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Effect of *Rhizobium* mechanisms in improving tolerance to saline stress in lettuce plants

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Abstract

Background Soils affected by salinity are a recurring problem that is continually increasing due to the impact of climate change on weather conditions and ineffective agricultural management practices. The use of plant growth promoting (PGP) Bacteria can alleviate its effects. In this regard, the genus *Rhizobium* has demonstrated excellent PGP capabilities through various plant growth promotion mechanisms and may therefore be a promising biofortifier under saline conditions. However, little is known about the production of volatile organic compounds (VOCs) by bacteria of this genus and their effects on plant development. Here, we aim to characterize the volatilome (the set of volatile metabolites synthesized by an organism) of *Rhizobium* for the first time and to further investigate the direct and VOC-mediated interaction between a strain of this genus and lettuce, a crop severely affected by salinity, both under saline and non-saline conditions.

Results In this study, it was shown that the use of *Rhizobium* sp. GPTR29 was able to increase the production of lettuce (*Lactuca sativa* L.) under normal and saline conditions. We analyzed the *Rhizobium* volatilome under non-saline (0 mM NaCl) and saline (100 mM NaCl) conditions by HS-SPME-GC−MS and found a differential composition in response to salinity. We detected 20 different compounds, where 3-methyl-1-butanol, 2-methyl-1-butanol, and α-pinene were the backbone of the *Rhizobium* volatilome. Exposure to these compounds in bicameral plates under salt stress resulted in increases in plant development of 17.1%, 16.0% and 33.1% in aerial part size, number of leaves and root length, respectively. Under greenhouse conditions and salinity, the inoculation of *Rhizobium* sp. GPTR29 resulted in an increase of 17.8% and 27.4% in shoot fresh and dry weight, respectively. Phenolic compounds were analyzed by HPLC–DAD-MS, revealing an increase in total flavonoid content under salinity conditions (100 mM NaCl) and apigenin derivative, luteolin 7-*O*-glucoside and quercetin 3-*O*-glucuronide individually.

Conclusions These results provide new avenues for the study of PGP mechanisms in this bacterial genus, such as VOCs and their effects on plant growth, which play an important role in mediating plant–microorganism interactions.

Keywords *Rhizobium*, Lettuce, Salinity, Volatile organic compounds (VOCs), Phenolics, Flavonoids

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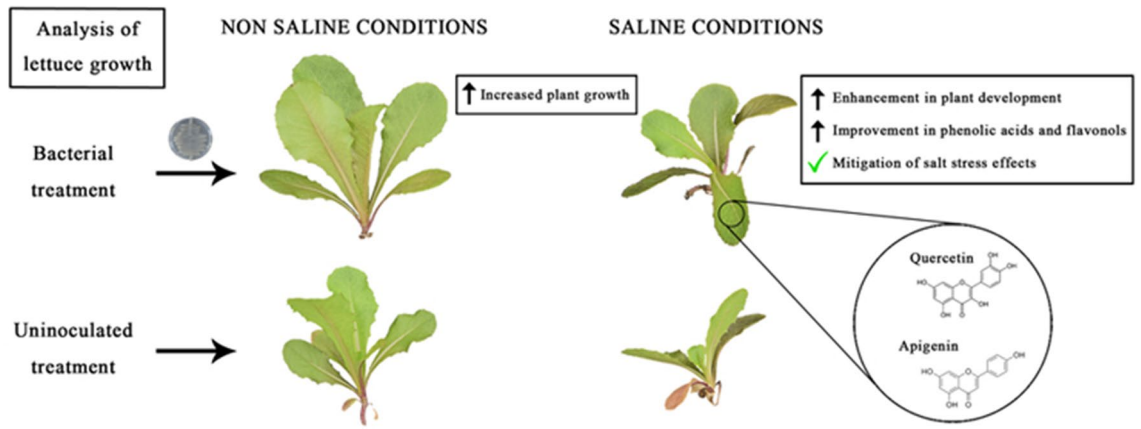
Graphical abstract



Introduction

The accumulation of soluble salts in the soil due to natural or anthropogenic factors leads to soil salinity, which is considered the second most important cause of soil degradation after soil erosion, mainly in arid and semi-arid regions [48]. According to the Food and Agriculture Organization (FAO) of the United Nations and other scientific literature, more than 900 million hectares could be affected by salt, and approximately 240 million hectares could be affected in the Eurasian region [2, 15, 34]. Moreover, the rate of land degraded by salinity is estimated to increase annually by 10% due to factors such as climate change, overuse of fertilizers, inappropriate irrigation practices, sea water intrusion, and other natural processes (weathering of rocks or saline parent material) [23, 48]. The economic impact of this abiotic stress on agricultural activity has been estimated to be $27.3 billion per year due to lost crop production [34, 48].

The most important ions associated with soil salinity are Na^+ and Cl^- due to their negative effects on plants and soil, although other cations and anions (Ca^{2+}, Mg^{2+}, SO_4^{2-} or HCO_3^-) may also have an influence [46]. In this sense, soil salinity affects various aspects of plant growth and development by imposing osmotic stress, ion toxicity, oxidative stress, and nutrient deficit (limiting the uptake of macro- and micronutrients) [5, 7, 34, 35]. All these negative effects alter different aspects of plant development, such as germination, vegetative growth, or reproductive development [35].

Crop yield usually decreases when salt concentrations exceed the threshold of 4 dS m^{-1}, with a wide range between plant species and cultivars: halophytes are plants able to grow in highly saline soils (tolerant plants), while glycophytes are plants unable to withstand certain saline conditions (sensitive plants) [5, 23, 35, 52]. Glycophytes include most food crops, where growth inhibition or death occurs from 100 to 200 mM salt, as in the case of rice or wheat [35, 44]. Studies in India estimate that the yield loss of these crops in saline soils is more than 40% compared to non-saline soils [40]. This also occurs in different horticultural crops, therefore this study focuses on lettuce (*Lactuca sativa* L.), a vegetable crop considered to be sensitive to salinity, as it has a salinity stress tolerance threshold of 1.3 dS m^{-1} [46]. Nevertheless, it is one of the most economically important leafy vegetable crops in the world due to its outstanding nutritional composition: a high content of antioxidant compounds, primarily vitamin C and polyphenols, as well as fiber [28, 47]. It is widely cultivated in temperate areas around the globe, and more than 25 million tons are produced annually [14].

In the face of this unrelenting problem, the search for environmentally sustainable alternatives that can meet the demand for food must be considered a top priority. The use of viable and cost-effective strategies, such as the application of bacterial biofortifiers, is therefore a critical issue. These are microorganisms that are able to improve the nutritional capacity of crops by different plant growth promoting (PGP) mechanisms, increasing nutritional value and quality of the final products [21]. *Rhizobium* genus has been widely described for improving the productivity and nutritional content of different crops with which they are associated, not only legumes, but also horticultural crops [7, 17–19, 24, 25]. All these articles highlight the role of strains belonging to this genus as probiotics of non-legumes, thanks to the effect

of different PGP mechanisms, such as phosphate solubilization, production of siderophores, phytohormones or exopolysaccharides, among others. This genus has also demonstrated its ability to reduce salinity incidence in legume crops by regulating oxidative stress, balancing phytohormone levels and secreting osmolytes [8, 56]. Although a small number of studies have been carried out with non-legume crops and saline conditions, such as lettuce [6] and canola [45], revealing excellent capabilities between salt-tolerant strains.

However, there is a lack of knowledge about which VOCs *Rhizobium* strains are able to produce and their effect as plant growth promoters. VOCs are carbon-containing chemical substances with low molecular masses that can be emitted by rhizobacteria [5]. It has been reported that microbial volatiles can induce resistance to diseases and stimulate plant growth, and other studies have indicated the effect of these substances in alleviating salt stress in plants [5, 11, 52, 53]. All these findings support the hypothesis that VOCs production is relevant for the promotion of plant growth and tolerance to salt stress. Therefore, characterization of the volatilome profile produced by strains of this genus, as well as their possible effects on plants, could provide further insight into mechanisms to help with this serious problem.

Furthermore, plants developed under conditions of abiotic stresses induce the synthesis of ROS in response to these conditions, which in turn leads to an accumulation of phenolic compounds with antioxidant activity [36]. In recent years, it has also been described how inoculation with PGPR is capable of inducing an enhancement in the concentration of phenolic compounds under non-stress conditions, as well as under salinity conditions [6, 17, 20, 25, 26]. However, the molecular mechanisms that mediate this response are not clear and have not been studied in depth, as the response in antioxidant composition varies depending on the inoculum used or the growth conditions of the plants (such as the salinity level) [6, 25, 26, 29, 37, 47]. Hence the importance of studying each interaction individually.

In this study, we aimed to unveil the ability of a potential new species within the genus *Rhizobium* to promote lettuce growth and help mitigate the effects of salt stress on this horticultural crop. To do that, we evaluated in vitro classical PGP mechanisms of this strain, such as phosphate solubilization, colonization of the lettuce root system, or production of indole acetic acid (IAA) and siderophores. Additionally, we were able to identify for the first time some VOCs produced by a strain of the genus *Rhizobium* under normal (0 mM NaCl) and saline (100 mM NaCl) growth conditions, also analyzing the effect of these volatiles on lettuce growth. We further identified the potential genetic machinery involved in these processes. Our experiments also evaluated the effect of direct inoculation of this isolate on different productive variables in lettuce plants, with and without salinity, as well as the possible change in the content of bioactive compounds under the different growth conditions. Taken together, our results suggest that the direct effect of *Rhizobium* sp. GPTR29, as well as its emitted VOCs, could have a positive effect on lettuce growth and nutritional content, even under salt stress conditions.

Materials and methods

Isolation, draft genome sequencing, annotation, and phylogenomic analysis of the strain

For the present study, the GPTR29 strain was isolated from within a surface-sterilized effective nodule (pink or dark pink color) of *Trifolium repens* L. grown for 40 days in a soil of Golpejas, Salamanca, Spain (41° 0′ 56.94″N; 5° 55′ 20.07″W). The isolation method was previously described by Vincent [55] on YMA (yeast mannitol agar) plates that were maintained at 28 °C. This medium was employed until pure cultures were obtained.

Bacterial genomic DNA was obtained from pure colonies of the GPTR29 strain, which were grown on TY plates and collected after 48 h at 28 °C, using the Quick-DNA™ Fungal/Bacterial MiniPrep Kit (Zymo Research, Orange, CA, USA).

The Illumina MiSeq platform was used to obtain the draft genome sequence via a paired-end run (2×250 bp) by shotgun sequencing at Microbes NG Ltd. (Birmingham, UK). The sequence data were assembled using Velvet 1.2.10 [59]. Identification of the strain using genomic information was conducted using the Type (Strain) Genome Server [33], which is a platform that employs the Genome-BLAST Distance Phylogeny method (GBDP) [32] to compare whole genome sequences at the nucleotide level. Gene annotation, analysis of potential plant–microbe interactions and stress resistance were performed using RAST 2.0 (Rapid Annotation using Subsystem Technology) (http://rast.theseed.org) [38]. AntiSMASH (v5.1.0) was used as a specific complement for the annotation of secondary metabolite biosynthetic gene clusters (BGCs) [9].

Analysis of in vitro *PGPR mechanisms and salt tolerance of the bacterial isolate*

PGPR mechanisms and salt tolerance of GPTR29 were analyzed by in vitro traits. All experiments were performed three times. The ability to solubilize insoluble forms of phosphate (P) was assessed with Pikovskaya agar medium [39] using 0.5% $CaHPO_4$ as the P source. Plates were inoculated with 10 µl of a GPTR29 solution at a concentration of 10^8 CFU mL^{-1} and then incubated for

15 days at 28 °C, and the presence of clear solubilization halos around the colonies was examined after that time.

M9-CAS-AGAR, modified with an additional cationic solvent (HDMTA) according to Alexander and Zuberer [3], was used to evaluate siderophore production. The strain was inoculated in this medium, as described in the previous paragraph. Five days after inoculation, the presence of yellow–orange halos around the colonies were analyzed.

The IAA production of GPTR29 was measured in two ways. In the first method, JMM medium (John Howieson minimal medium) supplemented with tryptophan (0.17 g L^{-1}) was used to measure IAA production, according to the protocol described by García-Fraile et al. [19]. In the second method, IAA production was detected by HPLC at the Elemental Analysis, Chromatography and Mass Spectrometry Service of NUCLEUS (University of Salamanca, Spain), as described in Jimenez-Gómez et al. [24].

To evaluate the salinity tolerance of GPTR29, the strain was inoculated in TY medium supplemented with 0.0%, 1.0%, 2.5%, and 5.0% NaCl, as described above. Five days after inoculation, the presence of bacterial growth on the plates was assessed.

Plant colonization assays under normal and salt-stress conditions

In order to assess the colonization ability of the strain, the GFP-tagged GPTR29 derivative was obtained by biparental mating with *Escherichia coli* S17.1 as a donor of the pHC60 plasmid, according to García-Fraile et al. [19]. *E. coli* S17.1 was grown in Luria–Bertani (LB) medium at 37 °C, and the recombinant strain was grown in TY (tryptone yeast extract) medium at 28 °C, and both media were supplemented with tetracycline (10 μg ml^{-1}).

Seeds of lettuce (*L. sativa* L. var Romaine) were surface-sterilized and germinated according to a previously described protocol: seeds were surface-sterilized by immersion in ethanol 70% for 30 s and sodium hypochlorite solution (5%) for 5 min, followed by 5 washes with sterile distilled water and germinated on water-agar plates, keeping them in darkness for 24 h. [6]. Subsequently, 2 days after germination the seedlings were transferred to square plates (12×12 cm) with Rigaud and Puppo medium (1.5% agar) [41], supplemented with 0 mM and 100 mM NaCl, with five seedlings per plate (15 seedlings per treatment).

For inoculation, 250 μL of the GFP-tagged bacterial suspension (10^8 CFU mL^{-1}) was inoculated on the roots of each seedling. Uninoculated controls were also included in the study. The plates were maintained in a growth chamber and observed at 7 days after inoculation.

For microscopy analysis, the roots inoculated with GFP-labeled GPTR29 were washed with sterile distilled water and stained with a solution of propidium iodide (10 μM). A Nikon Eclipse 80i fluorescence microscope with an LED lamp (Nikon, Tokyo, Japan) was used to observe GPTR29 colonization.

Volatile organic compounds production analyzed by GC–MS

To evaluate the ability of GPTR29 to produce VOCs under normal conditions and salinity stress, the strain was grown in TY liquid medium supplemented with 0 mM and 100 mM NaCl, respectively. One hundred μL of GPTR29 suspension adjusted to a final concentration of 10^8 CFU mL^{-1} (optical density of 0.6 at 600 nm) was inoculated in 4 mL of the medium. Then, it was incubated on an orbital shaker at 28 °C for two and five days at 180 rpm in a 20 mL headspace vial with a screw cap (Merck KGaA, Darmstadt, Germany).

After each time, the VOCs production of GPTR29 was determined by headspace solid phase microextraction (HS-SPME-GC–MS) according to the methodology previously developed with minimal modifications [4]. Briefly, VOCs present in 4 mL of culture medium were extracted by HS-SPME using a divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber under continuous stirring (250 rpm) with an incubation time of 5 min and extraction time of 30 min at 45 °C. Then, detection was accomplished using a 436-GC system (Bruker Daltonics, Fremont, CA) coupled to a SCION Single Quadrupole (SQ) mass detector and Bruker Daltonics MS workstation software (version 8.2). Analyses were performed using a Combi-PAL autosampler (Varian Pal Autosampler, Switzerland). A GC fused silica capillary column Rxi-5Sil MS (30 m×0.25 mm I.D.×0.25 μm film thickness, RESTEK Corporation, U.S., Bellefonte, Pennsylvania) was used for chromatographic separation. Helium C-60 (Gasin, Portugal) was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The injections were in split mode with a 1:5 ratio. The oven temperature program was 40 °C (1 min) to 250 °C (5 min) at 5 °C min^{-1}, followed by increasing to 300 °C (1 min) at 5 °C/min. The MS detector was operated in electron impact (EI) mode (70 eV). The transfer line temperature was 250 °C, the manifold temperature was 40 °C, and the EI temperature was 260 °C. The mass range was 40–250 m/*z*, with a scan rate of 6 scan/s. The chromatographic analysis was performed in full scan mode. VOCs were identified through chemical reference standards or by

comparison between the obtained MS fragmentation with the mass spectra present in the National Institute of Standards and Technology (NIST 14) database and through comparison with the Kovats retention index.

In vitro *seedling inoculation and* in vitro *VOCs exposure*

Sterilization and germination of lettuce (*L. sativa* L. var Romaine) seeds were carried out as previously described for different in vitro trials: inoculation of lettuce seedlings under normal and saline conditions and exposure of lettuce seedlings to GPTR29 VOCs under normal conditions and salinity. Each experiment was performed by triplicate.

The ability of GPTR29 to promote lettuce growth during its early developmental stages was assessed by in vitro assays under normal conditions and salinity stress. Five lettuce seedlings were placed on each Rigaud and Puppo square plate (15 seedlings per treatment). The first experiment was performed under non-saline conditions by inoculating 250 µL of a GPTR29 suspension (10^8 CFU mL^{-1}) into the roots of each seedling and 250 µL of sterile distilled water for the uninoculated controls. The second experiment was developed under salinity conditions, placing the seedlings on Rigaud and Puppo medium supplemented with 100 mM NaCl, with the same inoculation methodology. The seedlings were maintained in a growth chamber (incandescent and fluorescent lighting of 400 microeinsteins m^{-2} s^{-1} and 400–700 nm, programmed for a day–night cycle of 16 h photoperiod, with a constant temperature varying between 23–25 °C and 50–60% relative humidity) and observed at 7 and 15 days post-inoculation (dpi). At these times, the length of the shoots and the roots and the number of leaves, and secondary roots of seedlings were measured.

The effects of GPTR29 VOCs exposure on lettuce seedlings under non-saline and saline conditions were measured using the following methodology. Five lettuce seedlings were placed on one part of a bicompartmentalized plate (90×90 mm) containing Rigaud and Puppo medium (15 seedlings per treatment) supplemented with 100 mM NaCl in the case of the salinity test. The other compartment of the plate contained TY medium (also supplemented with 100 mM NaCl under saline conditions), where 100 µL of a GPTR29 suspension (10^8 CFU mL^{-1}) was inoculated. Uninoculated controls were also included in the study. The plates were sealed with Parafilm® (Bemis™, Zurich, Switzerland), and lettuce seedlings were maintained in a growth chamber (with the same conditions as mentioned above) and observed at 5 and 10 dpi to determine the effects of VOCs exposure. At these times, the same parameters as above were measured.

Growth promotion and saline tolerance assays in microcosm conditions

The ability of GPTR29 to promote plant growth in *L. sativa* L. var Romaine and alleviate the effects of salt stress was evaluated under greenhouse conditions using a mix of nonsterilized soil and vermiculite "SEED PRO 6040"/vermiculite (3:1 *v*/*v*) (PROJAR, Madrid, Spain) as substrate in plastic pots with a capacity of 2.4 L. Soil characteristics were silty clay (clay: 19.80%; silt: 56.23%; sand: 23.97%) with pH 8.04, EC 0.1 dS m^{-1}, organic carbon 1.44%, N 0.157%, organic matter 2.49%, C/N ratio 9.2, available P 0.10 cmol (+) kg^{-1}, available K 0.30 cmol (+) kg^{-1}, available Mg 0.51 cmol (+) kg^{-1}.

Lettuce seeds were previously surface-sterilized and germinated as explained above. The seedlings were transferred to the substrate and then inoculated with 5 mL of the strain suspension with a final concentration of 10^8 CFU mL^{-1} after 7 days. The assay was performed under normal and salinity conditions, including GPTR29 inoculated and uninoculated treatments, in both conditions. Fifteen plants were included in each treatment and condition with a randomized distribution in the greenhouse and weekly rotations were made. To obtain saline stress conditions, plants were irrigated with 50 mL of an aqueous solution of NaCl (100 mM) every 72 h, whereas the plants developed under normal conditions were irrigated only with water under the same conditions.

The plants were maintained for 27 days in a greenhouse illuminated with natural light, 50–60% relative humidity, a temperature between 20 and 25 °C, and a photoperiod of 16/8. After this time, the number of leaves, shoot fresh weight, shoot dry weight, and chlorophyll content of the leaves were analyzed with a SPAD-502PLUS chlorophyll meter (Soil Plant Analysis Development) (Konica Minolta, Osaka, Japan).

Dry plant samples were analyzed in the Analysis and Instrumentation Service at IRNASA-CSIC (Salamanca, Spain). Total carbon and nitrogen were both determined by the Dumas method using a LECO CN628 Combustion Analyzer. Mineral composition in plant tissues was determined by inductively coupled plasma–optical emission spectrometry (ICP–OES, Varian 720-ES, Mulgrave, Australia) after digestion in a microwave oven (Milestone ETHOS UP, Sorisole, Italy) using diluted nitric acid and hydrogen peroxide.

Phenolic compound analysis of lettuce leaves

Lettuce leaves were analyzed 20 days post-inoculation to determine the phenolic composition, as previously reported [26]. Briefly, leaves were extracted (5 mg) using MeOH:H_2O 80:20, and after removing chlorophylls, the extracts were analyzed by HPLC–DAD–MS to determine the phenolic composition. A Hewlett–Packard 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) was employed to perform the analysis as previously described [26]. The API 3200 Qtrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) was connected to the HPLC system via the DAD cell outlet [13], and spectra were recorded in negative ion mode using the same conditions as described previously [26]. The UV–vis spectra, retention times, and mass spectra were employed for compound identification. Quantification was performed from the peak area values obtained in the chromatograms recorded at 280 nm (protocatechuic acid derivative), 330 nm (caffeic acid and its derivatives, including cichoric acid) or 360 nm (flavone and flavonol derivatives). Commercial standards of coumaric acid, quercetin 3-*O*-glucoside and luteolin 7-*O*-glucoside were used to quantify those compounds, whereas the contents of caffeic acid derivatives, protocatechuic acid glucoside, apigenin derivative and quercetin derivatives were expressed as caffeic acid, protocatechuic acid, apigenin 7-*O*-glucoside, and quercetin 3-*O*-glucoside equivalents, respectively. The results, expressed in g kg^{-1} of plant dry weight, were the mean value of three independent analyses.

Statistical analysis

Data were statistically analyzed with GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA), mean values were compared with unpaired Student's *t* test ($p \leq 0.05$). Two-way analysis of variance (ANOVA) was performed to analyze the effect of salinity and inoculation on the evaluated parameters ($p \leq 0.05$) (Additional file 1: Tables S1, S2, S3, S4).

Results

Isolation and phylogenomic analysis of GPTR29 strains

The GPTR29 strain was isolated from inside surface-sterilized white clover nodules. Based on the obtained genome, dDDH comparison reveals that strain GPTR29 can be assigned to the genus *Rhizobium* but not within some species described due to the obtained values. The genome of strain GPTR29 presents dDDH values of 59.00% (*R. ruizarguesonis* UPM1133^T), 52.90% (*Rhizobium indicum* MCC 3961^T), 52.50% (*Rhizobium laguerreae* FB206^T) and 51.00% (*Rhizobium leguminosarum* USDA 2370^T) with the type strain of close species of the genus *Rhizobium*, with these values cut off from 70%, which is the value used to delimit genomic species by dDDH comparison [57].

GPTR29 genome analysis

The draft genome obtained for the GPTR29 strain contains 51 contigs with a genome length of ~ 7 Mbp, a G + C content of 60.5%, and 6411 predicted coding sequences (Fig. 1). The draft genome sequence of GPTR29 was deposited in the NCBI GenBank database under Bioproject PRJNA603738 (accession number JABERB000000000).

Based on in silico analysis with the SEED-viewer 2.0 framework, it has been shown that the GPTR29 genome has annotated genes related to the biosynthesis of molecules with plant growth-promoting effects. The coding sequences of enzymes related to indole acetic acid synthesis have been found: (EC 4.1.1.48), (EC 3.5.1.4), (EC 1.2.1.3), (EC 4.2.1.84). Genes involved in siderophore biosynthesis and transport, such as *iucB* and *iucD* (aerobactin biosynthesis) or the *FhuCDB* system (ferric hydroxamate transporter), have been observed. The GPTR29 genome also encodes enzymes related to the solubilization of insoluble forms of phosphate, such as phosphatases (EC 3.69.1.1, EC 3.1.3.1, EC 3.6.1.11, and EC 2.7.4.1) or citrate synthase (EC 2.3.3.1). In this regard, the presence of genes associated with biological nitrogen fixation has also been determined, such as the master regulator *nifA*, as well as *nifB* and the *nifHDKE* cluster. Furthermore, genes involved in colonization of the plant root system have been annotated, such as *lptABCDFG* system for the transport and assembly of lipopolysaccharide (LPS), or the gene implicated in cellulose production, beta-1,4-glucanase (cellulase) (EC 3.2.1.4) [6, 30, 42]. Finally, the sequence of enzymes involved in the final stages of VOCs biosynthetic pathways, such as EC 1.1.1.1, EC 4.2.1.33, EC 1.1.1.85, EC 2.3.3.13 (2-methyl-1-butanol and 3-methyl-1-butanol), or EC 2.2.1.6 (acetoin), have also been found.

Related to salinity tolerance strategies, the presence of the nucleotide sequences of enzymes involved in trehalose biosynthesis has been detected: trehalose synthase (EC 5.4.99.16), alpha-trehalose-phosphate synthase (EC 2.4.1.15), or trehalose-6-phosphate phosphatase (EC 3.1.3.12). Proline iminopeptidase (EC 3.4.11.5) and the system *proVWX*, both identified as proline uptake and

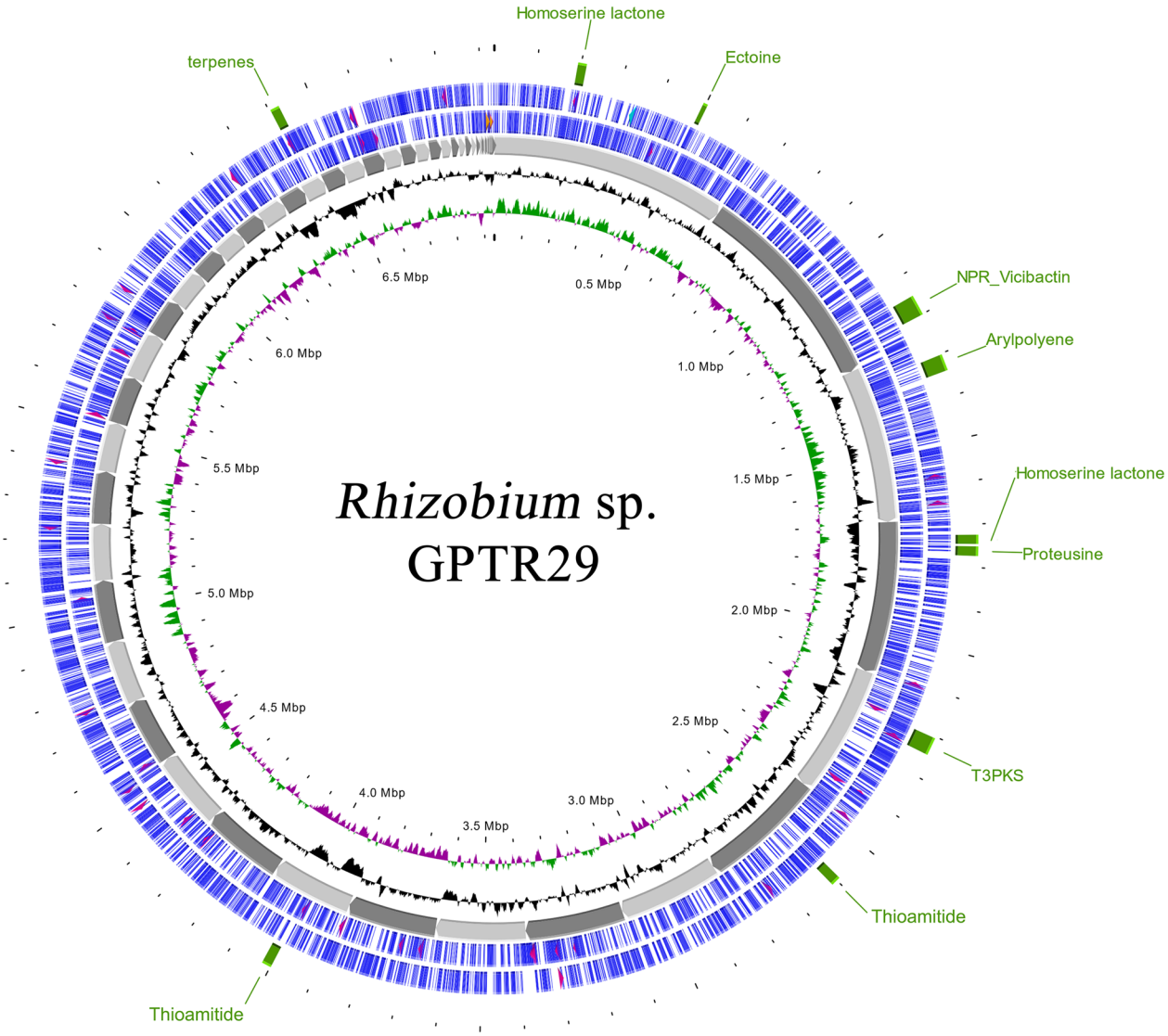


Fig. 1 Circular genome representation of *Rhizobium* sp. GPTR29. The inner to outer circles show GC skew curves (+/−; green/purple), GC content (black), contigs (light gray), coding sequences (CDSs; blue), rRNAs (violet), tmRNAs (turquoise) and antiSMASH annotated regions (green)

synthesis systems, have been annotated. In addition, the GPTR29 genome exhibits *betABC* and *soxABDG* systems, which encode enzymes related to choline and glycine-betaine synthesis, other osmolytes relevant for coping with salt-stress conditions.

On the other hand, the production of substances with important redox properties is necessary for the fight against oxidative stress. For example, the genes *ghsA*, *gshB*, or *gltT*, which are involved in the biosynthetic cycle of the tripeptide glutathione (GSH), have been annotated in the GPTR29 genome. Glutaredoxins (*grx1*, *grx2* and *grx3*) or superoxide dismutases (encoded by the *sodABC* system) are also present. In addition, the presence of genes associated with the synthetic pathways of phenolic compounds, which are also described for their important antioxidant activity, has been detected [13, 28]. The GPTR29 genome encoded genes related to the biosynthesis and utilization of phenylalanine, the main precursor for the synthesis of phenolic compounds, such as the enzymes aromatic amino acid transaminase (EC 2.6.1.57) and naringenin-chalcone synthase (EC 2.3.1.74).

The AntiSMASH program predicted various BGCs (Fig. 1), including (i) a thioamitide cluster, in which 33% of genes show similarity to the exopolysaccharide

biosynthetic gene cluster from *R. leguminosarum*; (ii) a terpene cluster that does not resemble any known cluster in the antiSMASH database but, according to the BLASTp comparison, the core gene shows 99.65% similarity to a phytoene/squalene synthase family protein of *R. leguminosarum*; (iii) a non-ribosomal peptide synthetase-like (NRPS-Like) cluster, in which 88% of genes show similarity to the vicibactin biosynthetic gene cluster from *Rhizobium etli*; and (iv) an ectoine cluster that does not resemble any known cluster in the antiSMASH database but, according to the BLASTp comparison, the core gene shows 98.44% similarity to the ectoine synthase protein of *R. leguminosarum.*

Analysis of in vitro *PGPR mechanisms and salt tolerance of the bacterial isolate*

The results of in vitro plant growth promotion analysis showed that GPTR29 was able to solubilize $CaHPO_4$, forming 2.0 mm solubilization halos around the colonies 15 days after inoculation. GPTR29 was also able to grow in M9-CAS-AGAR, where the colonies were surrounded by a yellow–orange halo (2.5 mm periphery around colonies) indicative of siderophore production. In addition, GPTR29 produced 74.2 mg L^{-1} indole acetic acid when grown in JMM liquid medium supplemented with tryptophan; however, the concentration of IAA was 0.77 mg L^{-1} when measured by HPLC. In terms of

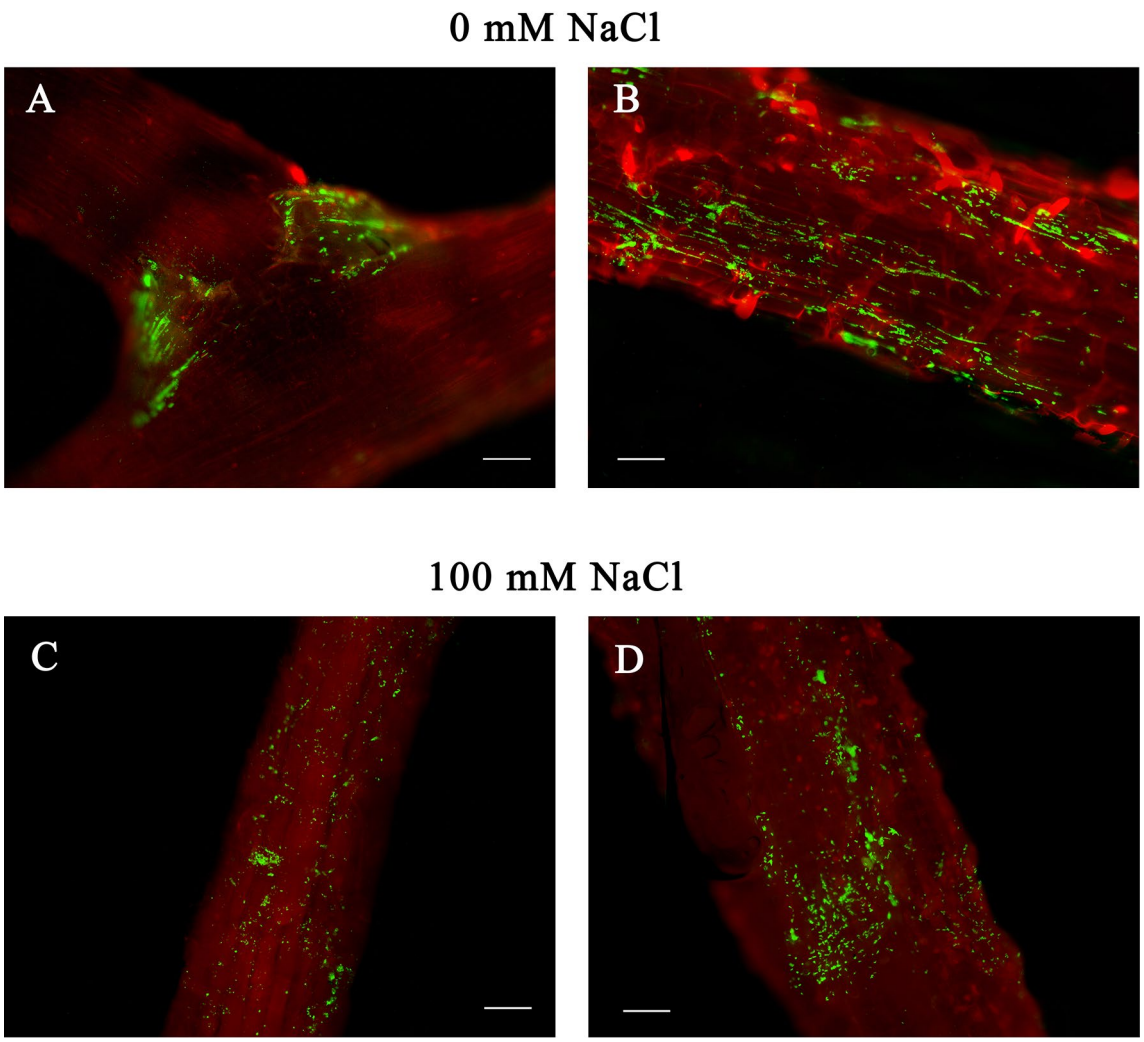


Fig. 2 Fluorescence optical micrographs of lettuce seedling roots inoculated with GPTR29 green fluorescent protein (GFP)-tagged strain contrast stained with propidium iodide (red) seven days post-inoculation, under non-saline (0 mM NaCl) (**A** and **B**) and saline (100 mM NaCl) (**C** and **D**) conditions: **A** shows the ability of GPTR29 to colonize lateral root primordia (bar 100 µm); **B** shows the biofilm formation of GPTR29 (bar 100 µm); **C** and **D** also show the biofilm structures (bar 100 µm)

salinity tolerance, GPTR29 was found to be able to grow between a salinity range of 0–2.5% NaCl.

Colonization of lettuce roots assays

Bacterial colonization of lettuce roots was analyzed by fluorescence microscopy in non-saline and salt-stressed systems. Under normal conditions and 7 days after inoculation with GFP-tagged GPTR29, it was observed that the strain was able to colonize root surfaces, aggregating in the intercellular spaces of the epidermis, as well as between root hairs. Moreover, different polysaccharide structures, such as typical biofilm-initiating microcolonies and the biofilm itself, were observed both on the surface of the main roots and at the beginning of secondary root formation (Fig. 2a, b). Bacterial colonization in salt-stressed root system was also analyzed 7 dpi, showing that strain GPTR29 was still able to form microcolonies and biofilms on the epidermal surface (Fig. 2c, d).

Production of volatile organic compounds

The VOCs of GPTR29 were analyzed by GC–MS, and only those compounds that were not found in the chromatographic profiles of the uninoculated salt-stressed

Table 1 *Rhizobium* sp. GPTR29 volatiles profiling analyzed by GC–MS produced at exponential phase (2 dpi) and stationary phase (5 dpi)

Compounds	RT (min)	Identifier m/z	R match	2 days post-inoculation		5 days post-inoculation	
				Normal conditions Area (AU) (10^7)	100 mM NaCl Area (AU) (10^7)	Normal conditions Area (AU) (10^7)	100 mM NaCl Area (AU) (10^7)
Amides							
2-Methylpropionamide[L2]	2.17	59	726	n.d	n.d	n.d	3.94
Pyrazoles							
6,6-Dimethyltetrahydro-2H-pyran-2-one[L2]	2.54	56	854	n.d	n.d	n.d	4.83
Alkanes							
3-Methylhexane[L2]	2.64	55, 56	895	n.d	n.d	n.d	2.38
3-Ethyl-heptane[L2]	10.43	43, 57, 60	871	0.28	n.d	n.d	n.d
3,3-Dimethyl-octane[L2]	11.85	43, 57, 70, 71	892	82.0	n.d	n.d	n.d
Ketones							
3-Pentanone[L1]	2.88	57	871	n.d	n.d	3.17	6.62
1-Mercapto-2-propanone[L2]	3.94	43	920	4.86	n.d	n.d	n.d
Acetoin[L1]	4.33	45	826	n.d	1.16	n.d	n.d
5-Methyl-3-hexanone[L2]	5.36	57	855	n.d	n.d	1.75	5.75
3-Heptanone[L1]	8.20	44, 57	762	n.d	0.13	n.d	n.d
6-Methyl-2-heptanone[L2]	9.56	43	809	n.d	0.46	n.d	n.d
6-Methyl-5-hepten-2-one[L2]	11.17	41, 43, 69, 108	853	0.93	0.26	n.d	n.d
Alcohols							
3-Methyl-1-butanol[L1]	3.44	55, 70	825	2.91	4.67	3.28	6.57
2-Methyl-1-butanol[L1]	3.49	57	864	3.51	1.91	1.96	4.22
3-Methyl-1-butanol, acetate[L1]	7.93	43, 70	849	1.89	n.d	n.d	n.d
2-Ethyl-4-methylpentan-1-ol[L2]	10.83	55, 57	786	n.d	n.d	0.69	2.10
Sulfides							
2,4-Dithiapentane[L2]	6.74	58, 61, 108	804	n.d	n.d	n.d	1.07
Benzyl methyl sulfide[L2]	14.78	65, 91	785	n.d	n.d	n.d	0.27
Terpenes							
α-Pinene[L1]	7.98	77, 93	887	0.76	0.05	0.79	2.05
Thioethers							
2-Methyl-3-(methylthio)furan[L2]	8.35	53, 69, 99, 113	885	n.d	n.d	0.24	1.66

RT, retention time; AU, arbitrary units. n.d., not detected

[L1] Compounds (identification by comparison with chemical reference standards under identical analytical conditions). [L2]Putatively annotated compounds (comparison with MS spectral similarity to NIST 14) [54]

or normal controls were considered, respectively, for each type of condition. Differential VOCs emission were observed between saline and non-saline conditions and between the exponential phase (2 dpi) and stationary phase (5 dpi), as shown in Table 1.

On the one hand, after two days of growth under normal conditions (0 mM NaCl), 8 compounds were identified: 3-ethyl-heptane, 3,3-dimethyl-octane, 1-mercapto-2-propanone, 6-methyl-5-hepten-2-one, 3-methyl-1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol acetate, and α-pinene. The compounds 6-methyl-5-hepten-2-one, 3-methyl-1-butanol, and 2-methyl-1-butanol were also found 2 days after inoculation under saline conditions (100 mM NaCl), but a further 3 different compounds were identified under salinity conditions that were not present under non-saline conditions: acetoin, 3-heptanone, and 6-methyl-2-heptanone.

On the other hand, for the growth of GPTR29 under normal conditions and 5 dpi, a total of 6 compounds were identified: 3-pentanone, 5-methyl-3-hexanone, 2-methyl-1-butanol, 2-ethyl-4-methylpentan-1-ol, α-pinene, and 2-methyl-3-(methylthio) furan. However, in the case of GPTR29 growth under salt stress at the same time, apart from these 6 compounds, 6 more compounds were detected, namely, 2-methylpropionamide, 6,6-dimethyl-tetrahydro-2H-pyran-2-one, 3-methylhexane, 3-methyl-1-butanol, 2,4-dithiapentane, and benzyl methyl sulfide.

In vitro *growth promotion of lettuce seedlings and VOCs exposure effects*

The results of the plant growth promotion in vitro experiments under normal conditions showed that strain GPTR29 positively promotes several parameters of lettuce growth (Table 2). In addition, it was also observed that GPTR29 helps to tolerate salt-stress conditions during the early stages of lettuce growth (Table 2). In both conditions, the length of the aerial part of lettuce seedlings was longer in GPTR29-inoculated plants than in control plants. The stem length increased by 32.5% (statistically significant) and 5.5%, 7 and 15 dpi, respectively, under normal conditions and significantly by 41.1% and 35.0%, respectively, under saline stress (7 and 15 dpi). In the same way, the length of the radicular system was significantly higher in GPTR29 inoculated plants compared to the respective uninoculated control: 23.6% under normal conditions and 7 dpi, and 98.4% and 52.7% under salinity (7 and 15 dpi) (Fig. 3a–d). Additionally, the greatest improvements in the number of secondary roots were observed after 15 days under normal conditions, with a significant increase of 44.0% in GPTR29-inoculated seedlings (Fig. 3a, b).

Exposure of lettuce seedlings to GPTR29 VOCs was also carried out under normal and 100 mM NaCl saline conditions in bicameral plates (Fig. 3e–h), showing very promising results. On the one hand, under normal conditions, a significant improvement in stem and root length was observed at both measurement times, with increases of more than 30.0%, due to exposure to GPTR29 volatiles. Similarly, the number of secondary roots was significantly increased at 5 and 10 dpi in lettuce exposed to GPTR29 volatiles, with increases of more than 64.0% in both cases. For the number of leaves, the results were also higher than the controls not exposed to GPTR29 VOCs, with a significant increase at the first measurement time (13.7%). On the other hand, under salinity conditions, GPTR29 volatiles significantly increased the aerial part of lettuce seedlings by 21.6% and 17.3%, 5 and 10 days after exposure, respectively. Likewise, root length was higher and statistically significant in plants exposed to GPTR29 VOCs compared to unexposed controls, 31.9% and 33.1% at both measurement times, respectively. Significant increases of more than 15% were also observed for the number of leaves in 5- and 10-day-old lettuces exposed to the strain VOCs (Table 3).

Table 2 Results from in vitro growth promotion assays in lettuce inoculated with *Rhizobium* sp. GPTR29 strain under non-saline (0 nM) and saline (100 nM) conditions

	In vitro growth promotion assays								
	Treatment	Shoot length (± S.D.) (mm)	Root length (± S.D.) (mm)	Number of secondary roots (± S.D.)	Number of leaves (± S.D.)	Shoot length (± S.D.) (mm)	Root length (± S.D.) (mm)	Number of secondary roots (± S.D.)	Number of leaves (± S.D.)
		7 dpi				14 dpi			
Non-saline conditions	Control	2.61 (± 0.72)a	2.75 (± 1.01)a	1.87 (± 0.99)a	2.87 (± 0.35)a	4.99 (± 0.51)a	3.44 (± 0.69)a	2.83 (± 1.47)a	3.77 (± 0.44)a
	GPTR29	3.45 (± 0.48)b	3.39 (± 0.43)b	1.87 (± 0.74)a	2.87 (± 0.35)a	5.26 (± 0.82)a	3.96 (± 0.18)a	4.08 (± 1.32)b	3.81 (± 0.40)a
100 mM NaCl	Control	1.38 (± 0.36)a	0.66 (± 0.29)a	2.13 (± 1.06)a	2.47 (± 0. 52)a	1.93 (± 0.34)a	0.89 (± 0.32)a	3.53 (± 1.36)a	3.33 (± 0.49)a
	GPTR29	1.95 (± 0.41)b	1.32 (± 0.42)b	2.20 (± 1.01)a	2.60 (± 0.51)a	2.61 (± 0.50)b	1.36 (± 0.33)b	3.67 (± 1.11)a	3.67 (± 0.49)a

Treatment sharing the same letter belongs to the same subgroup, according to the Student's *t* test ($p \leq 0.05$). S.D. = standard deviation; dpi = days post-inoculation

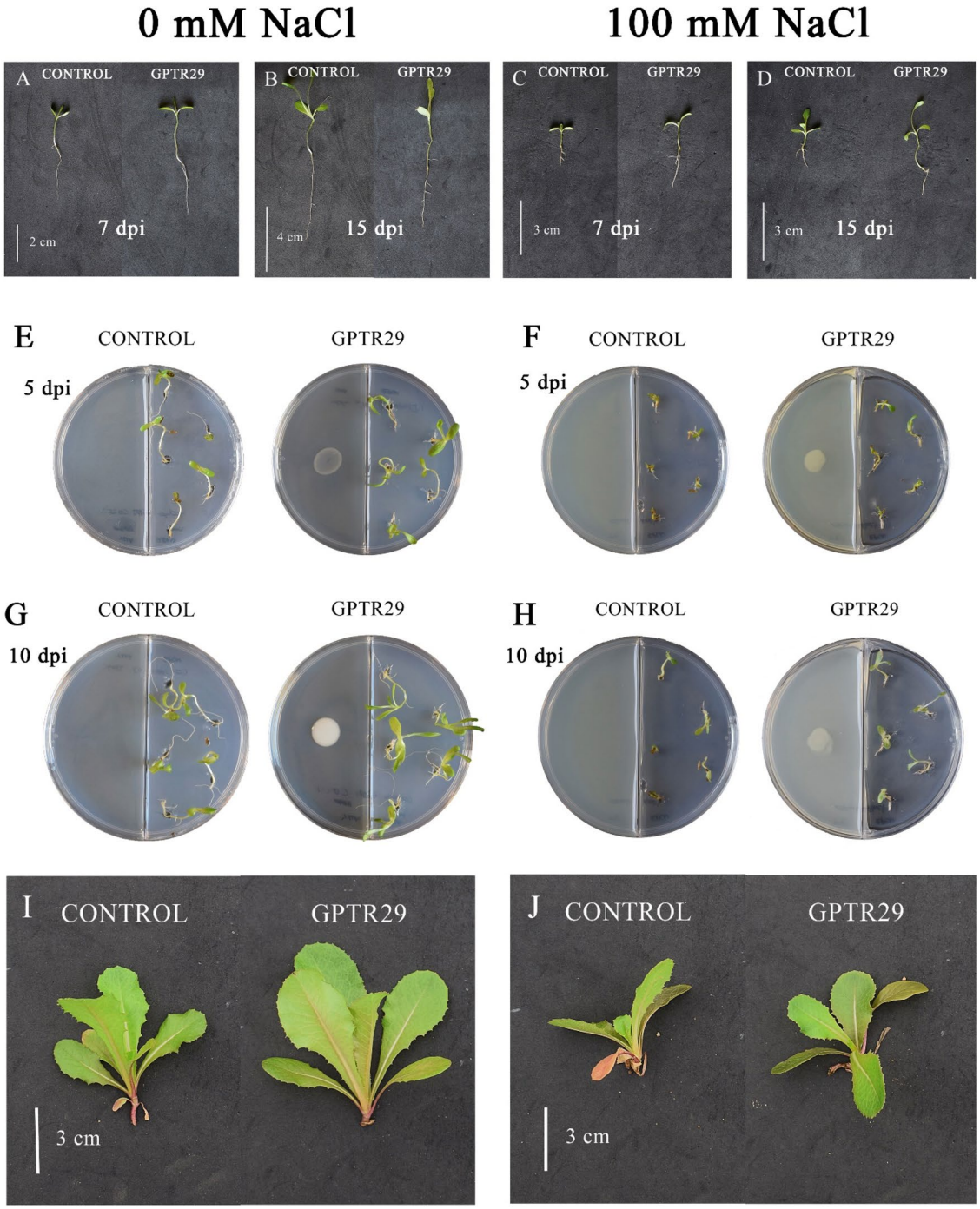


Fig. 3 Plant inoculation assays. General appearance of the lettuce seedlings of in vitro growth promotion assays under normal conditions (**A** and **B**) and 100 mM NaCl (**C** and **D**) at 7 dpi (**A** and **C**) and 15 dpi (**B** and **D**). Experimental setup for GPTR29 VOCs exposure to lettuce plant growth under normal conditions (**E** and **G**) and 100 mM NaCl (**F** and **H**) after 5 (**E** and **F**) and 10 days (**G** and **H**). Lettuce growth promotion in greenhouse experiments under normal conditions (**I**) and 100 mM NaCl (**J**). Uninoculated control on the left and treated with GPTR29 on the right

Table 3 Effects of exposure to *Rhizobium* sp. GPTR29 VOCs on lettuce in vitro growth under non-saline (0 nM) and saline (100 nM) conditions

	Effects of VOCs exposure on plant growth under salinity								
	Treatment	Shoot length (± S.D.) (mm)	Root length (± S.D.) (mm)	Number of secondary roots (± S.D.)	Number of leaves (± S.D.)	Shoot length (± S.D.) (mm)	Root length (± S.D.) (mm)	Number of secondary roots (± S.D.)	Number of leaves (± S.D.)
		5 dpi				10 dpi			
Non-saline conditions	Control	1.83 (± 0.56)a	1.67 (± 0.62)a	2.67 (± 1.95)a	2.93 (± 0.26)a	2.40 (± 0.57)a	2.01 (± 0.80)a	3.00 (± 1.95)a	3.70 (± 0.47)a
	GPTR29	2.44 (± 0.48)b	2.26 (± 0.66)b	4.40 (± 1.99)b	3.33 (± 0.49)b	3.42 (± 0.75)b	2.74 (± 0.93)b	5.21 (± 2.72)b	3.93 (± 0.48)a
100 mM NaCl	Control	0.66 (± 0.09)a	0.42 (± 0.08)a	1.00 (± 0.00)a	2.05 (± 0.23)a	0.75 (± 0.11)a	0.43 (± 0.08)a	1.37 (± 0.68)a	2.63 (± 0.50)a
	GPTR29	0.80 (± 0.12)b	0.55 (± 0.12)b	1.00 (± 0.00)a	2.38 (± 0.50)b	0.88(± 0.18)b	0,57 (± 0.12)b	1.89 (± 1.08)a	3.06 (± 0.54)b

Treatment sharing the same letter belongs to the same subgroup, according to the Student's *t* test ($p \leq 0.05$). S.D. = standard deviation; dpi = days post-inoculation

Effects of GPTR29 inoculation on plant growth and salinity tolerance in a greenhouse

A microcosm condition assay to evaluate the ability of GPTR29 was performed in a greenhouse. Different variables related to the aerial part of the lettuce (number of leaves, shoot fresh weight, shoot dry weight, and chlorophyll content of the leaves), which is considered the marketable part of this crop, were analyzed. The results showed that inoculation with GPTR29 improved growth parameters under non-stress and salt-stress conditions (Fig. 3i, j). Chemical analysis of the nutrient content revealed an improvement in the content of ions of interest, as well as in the ionic balance under saline conditions.

Under normal conditions, bacterial inoculation significantly enhanced the shoot fresh weight by up to 29.0% compared to uninoculated plants. The dry weight of the shoots was also 9.4% higher than that of the controls. Moreover, a significant increase in the number of leaves of the inoculated lettuces was observed, with 14.1% more leaves than the control plants. Thus, the leaves of these plants treated with GPTR29 had a significantly higher chlorophyll content, with an increase of 7.4% (Table 4).

In accordance with the results of normal conditions, a significant increase in the size of the aerial part of the plants treated with strain GPTR29 was observed under 100 mM NaCl salinity compared to salt-stressed non-inoculated controls. Shoot fresh and dry weights were significantly higher in GPTR29-inoculated lettuce plants, with increases of 17.8% and 27.4%, respectively. Furthermore, leaves number and the content of chlorophyll pigment in the leaves of plants inoculated with GPTR29 showed the same significant improvement under salinity and non-saline conditions, up to 14.4% and 7.1%, respectively (Table 4).

In addition, inoculation with GPTR29 under normal and salinity conditions resulted in modification of the nutrient content of lettuce leaves (Table 4). In this regard, a higher content of P, N, and micronutrients Ca and Mg was observed in lettuces inoculated under normal conditions, by 11.8%, 0.9%, 4.8% and 6.5%, respectively; plants inoculated with GPTR29 under salt stress also showed improvements in the content of these elements, being higher than the control by 1.8%, 1.7%, 2.2%, and 5.8%, respectively. In addition, the contents of K and Na, the main players in the ion imbalance caused by salt stress, were also modified due to inoculation with GPTR29. Thus, the potassium content of leaves from lettuce inoculated under normal conditions was 17.5% significantly higher than that of non-saline control lettuce. In lettuces grown under salinity conditions, the increase in K content was 8.8% for lettuces treated with GPTR29. A 15.6% increase in sodium content was also observed in plants inoculated with GPTR29 under normal conditions. However, under salinity stress, the Na content was 3.3% higher in non-inoculated plants than in those inoculated with GPTR29.

Phenolic composition of lettuce leaves

Twelve different phenolic compounds were determined in the leaves of lettuces grown in greenhouses (7 phenolic acids and 5 flavonoids, Table 5). Phenolic acids were the most abundant compounds in the lettuce leaves (approximately 73% of total phenolic compounds), and among them, cichoric acid was the most important, followed by the derivatives of caffeic acid (those with quinic, tartaric and malic acid residues esterified to caffeic acid). Among flavonoids, quercetin 3-*O*-malonyl glucoside and quercetin 3-*O*-glucuronide were the most abundant compounds. The obtained results indicated that saline stress leads to an increase in the content of total phenolic compounds in lettuce plants, since lettuce plants grown under salinity conditions showed contents of both phenolic acids and

Table 4 Results from greenhouse growth promotion assays, chlorophyll content and elemental analysis in lettuce inoculated with *Rhizobium* sp. GPTR29 strain under non-saline (0 nM) and saline (100 nM) conditions

Greenhouse assays											
Production parameters						**Nutritional content of lettuce plants**					
	Treatment	**Chlorophyll (± S.D.) (SPAD units)**	**Number of leaves (± S.D.)**	**SFW (± S.D.) (g)**	**SDW (± S.D.) (g)**	**N (%)**	**Ca (mg/plant kg)**	**K (mg/plant kg)**	**Mg (mg/plant kg)**	**Na (mg/plant kg)**	**P (mg/plant kg)**
Non-saline conditions	Control	25.34 (± 3.65)a	5.53 (± 1.02)a	2.39 (± 1.01)a	0.14 (± 0.03)a	5.68 (± 0.22)a	13,384.00 (± 1476.044)a	92,689.50 (± 1679.38)a	6211.50 (± 1253.70)a	3232.00 (± 449.72)a	8099.00 (± 374.77)a
	GPTR29	27.20 (± 2.98)b	6.30 (± 0.70)b	3.08 (± 0.63)b	0.15 (± 0.02)a	5.72 (± 0.16)a	14,026.33 (± 556.302)a	108,887.67 (± 6229.36)b	6614.67 (± 336.30)a	3735.00 (± 446.15)a	9049.67 (± 182.67)b
100 mM NaCl	Control	25.54 (± 2.81)a	5.29 (± 0.81)a	2.96 (± 0.56)a	0.12 (± 0.03)a	5.31 (± 0.08)a	11,298.00 (± 367.92)a	92,112,00 (± 5641.30)a	5214.33 (± 149.00)a	22,241.00 (± 384.67)a	8750.67 (± 417.08)a
	GPTR29	27.34 (± 2.81)b	6.05 (± 0.83)b	3.48 (± 0.34)b	0.15 (± 0.04)b	5.41 (± 0.05)a	11,539.67 (± 1242.54)a	100,160.00 (± 2675.69)a	5542.00 (± 859.09)a	21,535.00 (± 72.13)a	8907.00 (± 712.23)a

Treatment sharing the same letter belongs to the same subgroup, according to the Student's t-test ($p \leq 0.05$). S.D. = standard deviation; SFW = shoot fresh weight; SDW = shoot dry weight

Table 5 Phenolic acid and flavonoid content (g kg^{-1}) of control and GPTR29 inoculated lettuce plants yielded in the greenhouse assays under non-saline (0 nM) and saline (100 nM) conditions

	Normal conditions		100 mM NaCl	
	Control (± S.D.)	GPTR29 (± S.D.)	Control (± S.D.)	GPTR29 (± S.D.)
Phenolic acids (g kg^{-1})				
Caffeoyl malic acid	1.69 (± 0.06)b	1.53 (± 0.07)a	1.74 (± 0.15)a	1.97 (± 0.04)a
Caffeoyl quinic acid	3.57 (± 0.03)a	3.62 (± 0.15)a	4.49 (± 0.42)a	4.12 (± 0.09)a
Caffeoyl tartaric acid	1.87 (± 0.02)a	2.29 (± 0.08)b	3.70 (± 0.28)a	3.90 (± 0.11)a
Cichoric acid (dicaffeoyltartaric acid)	10.29 (± 0.10)a	9.94 (± 0.41)a	12.68 (± 1.27)a	12.03 (± 0.24)a
Coumaric acid	0.39 (± 0.02)b	0.34 (± 0.02)a	0.51 (± 0.06)a	1.32 (± 0.05)b
Dicaffeoyl quinic acid	0.77 (± 0.03)a	0.79 (± 0.03)a	0.72 (± 0.07)a	0.74 (± 0.05)a
Protocatechuic acid glucoside	0.37 (± 0.02)a	0.56 (± 0.06)b	0.52 (± 0.09)a	1.10 (± 0.04)b
Total phenolics acids	18.94 (± 0.14)a	19.07 (± 0.80)a	24.36 (± 2.34)a	25.19 (± 0.58)a
Flavonoids (g kg^{-1})				
Apigenin derivative	0.14 (± 0.01)a	0.21 (± 0.01)b	0.23 (± 0.02)a	0.37 (± 0.01)b
Luteolin 7-*O*-glucoside	0.23 (± 0.01)a	0.22 (± 0.02)a	0.32 (± 0.04)a	0.37 (± 0.01)a
Quercetin 3-*O*-glucuronide	2.37 (± 0.02)b	2.07 (± 0.07)a	2.86 (± 0.25)a	3.26 (± 0.05)a
Quercetin 3-*O*-malonyl glucoside	3.19 (± 0.04)a	3.13 (± 0.18)a	4.12 (± 0.16)a	4.42 (± 0.09)a
Quercetin 3-*O*-glucoside	0.81 (± 0.02)a	0.85 (± 0.05)a	1.44 (± 0.14)a	1.70 (± 0.01)b
Total flavonoids	6.73 (± 0.08)a	6.55 (± 0.27)a	8.97 (± 0.59)a	10.11 (± 0.09)b

Values are expressed in mean ± standard deviation. Treatment sharing the same letter belongs to the same subgroup, according to the Student's *t* test ($p \leq 0.05$).
S.D. = standard deviation

flavonoids approximately 130% higher than those plants grown under normal conditions.

On the one hand, inoculation of lettuce with *Rhizobium* sp. GPTR29, under 0 mM NaCl conditions did not lead to significant changes in the total phenolic acid content of the leaves. However, GPTR29-treated plants showed significantly higher levels of caffeoyl tartaric acid and protocatechuic acid glucoside and lower levels of cichoric acid and caffeoyl malic acid compared to the non-saline control (Table 5). On the other hand, under salt-stress conditions (100 mM NaCl), it was observed that, in general, lettuce inoculated with GPTR29 had higher levels of phenolic acids, although in this case, the saline control samples also showed higher levels of cichoric acid and caffeoyl quinic acid than the inoculated samples.

Similarly, the results obtained for flavonoid concentration follow a parallel trend (Table 5). Under normal conditions, no significant differences in the total content of this type of phenolic were observed between non-saline control and inoculated samples, except for a significantly higher content of one apigenin derivative and a lower content of quercetin 3-*O*-glucuronide in lettuces inoculated with GPTR29. However, under salt stress, the concentration of two flavonoids detected, as well as the total flavonoid content, was significantly higher in leaves from inoculated plants than in those from salt-stressed non-inoculated plants.

Discussion

Salinity is a primary abiotic stress that significantly impacts different scales: environmental, social, or economic. In the agricultural context, it has the main repercussion on the efficiency and development of crops, as it often results in reduced growth parameters [15, 35]. In this complex scenario, the search for sustainable and effective alternatives to improve plant growth in saline conditions is necessary [5]. Therefore, in this study, we describe the effect of inoculation and VOCs production by a new *Rhizobium* strain, GPTR29, on the growth promotion of lettuce under salinity conditions. The *Rhizobium* genus has been widely described as an important biofertilizer due to its positive interaction with leguminous and non-leguminous plants even under abiotic stress, as well as its harmlessness to humans and animals [6, 7, 19, 24, 25]. However, the role of volatile compounds synthetized by bacteria of this genus in the plant response to salinity remains unknown to date.

The genus *Rhizobium* is a complex and highly diverse taxon comprising a large number of species that has undergone numerous taxonomic revisions [57]. Given this phylogenetic complexity, the use of genomic tools is essential to differentiate the closest related species [57], as we observed in the identification of strain GPTR29 within the genus *Rhizobium* but not within any described species based on a dDDH threshold of 70%

[57]. Comparison indicated that the closest species were within the defined *Rhizobium leguminosarum* species complex [57]. Differences with respect to these species in the percentages of dDDH were similar to those present within this complex (not higher than 66.1%) [57].

Knowledge of the genomic basis of beneficial interactions between microorganisms and plants is currently considered a valuable tool. It allows the identification of genes and pathways related to many processes of mutualistic interactions [18]. In this respect, the development of genomic technologies in recent years is a major breakthrough for microbiology.

Based on in silico analysis, GPTR29 exhibited great potential for plant growth promotion through several mechanisms as also demonstrated by in vitro assays. The GPTR29 genome encodes key genes of indol-3-acetic acid biosynthesis pathways, which play important role in plant development and growth [5]. Additionally, genes associated with nutrient uptake, such as phosphorus and iron uptake, were also identified. Thus, the use of phosphatase enzymes to solubilize insoluble forms of this compound has been described as a valuable PGP mechanism [24, 51]. Also, the presence of genes involved in siderophores biosynthesis and transport was observed, been widely described as a way to provide plants with Fe [5, 19, 26]. In addition, the production of bacterial polysaccharides, especially in saline conditions, can improve soil structure and water retention or limit the toxicity of certain ions, enhancing plant growth [5, 6, 19, 24, 53]. The potential of this strain to colonize the root system of lettuce was confirmed by the presence of genes related to the synthesis of LPS or cellulose [18].

The presence of genes related to the synthesis of osmoprotectants (trehalose, glycine-betaine, choline or ectoine) indicated the potential ability of the isolate to tolerate salinity, which was confirmed by its ability to grow under high-salinity conditions. [5, 7]. Moreover, GPTR29 genome encodes for genes related to the synthesis of glutathione or phenolic compounds, involved in oxidative stress fight [46, 51]. Last, the production of VOCs is described as a mechanism for the stimulation of plant growth, as well as resistance to biotic and abiotic stresses [5, 11, 52, 53]. In this regard, in silico genome analysis predicted the potential capacity of GPTR29 related to metabolic pathways to produce terpenes, ketones, or branched-chain alcohols.

Additionally, it was observed that GFP-tagged GPTR29 was able to colonize the root system of lettuce in a homogeneous way and to form biofilms, under non-stressed and salt-stressed scenarios. The ability to colonize root parts of non-legume plants has already been described for this genus, and can help plants to fight against salt stress [6, 19, 24]. The polysaccharide components of the biofilm matrix act as a physical barrier around the root system, preventing the toxic effects of salinity, improving water retention, and facilitating soil porosity and, therefore, soil structure [5, 50].

It has been reported that in plant–bacteria interactions, mixtures of VOCs released by PGPR bacteria can be perceived by plants, triggering a response [11, 16, 52, 53, 60]. Such a response can result in plant growth promotion, induced systemic resistance (ISR), or even pathogen suppression [6, 16, 53]. In recent years, some studies have shown that plant–*Rhizobium* interactions are conditioned by the bacteria's production of VOCs, which induce physiological adaptations for enhanced nutrient uptake, although the depth of these relationships is unknown [49]. GC–MS analysis of the GPTR29 volatilome showed a differential response under normal and saline growth conditions and between two different growth times (2 and 5 dpi). In this regard, it has been described that the profile of emitted VOCs is highly dependent on the culture medium and growth conditions of the treatment, as in the case of saline media [11, 53]. In addition, the density of the bacterial inoculum also affects the blend of volatiles [10], as does the state or growth stage of the inoculum, as shown in this work.

Among the compounds emitted by GPTR29 were some branched-chain alcohols, such as 3-methyl-1-butanol and 2-methyl-1-butanol, already described as possible triggers of growth promotion in *Arabidopsis* [16]. Likewise, the ketone 5-methyl-3-hexanone was reported to enhance *Arabidopsis* growth and has been emitted by *Burkholderia ambifaria* LMG 19467 [22], as well as by GPTR29. The bioactivity of acetoin emission was previously demonstrated, not only in promoting plant growth, but also in increasing salinity tolerance [11, 16]. Monoterpenes, such as α-pinene, have important chemoecological functions in plants, and have been reported to stimulate root growth, germination, and mitochondrial respiration in maize at concentrations of 0.05–1.0 mM, and it was also found in GPTR29 emissions [1]. Thus, it is observed that the compounds 3-methyl-1-butanol, 2-methyl-1-butanol, or α-pinene, which have been previously described in the literature for their PGP capabilities, induction of systemic resistance and beneficial effects on salinity tolerance [1, 11, 16, 22] act as the backbone of the volatilome by being present at both measurement times under both saline and non-saline conditions. The compounds 3-heptanone, 3-methyl-1-butanol and α-pinene have been detected in the analysis of the volatile metabolome of *Rhizobium*, with an increase in their relative concentration under heavy metal stress conditions [12], also shown in our research with a higher relative concentration under stress conditions; whereas, the increased production of aromatic alcohols such as

3-methyl-1-butanol in *Rhizobium* appears to be linked to an enhanced response to oxidative stress [31]. This finding would show a common mechanism of response to oxidative stress generated under abiotic stress conditions.

One of the benefits of plant exposure to acetoin and α-pinene is the stimulation of the root system (under both saline and non-saline conditions) [11, 16], an effect that was also observed in the present work. However, the effects of VOCs on plants are associated with the mixture composition [11, 16, 52, 53], and not always have the same effect on plant growth and stress tolerance [11, 16]. The results obtained from these analyses show for the first time the blend of volatiles emitted by a strain belonging to the genus *Rhizobium* and its beneficial effects on the growth of lettuce under both normal and salt-stress conditions. This extends the knowledge on the PGP mechanisms of this bacterial genus, especially to promote plant tolerance to salinity.

The results obtained in the present study indicate that GPTR29 was able to improve lettuce development during the initial stages of growth, even under salinity conditions. The inoculated lettuce seedlings had longer stems than the uninoculated controls, as well as larger radicular systems (longer and with a greater number of secondary roots). These results are of great interest, as the early stages of development are a limiting factor for plant establishment [58]. In this sense, it has been shown that exposure to salinity in lettuce seedlings generally delays germination and reduces growth [58].

The increasing problem of soil salinity in agriculture calls for new approaches to mitigate salt stress in crops. Appropriate farm management practices, plant breeding, or transgenic approaches are expensive and time-consuming alternatives that are not always easy to implement [23]. Nevertheless, the use of rhizobacteria has emerged as a sustainable and environmentally friendly alternative to address this issue [6, 7, 11, 25, 46, 50–53, 60]. This work found that inoculation with GPTR29 resulted in superior production parameters under saline and non-saline conditions, including larger size of aerial part and higher chlorophyll content. Under salinity conditions, it has been observed that significant degradation of this pigment occurs due to the action of chlorophyllase enzymes, among others [26]. Therefore, GPTR29 not only promotes plant growth and marketable yield, but also helps reduce chlorophyll degradation, contributing to the plant's appearance and tolerance to adverse environmental conditions that have become increasingly frequent in agriculture. This is consistent with previous results demonstrating the ability of the genus *Rhizobium* to promote the growth of non-leguminous plants and help reduce the negative effects of salinity [6, 7, 24, 25]. Hence, GPTR29 can be considered a very interesting alternative to ameliorate the effects of salinity on horticultural crops.

Based on the results obtained on the composition of phenolics in lettuce leaves (Table 5), it has been observed that the total content of phenolic acids and flavonoids is in accordance with previous studies carried out on *Lactuca sativa* L. var Romaine [6, 28]. Comparing the content of phenolic compounds between plants grown under normal conditions to those grown under salinity conditions, it was observed that saline stress led to an increase in these compounds in lettuce leaves. In this regard, phenolic compounds have been described as having high antioxidant activity, which limits the effects of cellular and molecular damage caused by reactive oxygen species (ROS) [13, 28]. This increase is consistent with the results of other scientific literature, for example, Mahmoudi et al. [29] reported that the content of flavonoids and total phenolics increased significantly in lettuce var. Romaine leaves when exposed to 100 mM NaCl treatment, in agreement with our results. However, it was also observed that both the type of salinity to which lettuce was exposed (NaCl or Na_2SO_4) and the lettuce variety were key parameters influencing the behavior of the phenolic content under these conditions [29, 37, 47].

Moreover, *Rhizobium* sp. GPTR29 inoculation affected the phenolic content of lettuce but in a different way depending on salinity stress (Table 5). Thus, on the one hand, under normal conditions, no significant differences were observed due to inoculation with this strain, whereas the general response observed under salinity conditions (100 mM NaCl) was an increase in phenolic acid and flavonoid content (the latter significant) in the leaves of inoculated plants. It has been suggested that this alteration in phenolic content may be a response of the plant to colonization by PGPRs and may induce the biosynthesis of these compounds through elicitation of the induced systemic tolerance process (IST) [26, 27].

As previously mentioned, the variation in phenolic composition depends on the growing conditions, the plant variety or the plant itself, but also on the type of inoculum used. Thus, Santander et al. [46] found no increase in phenolic content when arbuscular mycorrhizal fungi were inoculated in two lettuce varieties. Conversely, in a previous work [6], we reported increased phenolic acid and flavonoid contents with *Rhizobium laguerreae* inoculation in lettuce var. Romaine under normal conditions. However, studies of inoculation with PGPRs in various crops exposed to salinity showed an increase in the levels of phenolic compounds and flavonoids [25, 26, 43]. These last results agree with those described in the present work, which suggests

the protective role of GPTR29 against the negative and inhibitory effects caused by salinity. In this sense, the increase in the accumulation of phenolic compounds is a positive result, as it allows the plant to cope with the harmful effects of salinity by presenting a more effective defense system against oxidative stress than non-inoculated plants [43, 47]. In addition, the increase in phenolic compounds leads to an improvement in the nutritional content of the plants, which has a positive effect on the consumer and his or her health, as the antioxidant activity of these compounds is associated with a lower risk of cancer and cardiovascular diseases [13, 28].

Conclusions

The present work highlights the unexplored potential of the genus *Rhizobium* in promoting plant growth and enhancing crop adaptation to salt stress. The strain GPTR29, which belongs to a putative new species of the genus *Rhizobium*, has shown a wide variety of PGP mechanisms such as phytohormone biosynthesis, siderophore production, exopolysaccharide synthesis, osmolyte biosynthesis or volatile compound production as evidenced by genome annotation. The characterization of the *Rhizobium* volatilome carried out genuinely in this work has shown a differential response to salinity conditions, although it can be established that the volatilome of *Rhizobium* sp. GPTR29 presents a series of compounds (3-methyl-1-butanol, 2-methyl-1-butanol, and α-pinene) that are produced under all condition and which have the capacity to improve plant production, adding these mechanisms to those already known for this bacterial genus. In addition, inoculation and volatile emissions from this strain, GPTR29, have also been shown to have a positive effect on lettuce growth both under normal and saline conditions, improving plant productivity and mineral content under adverse environmental conditions. It has also been demonstrated that plants treated with GPTR29 significantly increase their nutritional content under salinity stress conditions, exhibiting a significantly higher concentration of phenolic acids and flavonoids than control plants. Furthermore, these results provide new avenues for the study of PGP mechanisms in this bacterial genus, such as VOCs and their effects on plant growth, which play an important role in mediating plant–microorganism interactions. For all these reasons, and in the face of climate threats and consequent adverse environmental conditions, the use of biofertilizers is a sustainable and safe alternative to maximize crop yield and quality.

Supplementary Information

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Additional file 1: Results of two-way ANOVA performed to assess the effect of salinity and inoculation on the parameters analysed in the experiments of the manuscript. The information is presented in Tables S1, S2, S3 and S4 according to each experiment.

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Author contributions

Conceptualization, JDF-F and RR; methodology, JDF-F, AJ-G and MA-C; software, JDF-F, FA, IG-E and MA-C; validation, PGP, MTE-B, RR and JDF-F; formal analysis, JDF-F; investigation, MA-C, FA, AJ-G, IG-E, and JDF-F; resources, PGP, MTE-B and RR; data curation, IG-E, PGP, MTE-B, RR and JDF-F; writing—original draft preparation, MA-C; writing—review and editing, IG-E, PGP, MTE-B, RR and JDF-F; visualization, PGP, MTE-B, RR and JDF-F; supervision, PGP, MTE-B, RR and JDF-F; project administration, RR; funding acquisition, PGP, MTE-B and RR. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

Genome data sequences are available in the GenBank under the following accessions: Bioproject: PRJNA603738; Biosample: SAMN14770944; Assembly: ASM1300415v1; Accession number: JABERB000000000.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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