that the LYK10 proteins of legumes form a single cluster, indicating their evolutionary relationship.

In the next step, experiments with the identified pea PsLYK10 homologue were performed and aimed to evaluate the effect of *PsLYK10* overexpression in legume (pea *P. sativum* L.) and non-legume (tomato *S. lycopersicum*) plants. To estimate the influence of this receptor on nodulation in legume plants, the composite pea plants carrying the construct p35S::*PsLYK10* in the transgenic roots (PsLYK10-OE) were obtained using *Agrobacterium rhizogenes* transformation

(Fig. 6a). Stimulation of *PsLYK10* expression in transgenic roots and nodules was about 3–4 times (Fig. 6a). Although we did not reveal a significant effect of *PsLYK10* overexpression on nodule number and root length in transgenic pea roots (Fig. 6b,c), our experiments showed the strong stimulation of the *PsDNF2*, *PsNADI*, *PsRSD*, and *PsSYMCRK* gene expression in PsLYK10-OE pea plants compared with control beta-glucuronidase overexpressing (GUS-OE) plants (Fig. 6d). These regulators are involved in the suppression of immune response in model legumes *M. truncatula* and *L. japonicus* [47]. Therefore, it demonstrates the importance of rhizobial EPS recognition in suppression of defense reactions in legume pea plants.

The experiments with overexpression of the pea *PsLYK10* gene under the constitutive promoter p35S were also performed in tomato *S. lycopersicum* (Fig. S4), but they did not reveal a significant influence the level of rhizobial colonization in such composite plants, in contrast to the effect of increased EPS synthesis in rhizobia upon tomato inoculation. As a consequence of approximately similar surface colonization by rhizobia, there was no essential difference in resistance to the phytopathogenic fungus *F. oxysporum* between tomato plants with overexpression of the *PsLYK10* gene (LYK10-OE) and GUS-control (GUS-OE) plants (Fig. S4). Probably, this result could be a consequence of the high enough content of the endogenous EPS receptor (the SILYK homologue from tomato) on the root surface in this plant. Indeed, in previous studies, a high level of similarity was revealed between MtLYK10/LjEPR3 from legume and their homologues from non-legume plants [28]. In addition, it may reflect the different influences of EPS perception on surface colonization by rhizobia in non-legume and intercellular rhizobial infection in legume plants.

Discussion

The interest in the investigation of rhizobial EPS is determined by their possible essential influence on the interaction between rhizobia and legume or non-legume plants. Previously, experiments with *exo*⁻ rhizobial mutants showed that production of EPS is required for the effective symbiosis of *R. leguminosarum* bv. *viciae* strains with host plants, pea *Pisum sativum*, and vetch *Vicia sativa*, which form indeterminate nodules [48–50]. Therefore, here we focus on a more detailed analysis of the effect of EPS in pea plants using an EPS-overproducing rhizobial strain and plants with overexpression of the gene that encodes the putative receptor to EPS. To obtain EPS-overproducing rhizobial strain, an additional copy of the gene encoding the transcriptional regulator RosR and controlling EPS biosynthesis was overproduced in the *R. ruizarguesonis* RCAM 1026 rhizobial strain.

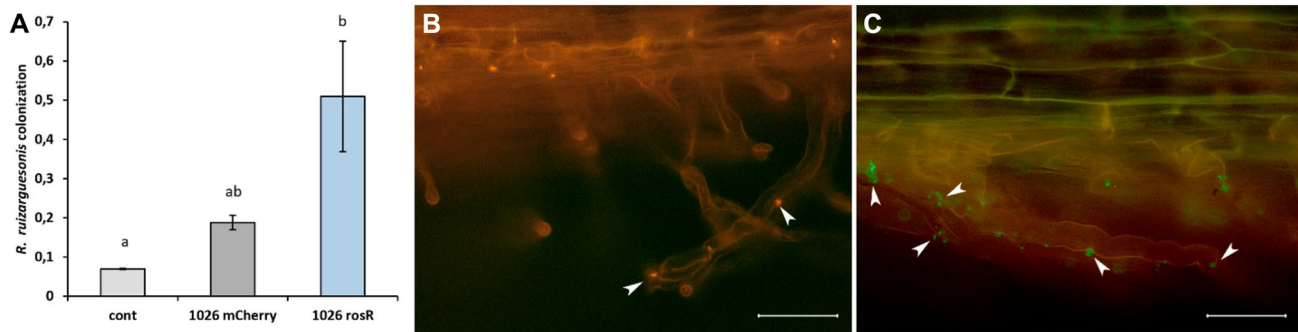


Fig. 4 Colonization of the surface of the tomato root by rhizobia *Rhizobium ruizarguesonis* RCAM 1026 pHC60-*rosR* compared to *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry* (without *rosR*), using qPCR (**a**) and fluorescent microscopy (**b**, **c**). Rhizobial colonization was determined by quantifying the expression of the *R. ruizarguesonis nodA* gene by qRT-PCR and calculating the number of gene copies, then normalizing these values to tomato *GAPDH* normalization factor was done. **B**—fluorescent image of tomato roots inocu-

lated with *R. ruizarguesonis* RCAM 1026 pHC60-*mCherry* (control). **C**—fluorescent image of tomato roots inoculated with *R. ruizarguesonis* RCAM 1026 pHC60-*rosR*. Scale bars=50 μ m. Standard one-way analysis of variance (ANOVA) and Tukey post hoc test were performed. Different lower-case letters indicate significant difference among the various variants. Bars indicate standard error of the mean for 5–6 plants from 2 independent biological experiments. White arrows indicate labeled rhizobia on the surface of the root

Indeed, our experiments showed the stimulating effect of enhanced EPS production in the RCAM 1026 strain carrying pHC60-*rosR* on nodulation. Pea inoculation with this recombinant strain stimulated the development of infection and resulted in increased amounts of nodules as well as stimulation of plant weight in these plants. These results were in accordance with the effect of *R. leguminosarum* bv. *trifolii* on clover nodulation, which also forms indeterminate nodules [23, 30, 51]. Furthermore, co-inoculation of the pea plant with the strain *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* significantly reduced disease symptoms caused by infection with highly aggressive strain 334 of *F. culmorum*. It demonstrates that inoculation of pea plants by rhizobia with increased EPS production may stimulate their resistance to phytopathogenic fungi. Therefore, these rhizobial strains may be applied as one of the biological control agents.

It was previously suggested that the *R. ruizarguesonis* RCAM 1064 strain forming ineffective white nodules in pea roots appears to be impaired in the synthesis of EPS or LPS [44]. Indeed, in this work the introduction of an additional copy of the *rosR* gene in this rhizobial strain resulted in complete blocking of the development of symbiosis in plants inoculated with *R. ruizarguesonis* RCAM 1064 pHC60-*rosR*. A possible reason for this effect may be the synthesis of structurally changed EPS in RCAM 1064 due to the mutation in the gene controlling EPS biosynthesis, which in the case of overproduction driven by the RosR transcription regulator may completely prevent nodulation. Therefore, it suggests that *R. ruizarguesonis* RCAM 1064 may be an *exo*⁻ rhizobial mutant, which should be verified by future genome sequencing for this strain. In any case, an *exo*⁻ rhizobial mutant can be a very useful for future

experiments aimed on the evaluation of EPS influence on symbiosis in pea plants.

Rhizobia are able not only to colonize the surface of the roots of legume plants, but may form an association with non-legume plants, stimulating their growth and development. The enhanced EPS synthesis in rhizobia increased the colonization of the root surface of non-legume plants, as shown in our experiments. Moreover, it resulted in stimulation of the shoot mass of inoculated plants as well as other growth parameters. Inoculation with EPS-enhanced *R. ruizarguesonis* strain essentially increased plant resistance to phytopathogenic fungus *F. oxysporum* infection. It allows us to consider the *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* rhizobial strain as a perspective biocontrol agent not only for legume but also for non-legume plants.

In recognition of rhizobial EPS in legume plants, specific receptor-like kinases are involved that have been recently described in *Lotus japonicus* and *Medicago truncatula* [24, 26]. They belong to the Lysin motif type (LysM type) of receptors, but have a specific structure of the ectodomain made up of three putative carbohydrate-binding modules (M1, M2, and LysM3), where M1 and M2 have unique $\beta\alpha\beta$ and $\beta\alpha\beta$ folds, while LysM3 has a canonical $\beta\alpha\alpha\beta$ fold [28]. We suggested that overexpression of this type of receptor in legume and non-legume plants may stimulate symbiotic nodulation and surface colonization in non-legume plants. Indeed, stimulation of well-known regulators such as DNF2, NAD1, RSD, and SYMCRK involved in the suppression of immune response was found to be activated in pea plants with overexpression of PsLYK10 LysM-RLK to EPSs. It demonstrates the importance of rhizobial EPS recognition in suppression of defense reactions in legume plants. However, it should be

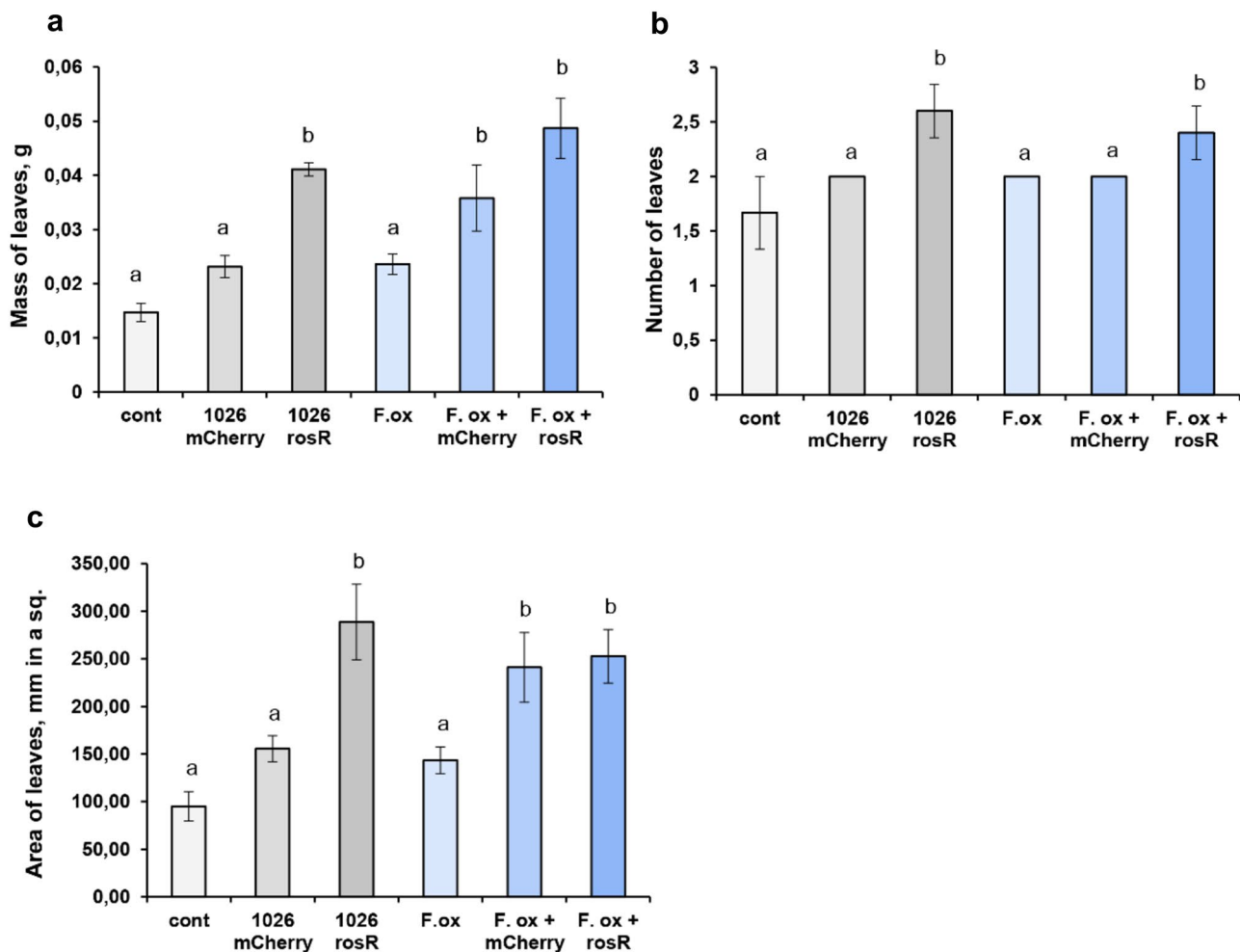


Fig. 5 Tomato plant susceptibility to *Fusarium oxysporum* Schldl. MFG 58284 infection. Mass of leaves (a), number of leaves (b), and area of leaves (c) in plants inoculated with the *R. ruizarguesonis* RCAM 1026 pHC60-*rosR* strain and *R. ruizarguesonis* RCAM 1026 pHC60-mCherry along (gray columns) and with the phytopathogenic

fungus *F. oxysporum* MFG 58284 (blue columns). One-way analysis of variance (ANOVA) and Tukey post hoc test were performed. Different lower-case letters indicate significant difference among the various variants. Error bars indicate standard error of the mean for 15–16 plants

noted that although some growth stimulation of pea plants with overexpression of the *PsLYK10* gene was found, it did not result in a significantly increased number of nodules in such plants compared with the EPS-overproducing rhizobial strain.

In non-legume composite tomato plants, carrying the *PsLYK10* gene from pea in transgenic roots, we also did not find any significant difference in root surface colonization compared to control plants transformed with the *GUS* gene under constitutive promoter (control GUS-OE). Additionally, there were no essential differences in resistance to the phytopathogenic fungus *F. oxysporum* between tomato plants with overexpression of the *PsLYK10* gene (LYK10-OE) and GUS control plants (GUS-OE). Finally, it may be concluded that biotechnological approaches aimed at stimulating EPS synthesis in rhizobia may be more effective

compared to those connected with plant modification to increase their susceptibility to EPS recognition.

Conclusion

Bacterial EPS provide stabilization of membranes against external factors, as well as improved surface adhesion. In this work, the transcriptional regulator RosR of *R. ruizarguesonis*, which regulates the synthesis of EPS, was overproduced in a pHC60 plasmid and expressed in the rhizobia. This resulted in an improved production of EPS by this recombinant strain. The number of nodules as well as root length and biomass were shown to increase significantly in pea plants inoculated with RCAM 1026 pHC60-*rosR*. It suggests that the enhanced EPS synthesis

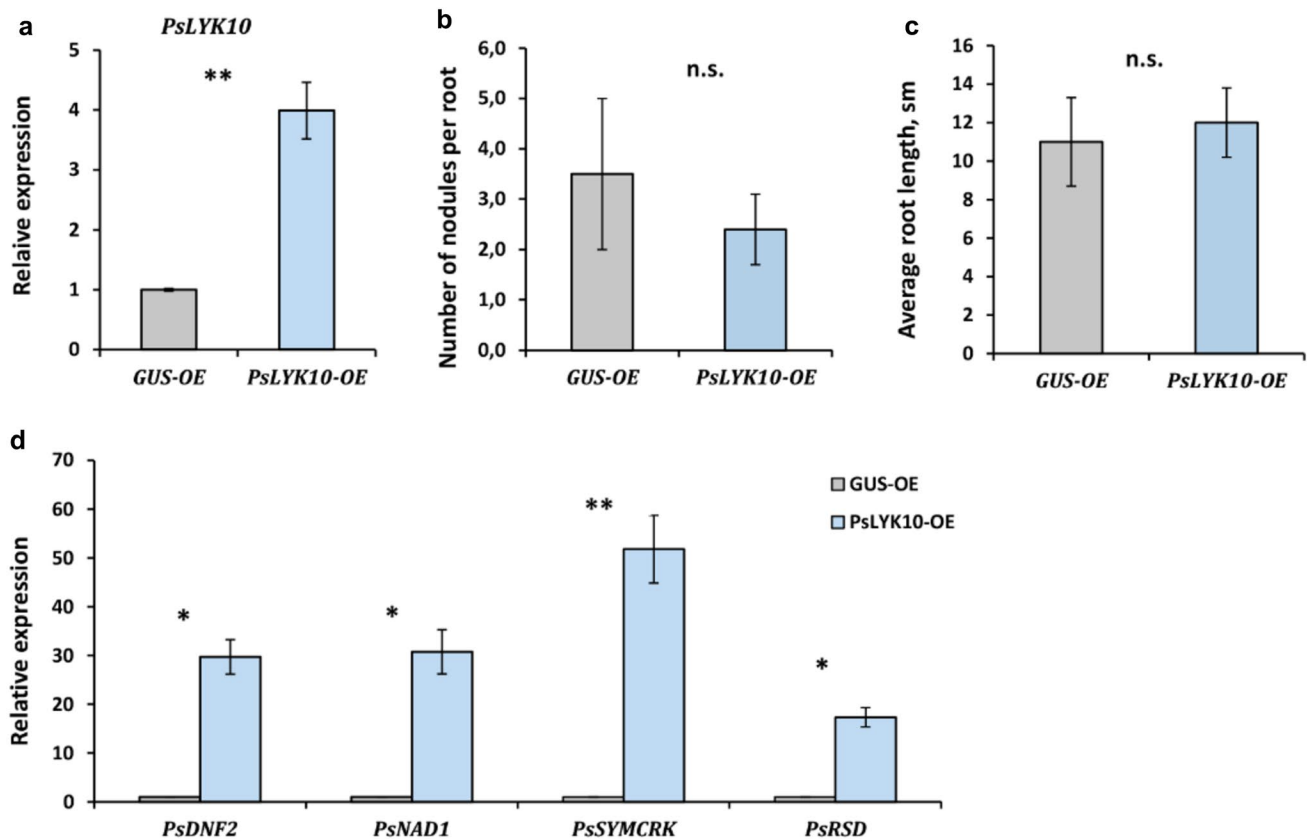


Fig. 6 Number of nodules, root length, and *PsDNF2*, *PsNAD1*, *PsSymCRK*, and *PsRSD* gene expression in *Pisum sativum* plants cv. Frisson with overexpression of the *PsLYK10* gene that encodes the receptor for EPS under the constitutive promoter p35S (*PsLYK10*-OE) compared to control plants (*GUS*-OE) 21 days after inoculation with *R. ruizarguesonis* RCAM 1026. Number of nodules (**b**), average root length (**c**), and *PsDNF2*, *PsNAD1*, *PsSymCRK*, and *PsRSD* gene

expression (**d**). The number of nodules and root length were scored only in transgenic fluorescent roots. Gene expression was evaluated in transgenic nodules of *GUS*-OE and *PsLYK10*-OE plants. Error bars indicate standard error of the mean for 5–6 composite plants. *—significant difference at $P \leq 0.05$; **—significant difference at $P \leq 0.01$; n.s.—non-significant difference

and secretion by rhizobia may stimulate plant root colonization and subsequent nodule formation in pea plants. In accordance with this, the introduction of additional copy of *rosR* gene in putative *exo*⁻ rhizobial strain RCAM 1064 completely abolished symbiosis development. The influence of enhanced EPS synthesis in rhizobia on colonizing activity was also estimated in non-legume plant tomato *S. lycopersicum*. Our findings shown the increased colonization of the root surface and stimulation of the shoot biomass of inoculated tomato plants. Finally, inoculation of pea and tomato with EPS-overproducing *R. ruizarguesonis* strain essentially increased plant resistance to phytopathogenic fungi in both legume and non-legume plants, demonstrating a significant biocontrol effect of this recombinant strain. At the same time, overexpression of *PsLYK10* gene that encodes a putative EPS receptor showed an essential effect on suppression of defense reactions in legume plants.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00284-024-03929-w>.

Acknowledgements The research was carried out using equipment from the Core Centrum ‘Genomic Technologies, Proteomics and Cell Biology’ in ARRIAM.

Author Contributions E.K. wrote the main manuscript text, prepared figures. A.B. obtained the recombinant strain, prepared figures. A.D. made plant transformation. N.K. performed the experiments, data analysis. A.S. performed the experiments with plants. E.S. contributed to data analysis. O.P. made the gene expression analysis. O.Y. performed gas chromatography. N.V. analyzed the plant disease severity. E.D. contributed to conceptualization, writing and editing of the manuscript, and supervision. All authors reviewed the manuscript.

Funding The article was made with the support of the Ministry of Science and Higher Education of the Russian Federation in accordance with the agreement. N 075–15–2022–320 date 20 April 2022, on providing a grant in the form of subsidies from the Federal budget of the Russian Federation. The grant was provided for state support

for the creation and development of a World-class Scientific Center “Agrotechnologies for the Future.”

Data Availability The authors declare that all data supporting the findings of this study are available in this article and its Supplementary Information files.

Declarations

Competing Interests The authors declare no competing interests.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

References

- Flores-Félix J-D, Marcos-García M, Silva LR et al (2015) Rhizobium as plant probiotic for strawberry production under microcosm conditions. *Symbiosis* 67:25–32. <https://doi.org/10.1007/s13199-015-0373-8>
- Flores-Félix JD, Menéndez E, Rivera LP et al (2013) Use of Rhizobium leguminosarum as a potential biofertilizer for Lactuca sativa and Daucus carota crops. *J Plant Nutr Soil Sci* 176:876–882. <https://doi.org/10.1002/jpln.201300116>
- Jiménez-Gómez A, Flores-Félix JD, García-Fraile P et al (2018) Probiotic activities of Rhizobium laguerreae on growth and quality of spinach. *Sci Rep* 8:295. <https://doi.org/10.1038/s41598-017-18632-z>
- Reddy PM, Ladha JK, So RB et al (1997) Rhizobial communication with rice roots: Induction of phenotypic changes, mode of invasion and extent of colonization. *Opportunities for Biological Nitrogen Fixation in Rice and Other Non-Legumes*. Springer, Dordrecht, pp 81–98
- García-Fraile P, Carro L, Robledo M et al (2012) Rhizobium promotes non-legumes growth and quality in several production steps: towards a biofertilization of edible raw vegetables healthy for humans. *PLoS ONE* 7:e38122. <https://doi.org/10.1371/journal.pone.0038122>
- Little BJ, Wagner P, Maki JS et al (1986) Factors influencing the adhesion of microorganisms to surfaces. *J Adhes* 20:187–210. <https://doi.org/10.1080/00218468608071236>
- Smit G, Swart S, Lugtenberg BJJ, Kijne JW (1992) Molecular mechanisms of attachment of Rhizobium bacteria to plant roots. *Mol Microbiol* 6:2897–2903. <https://doi.org/10.1111/j.1365-2958.1992.tb01748.x>
- Ausmees N, Jacobsson K, Lindberg M (2001) A unipolarly located, cell-surface-associated agglutinin, RapA, belongs to a family of Rhizobium-adhering proteins (Rap) in Rhizobium leguminosarum bv. trifolii. *Microbiology* 147:549–559. <https://doi.org/10.1099/00221287-147-3-549>
- Mongiardini EJ, Pérez-Giménez J, Althabegoiti MJ et al (2009) Overproduction of the rhizobial adhesin RapA1 increases competitiveness for nodulation. *Soil Biol Biochem* 41:2017–2020. <https://doi.org/10.1016/j.soilbio.2009.07.016>
- Dazzo FB, Truchet GL, Sherwood JE et al (1984) Specific phases of root hair attachment in the Rhizobium trifolii-clover symbiosis. *Appl Environ Microbiol* 48:1140–1150. <https://doi.org/10.1128/aem.48.6.1140-1150.1984>
- Via VD, Zanetti ME, Blanco F (2016) How legumes recognize rhizobia. *Plant Signal Behav* 11:e1120396. <https://doi.org/10.1080/15592324.2015.1120396>
- Hirsch AM (1999) Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Curr Opin Plant Biol* 2:320–326. [https://doi.org/10.1016/S1369-5266\(99\)80056-9](https://doi.org/10.1016/S1369-5266(99)80056-9)
- Laus MC, Logman TJ, Lamers GE et al (2006) A novel polar surface polysaccharide from Rhizobium leguminosarum binds host plant lectin. *Mol Microbiol* 59:1704–1713. <https://doi.org/10.1111/j.1365-2958.2006.05057.x>
- Hoedemaeker FJ, Richardson M, Díaz CL et al (1994) Pea (Pisum sativum L.) seed isolectins 1 and 2 and pea root lectin result from carboxypeptidase-like processing of a single gene product. *Plant Mol Biol* 24:75–81. <https://doi.org/10.1007/BF00040575>
- Vershinina ZR, Baymiev AK, Blagova DK et al (2012) Artificial colonization of non-symbiotic plants roots with the use of lectins. *Symbiosis* 56:25–33. <https://doi.org/10.1007/s13199-012-0156-4>
- Mithöfer A (2002) Suppression of plant defence in rhizobia-legume symbiosis. *Trends Plant Sci* 7:440–444. [https://doi.org/10.1016/S1360-1385\(02\)02336-1](https://doi.org/10.1016/S1360-1385(02)02336-1)
- Oleńska E, Małek W, Kotowska U et al (2021) Exopolysaccharide carbohydrate structure and biofilm formation by Rhizobium leguminosarum bv. trifolii strains inhabiting nodules of trifolium-repens growing on an old Zn–Pb–Cd-polluted waste heap area. *Int J Mol Sci* 22:2808. <https://doi.org/10.3390/ijms22062808>
- Jones KM, Kobayashi H, Davies BW et al (2007) How rhizobial symbionts invade plants: the sinorhizobium—medicago model. *Nat Rev Microbiol* 5:619–633. <https://doi.org/10.1038/nrmicro1705>
- Dickstein R, Bisseling T, Reinhold VN, Ausubel FM (1988) Expression of nodule-specific genes in alfalfa root nodules blocked at an early stage of development. *Genes Dev* 2:677–687. <https://doi.org/10.1101/gad.2.6.677>
- Cheng HP, Walker GC (1998) 1998-succinoglycan is required for initiation and elongation of infection threads.pdf. *J Bacteriol* 180:5183–5191
- Janczarek M, Kutkowska J, Piersiak T, Skorupska A (2010) Rhizobium leguminosarum bv. trifolii rosR is required for interaction with clover, biofilm formation and adaptation to the environment. *BMC Microbiol* 10:284. <https://doi.org/10.1186/1471-2180-10-284>
- Jones KM, Kobayashi H, Davies BW et al (2007) How rhizobial symbionts invade plants: the sinorhizobium—medicago model. *Nat Rev Microbiol* 5:619–633. <https://doi.org/10.1038/nrmicro1705>
- Janczarek M, Jaroszuk-Ścisiel J, Skorupska A (2009) Multiple copies of rosR and pssA genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in Rhizobium leguminosarum bv. trifolii. *Antonie Van Leeuwenhoek* 96:471–486. <https://doi.org/10.1007/s10482-009-9362-3>
- Kawaharada Y, Kelly S, Nielsen MW et al (2015) Receptor-mediated exopolysaccharide perception controls bacterial infection. *Nature* 523:308–312. <https://doi.org/10.1038/nature14611>
- Kawaharada Y, Nielsen MW, Kelly S et al (2017) Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia. *Nat Commun* 8:14534. <https://doi.org/10.1038/ncomms14534>
- Maillet F, Fournier J, Mendis HC et al (2020) Sinorhizobium meliloti succinylated high-molecular-weight succinoglycan and the Medicago truncatula LysM receptor-like kinase MtLYK10 participate independently in symbiotic infection. *Plant J* 102:311–326. <https://doi.org/10.1111/tpj.14625>
- Dupin S, Klein J, Rutten L et al (2022) Pseudogenization of the rhizobium-responsive EXOPOLYSACCHARIDE RECEPTOR in Parasponia is a rare event in nodulating plants. *BMC Plant Biol* 22:225. <https://doi.org/10.1186/s12870-022-03606-9>
- Wong JEMM, Gysel K, Birkefeldt TG et al (2020) Structural signatures in EPR3 define a unique class of plant carbohydrate receptors. *Nat Commun* 11:3797. <https://doi.org/10.1038/s41467-020-17568-9>

29. Rachwał K, Matczyńska E, Janczarek M (2015) Transcriptome profiling of a *Rhizobium leguminosarum* bv. trifolii *rosR* mutant reveals the role of the transcriptional regulator *RosR* in motility, synthesis of cell-surface components, and other cellular processes. *BMC Genomics* 16:1111. <https://doi.org/10.1186/s12864-015-2332-4>
30. Rachwał K, Boguszewska A, Kopcińska J et al (2016) The regulatory protein *RosR* affects *rhizobium leguminosarum* bv. trifolii protein profiles, cell surface properties, and symbiosis with clover. *Front Microbiol* 7:1–21. <https://doi.org/10.3389/fmicb.2016.01302>
31. Janczarek M, Skorupska A (2007) The *rhizobium leguminosarum* bv. trifolii *RosR*: transcriptional regulator involved in exopolysaccharide production. *Mol Plant-Microbe Interact* 20:867–881. <https://doi.org/10.1094/MPMI-20-7-0867>
32. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant* 15:26
33. Al-Surhane AA, Afzal M, Bouqellah NA et al (2021) The antifungal activity of ag/chl nps against *rhizoctonia solani* linked with tomato plant health. *Plants* 10:1–19. <https://doi.org/10.3390/plant10112283>
34. Beccari G, Covarelli L, Nicholson P (2011) Infection processes and soft wheat response to root rot and crown rot caused by *Fusarium culmorum*. *Plant Pathol* 60:671–684. <https://doi.org/10.1111/j.1365-3059.2011.02425.x>
35. Syrova DS, Shaposhnikov AI, Makarova NM et al (2019) Prevalence of the ability to produce abscisic acid in phytopathogenic fungi. *Mikol I Fitopatol* 53:301–310. <https://doi.org/10.1134/S0026364819050064>
36. Krall L, Wiedemann U, Unsin G et al (2002) Detergent extraction identifies different *VirB* protein subassemblies of the type IV secretion machinery in the membranes of *Agrobacterium tumefaciens*. *Proc Natl Acad Sci* 99:11405–11410. <https://doi.org/10.1073/pnas.172390699>
37. van Brussel AA, Tak T, Wetselaar A et al (1982) Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other rhizobia and agrobacteria harbouring a leguminosarum sym-plasmid. *Plant Sci Lett* 27:317–325. [https://doi.org/10.1016/0304-4211\(82\)90134-1](https://doi.org/10.1016/0304-4211(82)90134-1)
38. Orosz L, Sváb Z, Kondorosi Á, Sik T (1973) Genetic studies on *Rhizobiophage* 16–3. *Mol Gen Genet MGG* 125:341–350. <https://doi.org/10.1007/BF00276589>
39. Loewus FA (1952) Improvement in anthrone method for determination of carbohydrates errors in volumetric analysis arising from adsorption. *Anal Chem* 24:219–219
40. Lullien V, Barker DG, de Lajudie P, Huguët T (1987) Plant gene expression in effective and ineffective root nodules of alfalfa (*Medicago sativa*). *Plant Mol Biol* 9:469–478. <https://doi.org/10.1007/BF00015878>
41. Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. Third Edition
42. Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. *Mol Biol Evol* 38:3022–3027. <https://doi.org/10.1093/molbev/msab120>
43. Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices. *Bioinformatics* 8:275–282
44. Kuzakova OV, Lomovatskaya LA, Goncharova AM, Romanenko AS (2019) Effects of *rhizobium leguminosarum* bv. *viciae* strains different in their symbiotic effectiveness on changes in cAMP and hydrogen peroxide concentrations in cells of pea seedlings. *Russ J Plant Physiol* 66:712–717. <https://doi.org/10.1134/S1021443719050121>
45. Leppänen IV, Shakhnazarova VY, Shtark OY et al (2017) Receptor-like kinase LYK9 in *Pisum sativum* L. Is the CERK1-like receptor that controls both plant immunity and AM symbiosis development. *Int J Mol Sci* 19:8. <https://doi.org/10.3390/ijms19010008>
46. Camacho C, Coulouris G, Avagyan V et al (2009) BLAST+: architecture and applications. *BMC Bioinformatics* 10:1–9. <https://doi.org/10.1186/1471-2105-10-421>
47. Berrabah F, Ratet P, Gourion B (2015) Multiple steps control immunity during the intracellular accommodation of rhizobia. *J Exp Bot* 66:1977–1985. <https://doi.org/10.1093/jxb/eru545>
48. Canter Cremers HC, Batley M, Redmond JW et al (1990) *Rhizobium leguminosarum* *exoB* mutants are deficient in the synthesis of UDP-glucose 4'-epimerase. *J Biol Chem* 265:21122–21127
49. Van Workum WAT, Van Slageren S, Van Brussel AAN, Kijne JW (1998) Role of exopolysaccharides of *Rhizobium leguminosarum* bv. *viciae* as host plant-specific molecules required for infection thread formation during nodulation of *Vicia sativa*. *Mol Plant-Microbe Interact* 11:1233–1241. <https://doi.org/10.1094/MPMI.1998.11.12.1233>
50. Kijne JW, Smit G, Díaz CL, Lugtenberg BJ (1988) Lectin-enhanced accumulation of manganese-limited *Rhizobium leguminosarum* cells on pea root hair tips. *J Bacteriol* 170:2994–3000. <https://doi.org/10.1128/jb.170.7.2994-3000.1988>
51. Vershinina ZR, Chubukova OV, Nikonov YM et al (2021) Effect of *rosR* gene overexpression on biofilm formation by *rhizobium leguminosarum*. *Microbiology* 90:198–209. <https://doi.org/10.1134/S0026261721020144>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.