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SOIL RESEARCH

Enhancing agriculture recovery of *Phaseolus vulgaris* L. and *Cucurbita pepo* L. with *Olea europaea* L. plant growth promoting rhizobacteria

R. Hadjouti[A,*], H. Mohand Kaci[A], F. Benzina[A] and J. N. Furze[B,C,D]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
R. Hadjouti
Laboratoire de Valorisation et Conservation des Ressources Biologiques (VALCOR), Faculté des Sciences, Département de Biologie, Université M'hamed Bougara de Boumerdes, BP35000 Boumerdes, Algeria
Email: r.hadjouti@univ-boumerdes.dz

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ABSTRACT

Context. The rhizosphere is an environment created by interactions between root exudates and microorganisms. Interactions are beneficial due to certain components having a plant growth promoting rhizobacteria (PGPR) effect. **Aims.** This study consists of the isolation, screening of PGPR from the rhizosphere of *Olea europaea* L. of a Mediterranean climatic region in Algeria and the study of their effects on growth of two agronomic vegetables *Phaseolus vulgaris* L. and *Cucurbita pepo* L. **Methods.** Based on their ability to produce the PGPR molecules indole-3-acetic acid (IAA), phosphatase and siderophores, three rhizobacteria (S25, S75, and S79) were chosen for *in vivo* tests and capacity to produce the cell wall degrading enzymes chitinase, lipase, protease, glucanase, cellulase, and and phospholipase. They were also examined using scanning electron microscopy (SEM) and analysed using matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) for identification. **Key results.** Bacterial strains identified as *Bacillus cereus* and *Bacillus thuringiensis* were able to enhance significantly germination of the two vegetables at $P < 0.001$. Vegetative parameters of *C. pepo* were significantly affected by the bacterial inoculation. We noted increases in stem length ($P < 0.05$), number of flowers ($P < 0.01$), and root length ($P < 0.001$). **Conclusion.** The bacterial isolates of this study provide biological options in treatments originating from alternate hosts. **Implications.** They provide hope for companion/intercrop planting schemes, leading to optimisation of agricultural yields in agroecological blends.

Keywords: *Bacillus*, cell wall degrading enzymes, indole-3-acetic acid, matrix-assisted laser desorption/ionisation time of flight mass spectrometry, phosphatase, plant growth promoting rhizobacteria, siderophores.

Introduction

The principle imperative facing us today is the feeding of a human population, which may reach in excess of 8.9 billion by 2050, with major global increased demand for food in developing countries such as those in Asia and Africa. Agricultural practise optimisation serves to meet our expanding nutritional requirements with limits to agricultural production; catastrophic scenarios are eminent. Conventional modern agriculture increases productivity through external chemical inputs, including fertilisers, pesticides, fungicides and herbicides (Mokrani *et al.* 2019; Fadiji and Babalola 2020). However, excessive chemical application causes environmental disorders that affect both soil quality and plant health. In addition, chemical application promotes resistant pathogen emergence and decreases beneficial organism populations in the edaphic environment (Pandey *et al.* 2019; Sabaté *et al.* 2020). Crucial interactions between plant soil and microfauna occur in edaphic settings. Three distinct components are recognised in plant rhizospheres; the rhizosphere, the rhizoplane, and the root itself. Rhizospheric soil zones are influenced by root exudates that effect microbial relation (activity). The rhizoplane is the root surface, including adhering root components. Herein, endophytic

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microorganisms are able to colonise inner root tissues (Compant *et al.* 2019). In the dynamic environment of the rhizosphere, microorganisms develop and interact (Rabbee *et al.* 2019; Chandra *et al.* 2020). Rhizospheric microorganisms have essential roles in plant–host ecological fitness. They complement plant growth and improve pathogenic resistance. Microorganisms uphold growth of plants and thereby have effects on soil and crop qualities (Zhu *et al.* 2020; Azizoglu *et al.* 2021). Plant growth promoting rhizobacteria (PGPR) are soil bacteria present around/on the root domain and are involved in promoting plant growth and development via secretion of various regulatory analogues predominant in the rhizospheric zone (da Silva *et al.* 2018; Rodriguez *et al.* 2019). Hence, they may decrease our dependence on agricultural chemicals (Ahemad and Kibret 2014; Pereira *et al.* 2019). The mode of action of PGPR that promotes plant growth comprises: (1) abiotic stress tolerance in plants; (2) nutrient fixation and uptake; (3) growth regulation; and (4) siderophores, volatile organic compounds, and protecting enzymes production such as chitinase, cellulase, glucanase, and 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase for the prevention of plant diseases (Minotto *et al.* 2014; Vejan *et al.* 2016; Duca *et al.* 2018).

Recently, consumers are exhibiting an increased interest in the relationship between their health and the nutritional aspects of their food (including vitamin content, mineral elements and antioxidants, etc.) (Chekanai *et al.* 2018; Neves *et al.* 2019). Common bean (*Phaseolus vulgaris* L.) and zucchini (*Cucurbita pepo* L.) are fresh grown vegetables with important nutritional and economic value. Common bean is the world's most important food legume for human consumption and is in great demand in Africa and Latin America (Myers and Kmiecik 2017). Beans provide a source of protein, dietary fibre, starch and minerals (including potassium, thiamine, vitamin B6 and folic acid) (Chekanai *et al.* 2018). Bean cultures are characterised by sensitivity to environmental factors such as sub-optimal availability of mineral nutrients. Low phosphorus (P) availability is considered to be the principal limiting factor for legume growth (Neila *et al.* 2014). Zucchini is an important crop of Mediterranean origin. Beneficial characteristics of zucchini include its nutrient content, short growing period, ease of storage and transportation and medicinal value (Liu *et al.* 2020). It contains a number of beneficial micronutrients such as minerals, carotenoids, vitamin C and phenolic compounds (Martínez-Valdivieso *et al.* 2017). Olive (*Olea europaea* L.) displays strong growth and productivity across northern Africa (Atrouz *et al.* 2021). This crop is not irrigated or chemically treated in Algeria, hence the need to preserve and valorise their rhizosphere, which constitutes a reservoir of biodiversity and provides a candidate for rhizobacterial flora with PGPR benefits to complement bean and zucchini growth.

The aim of this study to isolate and screen for new rhizobacteria from olive (*O. europaea*) with a PGPR effect in *C. pepo* and *P. vulgaris*. The objective of the current study is to demonstrate bacterial isolates production of plant growth molecules (IAA, siderophores, phosphatase) and to show acceleration of germination and vegetative development of the two plants. Further, we hope to confirm identification of isolates using basic laboratory testing, scanning electron microscopy (SEM) and matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectroscopy.

Materials and methods

Soil sampling and isolation of rhizobacteria

Olive rhizospheric soil and root samples were collected from an olive field (in January 2019) in Bir Khadem region, Algiers, Algeria (36°42′59.99″N, 3°03′60.00″E). Samples were taken 0.5–1 m around plants at 30–50 cm depth in the soil. Extracted root systems were carefully shaken by hand to remove soil. Roots and rhizospheric soil were placed in sterilised plastic bags. In the laboratory, serial dilutions were used to isolate bacteria from the three parts of the rhizosphere: (1) rhizospheric soil (RS); (2) rhizoplane (RP); and (3) endorhizosphere (E). A total of 10 g of soil and 10 g of roots were added separately to 90 mL of sterilised saline solution (0.9% NaCl) for isolation from RS and RP respectively. Whereas in endospheric isolation 10g of roots were sterilised with 2% sodium hypochlorite solution for 3 min, then washed five times with sterile distilled water in order to eliminate bacteria residing in the root surface which belong to RP. The roots were ground with a sterile mortar to free the inside of the roots and then added to 90 mL of sterilised saline solution (0.9% NaCl). The three resulting saline solutions were used for preparation of serial dilutions for isolation of rhizobacteria using nutrient agar and King B medium. After incubation for 24–72 h at 30°C, all morphologically different colonies according to their macro and microscopic characteristics (shape and texture of colonies, Gram colouration, spore presence, motility) were isolated, purified and tested for phytopathogenicity and PGPR traits.

Determination of gram strains

Thin microbial smears were air dried and fixed by heat. Smears were held using a slide rack then covered with crystal violet for 1 min. Each slide was washed with distilled water for a few seconds, then covered with an iodine solution for 30 s. The iodine was washed off with 95% ethyl alcohol solution. Fushine was applied to each smear for 1 min. Smears were washed with distilled water. The stained slides were air dried and observed (Aneja 2007).

Scanning electron microscopy

Bacteria were cryofixed and rapidly examined at very low temperatures (below −120°C) by cryo-SEM and metalised by cathodic spraying with gold alloy according to Kaláb *et al.* (2008).

Determination of phytopathogenic rhizobacteria

Isolated rhizobacteria were tested for their pathogenicity on plants by their hypersensitive reaction. A volume of 1 mL of bacterial suspension was injected in tobacco (*Nicotiana tabacum* L.) leaves at 20–25°C. A control was maintained with sterile physiologic water injection. After 24 h, the appearance of a collapse at the injection site indicated a positive hypersensitive reaction (Cooksey *et al.* 1990).

Phytopathogenic isolates were eliminated and were not subjected to determination of plant growth promoting attributes.

Determination of plant growth promoting attributes

The isolated rhizobacteria were screened for PGPR attributes by assessing the production of IAA, siderophore and phosphate solubilisation as follows.

IAA production

IAA production was estimated calorimetrically with the standard method described by Bric *et al.* (1991) with some modifications. In the current study, Luria–Bertani (LB) broth (g/L) was prepared with yeast extract (5 g/L), NaCl (5 g/L) in distilled water (1000 mL) and supplemented with tryptone (10 g/L). pH was adjusted to 7.5 and supplemented with 5 mM of L-tryptophan (LBT: LB supplemented with L-tryptophan), 0.06% of SDS and 1% glycerol. Bacterial suspensions (10^6 CFU mL^{-1}) were incubated for 3 days under continuous stirring at 180 rpm at 28 ± 2°C, pelleted through centrifugation at 10 000*g* for 10 min at 4°C). A volume of 1 mL of the supernatant was incubated with 2 mL of Salkowski reagent: $HClO_4$ (150 mL), distilled water (250 mL), and 0.5 M $FeCl_3{\cdot}6H_2O$ (7.5 mL) for 30 min in the dark at room temperature (30°C). The concentration of IAA produced was calculated using a standard curve. Optical density was recorded at 535 nm with known amounts of commercial IAA in Salkowski reagent and sterile LBT broth.

Siderophore production

Siderophore production was revealed using King B solid medium: (peptone 20 g); (K_2HPO_4 1.5 g); ($MgSO_4$ 1.5 g/L); glycerol (15 mL/L) and agar agar (15 g/L). After inoculation and incubation during 24–96 h at 30°C of bacteria isolates, florescent pigmentation was observed in siderophore producing bacteria with the naked eye and under ultraviolet (UV) light at wavelengths 254 and 366 nm (King *et al.* 2009).

Siderophores were also detected on Chrome Azurol-S (CAS) agar medium. Isolates were streaked on Petri plates containing CAS medium and incubated at 30°C for 48 h. A positive result was revealed by formation of orange halos (Alexander and Zuberer 1991).

Phosphate solubilisation

Phosphate solubilisation was indicated following Pikovskaya (1948). Bacteria were inoculated in Pikovskaya medium (PVK: 10 g/L glucose, 5 g/L, $Ca_3(PO_4)_2$, 0.2 g/L of KCl, 0.1 g/L of $MgSO_4{\cdot}7H_2O$, 0.2 g/L of NaCl, 0.5 g/L of $(NH_4)_2SO_4$, 0.02 g/L of $FeSO_4{\cdot}7H_2O$, 0.002 g/L of $MnSO_4{\cdot}2H_2O$, 20 g/L of agar agar), then incubated at 30°C during 72 h. Positive reactions were shown by the presence of a clear halo surrounding bacterial colonies.

Cell wall degrading enzymes production

The three bacterial isolates with greatest capacity to produce PGPR molecules were subjected to extracellular enzyme production tests.

Chitinase production

The ability of bacterial isolates to produce chitinase was ascertained by spotting them on a basal medium mixed with 2.4% chitin suspension (El-Masry *et al.* 2002).

Protease production

A growing bacterial culture was inoculated in the form of a line in skim milk agar for 48 h at 30°C. A clear zone around the line indicated a positive result (Smibert and Krieg 1994).

Lipase production

Spots of isolates were deposited in the surface of LB medium supplemented with 1% Tween 80 and incubated from 1 to 5 days at 28°C (Sierrea 1957). An opaque halo around colonies indicated a positive result.

Glucanase production

A specific agar medium was used composed of peptone (5 g/L), yeast extract (5 g/L) and barley (*Hordrum vulgare* L.) flour 10 (g/L). A colony of each strain was inoculated following Zouari *et al.* (2020).

Cellulase production

Cellulase production was determined with the method described by Prasad *et al.* (2012) using M9 agar (Miller 1972), supplemented with 10 g/L cellulose and 1.2 g/L yeast extract. Isolates were inoculated and incubated for 8 days at 28°C. Development of a clear halo around colonies indicated a positive response (Verma *et al.* 2007).

Phospholipase production

Nutrient agar was supplemented with 10 mL of sterile egg yolk emulsion in physiological water. Spots of isolates were

deposited in the surface of the prepared medium, then incubated at 30°C for 24 h. The appearance of a clear halo around the spots indicated a positive result (Thaler *et al.* 1998).

In vivo assays

Three of the best rhizobacteria from isolates with the highest level of IAA production and/or the highest number of positive result for production of PGP molecules were chosen for *in vivo* testing according to Lwin *et al.* (2012), including germination tests and vegetative growth assays. Seeds of zucchini and common bean were used for each treatment. The seeds used in the current experiment were supplied by the Algerian National Centre for Seed and Plant Control and Certification.

Germination test

Zucchini and common bean seeds were sterilised in 2% sodium hypochlorite solution for 3 min, then washed five times with sterile distilled water. Sterilised seeds were incubated in 50 mL of bacterial suspension (10^6 CFU mL^{-1}) at 28 °C for 24 h. Control preparation of seeds was maintained in sterile distilled water. After incubation, seeds were transferred into Petri plates containing sterile humid cotton and incubated at normal room temperature for 10 days (Lwin *et al.* 2012).

Vegetative growth assays

Sterilisation and inoculation of seeds for vegetative growth tests was carried out following the same steps described as for germination. After 24 h of incubation, inoculated seeds were sown in plastic pots (16 cm high and 19 cm diameter) containing sterilised soil (three times autoclaved for 20 min at 120°C with an interval of 24 h) in greenhouse conditions. The experiment was carried out three times with nine replicates per treatment. All pots were watered three times a week with 200 mL of sterile water. After 40 days, plants were separated and transferred. Measurements of root length, stem length, number of lateral roots, number of leaves, and number of flowers were recorded (Lwin *et al.* 2012).

Characterisation of efficient bacteria

MALDI-TOF MS (matrix-associated laser desorption/ionisation-time of flight mass spectrometry)

Matrix preparation was carried out by diluting a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 500 µL of 50% acetonitrile, 250 µL of 10% trifluoroacetic acid (TFA) and 250 µL of HPLC water. After vigorous stirring, sonication was carried out for 10 min, followed by centrifugation (13 000*g*, for 5 min). Samples were transferred to clean polypropylene tubes.

Each bacterial colony obtained from young cultures (18–24 h) was deposited in duplicate on the MALDI-TOF target plate and covered with 1.5 µL of the matrix solution. The matrix and target plate were dried at room temperature (28°C) for 5 min and analysed (Pfleiderer *et al.* 2013). A Microflex LT MALDI-TOF mass spectrometer was used for bacterial identification. The spectra of the bacteria obtained were compared with the Bruker computer database using the flex Analysis ver. 3.3 and MALDI-Biotyper ver. 3.0 software for data analysis. Isolates were assumed to be correctly identified at the species level when the logarithmic score value (LSV) was greater than or equal to 1.9 (Seng *et al.* 2009).

Statistical analysis

Germination and vegetative assays data were subjected to one-way and two-way ANOVA and the Tukey Test, using SPSS ver. 16. Differences were considered to be significant at $P < 0.05$.

Results

Isolation of rhizobacteria and observation of phenotypic characteristics

A total of 113 rhizobacteria were isolated from the three different part of the olive rhizophere. A total of

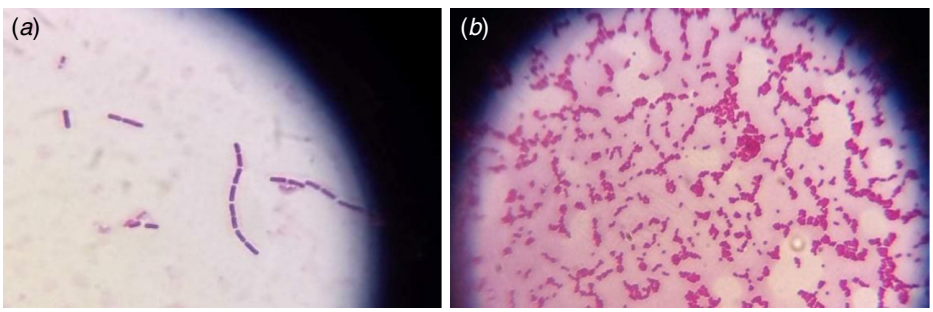

Fig. 1. Gram-positive and Gram-negative bacteria microscopic aspect under photonic microscope GX1000. (*a*) Strain S79, Gram-positive rod-shaped bacteria, (*b*) Strain S35, Gram-negative spherical-shaped bacteria.

28.32% of the rhizobacteria were endophytes; 36.28% are from the rhizoplane and 35.4% are from the rhizosphere.

The majority of the obtained colonies were white or beige, others were orange. The texture was opaque for the majority, sometimes shiny, flat or convex, mucoid or dry. The contour was regular or irregular.

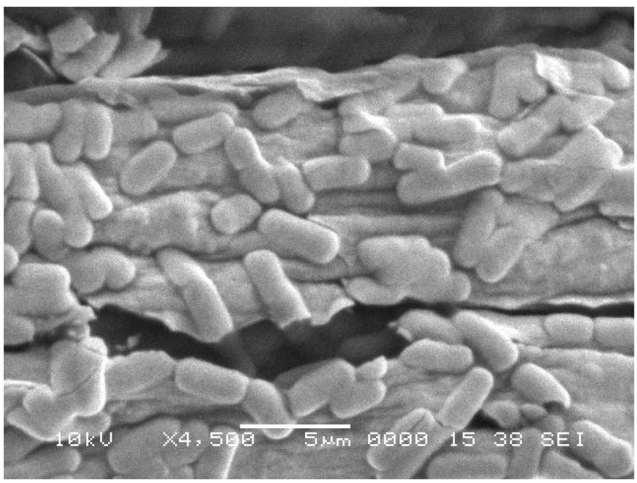

Fig. 2. Scanning electron microscopy of S25 showing the morphological rod-shaped aspect of isolated bacteria (GX 4500).

Gram colouration and scanning electron microscopy

The isolates were divided into four groups: (1) Gram-positive rod-shaped (46.90%); (2) Gram-positive spherical-shaped (8%); (3) Gram-negative rod-shaped (39.82%); and (4) Gram-negative spherical-shaped (4.42%) (Fig. 1*b*) based on the Gram staining analysis. Most of the Gram-positive bacteria were able to grow at 40°C and 55°C with presence of spores while Gram-negative isolates, which were mostly isolated in King B medium did not form endospores.

The three selected rhizobacteria for *in vivo* tests had rod-shaped form under photonic microscopy and scanning electron microscopy (Fig. 2). S75 was Gram-negative, whereas S25 and S79 were Gram-positive (Fig. 1*a*) with the presence of endospores.

Phytopathogenicity traits

Only two of the isolated rhizobacteria showed positive pathogenicity, they were eliminated from the study. The three selected rhizobacteria (S25, S75 and S79) were chosen according to their PGPR traits and showed negative reactions in the phytopathogenicity test.

Plant growth promoting attributes

IAA production

A total of 47.72% of bacteria tested for IAA production revealed positive results (Fig. 3). According to the realised

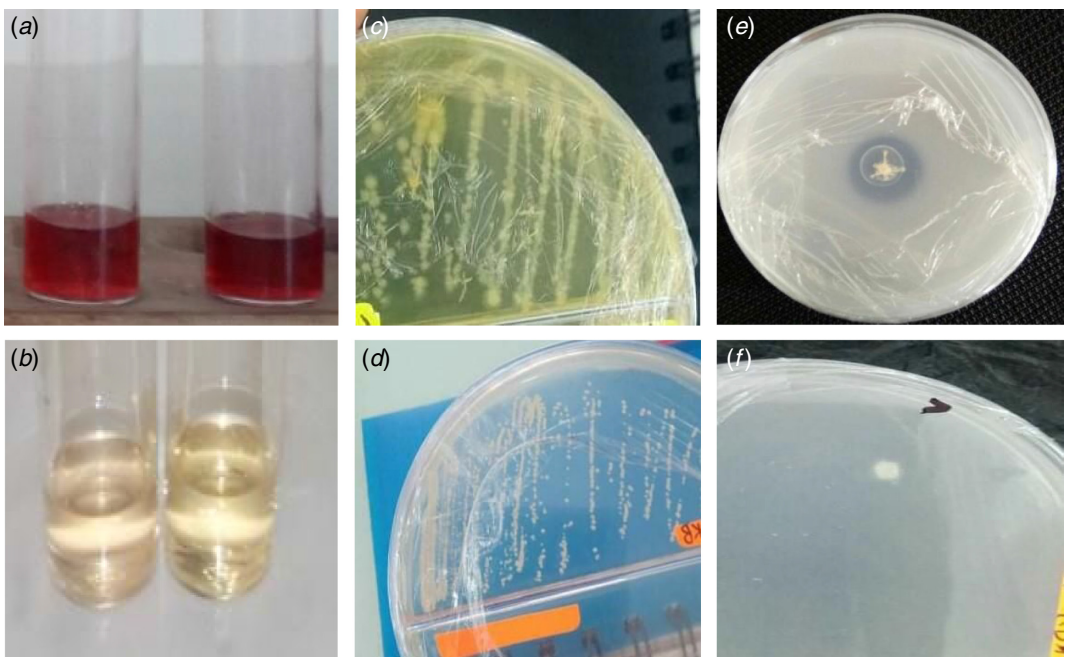

Fig. 3. Screening for PGPR activity results. (*a*) Positive result for IAA production, characterised by the appearance of red colour instead of yellow colour, which indicates a negative result as shown in (*b*); (*c*) positive result of siderophores production of types pyoverdine and pyocyanin appeared as fluorescent pigmentation on King B medium, absence of fluorescence indicates absence of production (*d*); (e) positive result of phosphate solubilisation revealed by a clear halo around colonies, the absence of halo indicated a negative result (*f*).

standard curve, quantitative IAA production on LB broth supplemented with (0.5%) of L-tryptophan gave a varying level of IAA production. The highest level recorded was by strains S1, S4, S24, S25, S26, S75 and S79 (Table 1).

Siderophore production

After 72 h of incubation, 79.54% of bacterial isolates developed a fluorescent pigmentation on King B medium indicating the production of siderophores (Fig. 3), they also showed positive reaction on CAS medium represented by the appearance of an orange halo around colonies .

Phosphate solubilisation

A total of 68.18% of tested bacteria induced solubilisation of tri-calcium phosphate on Pikovskaya's medium by forming clear zones around the colonies where the highest production level according to the clear zones was recorded by strains S26 and S72. However, 43% of isolates did not show any halo around their colonies, indicating that no inorganic phosphate solubilisation (Fig. 3).

Cell wall degrading enzymes

The three selected bacteria were tested for extracellular enzyme production and produced chitinase, protease, lipase, phospholipase, and cellulase. Strain S79 was the only producer of glucanase (Fig. 4 and Table 2).

Germination test

Three bacterial strains (S25, S75 and S79), were chosen according to their PGPR efficacy and were inoculated in common bean and zucchini seeds. These isolates showed significant effects at $P < 0.001$. Best effect on zucchini and bean seed germination was recorded with the strain S79 (Fig. 5).

Vegetative growth assays

Strain S79 had the highest effect on stem length (84.12%), number of leaves (85.71%) and number of flowers (100%), though the lowest effect on roots length and lateral roots. Strains with S25 and S75 enhanced roots length and lateral roots number. One-way ANOVA tests showed a significant effect at $P < 0.05$ on stem length and flowers number, and a significant effect at $P < 0.001$ on roots length, while a non-significant effect (n.s.) was shown on lateral roots number (Fig. 6).

MALDI-TOF MS

MALDI-TOF MS analysis of the three selected bacteria revealed that the strains S25 and S79 are both *Bacillus* genus, whereas S75 logarithmic score value was below the recommended value for identification (Table 3). The mass spectrometry profiles characteristics of each strain are mentioned in Fig. 7. Each peak in the respective profiles identifies specific bacterial proteins.

Table 1. Main PGPR screening results.

Strain	Origin	Phosphatase	Siderophore	IAA (µg/mL)	
S1	E	+	–	+	73.54
S3	E	+	+	+	54.74
S4	E	–	+	+	86.25
S5	E	+	–	+	62.11
S6	E	–	+	+	19.54
S7	E	–	+	+	37.41
S9	E	–	+	+	33.36
S11	E	–	+	+	19.17
S13	E	+	+	–	–
S17	E	+	+	–	–
S20	E	+	+	–	–
S22	E	+	+	–	–
S24	E	–	+	+	84.96
S25	E	+	+	+	98.97
S26	E	–	+	+	133.80
S27	E	+	+	+	14.74
S34	RP	+	–	+	28.94
S38	RP	+	+	–	–
S39	RP	+	+	–	–
S40	RP	+	+	–	–
S42	RP	+	+	–	–
S48	RP	+	+	–	–
S52	RP	+	+	–	–
S57	RP	+	+	+	25.80
S59	RP	+	+	–	–
S70	RP	+	+	+	31.33
S71	RP	+	+	+	46.63
S72	RP	+	+	+	24.88
S73	RP	+	+	–	–
S75	RS	+	+	+	90.01
S76	RS	+	+	–	–
S79	RS	+	–	+	166.60
S80	RS	–	+	+	27.07
S81	RS	+	+	–	–
S83	RS	+	+	–	–
S84	RS	–	+	+	22.48
S87	RS	+	+	–	–
S89	RS	+	+	–	–
S91	RS	+	+	–	–

The main results of PGPR traits of the isolated rhizobacteria. Only strains with at least two positive results are mentioned (35.13% of the strains).

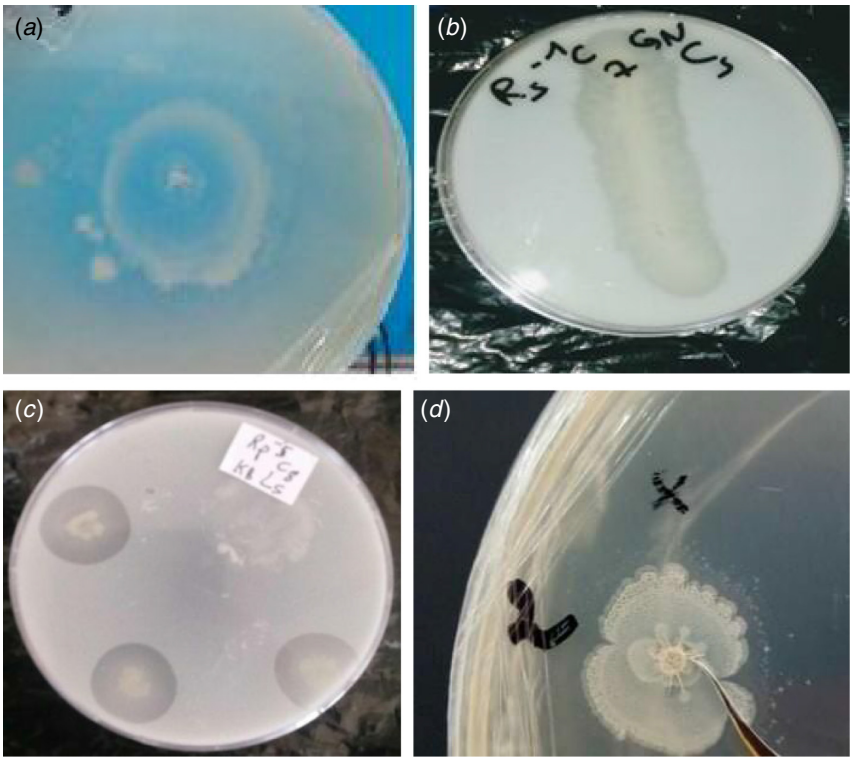

Fig. 4. Screening for cell wall degrading enzyme production. (*a*) Positive result for chitinase production, (*b*) positive result of protease production, (*c*) positive result of phospholipase production. All those three results were revealed by a clear halo around colonies. (*d*) Positive result for lipase production revealed by dark halo or formation of crystals around colonies.

Table 2. Cell wall degrading enzymes of the three selected rhizobacteria.

Strains	Chitinase	Protease	Glucanase	Lipase	Phospholipase	Cellulase
S25	+	+	−	+	+	+
S75	+	+	−	+	+	+
S79	+	+	+	+	+	+

−, indicated absence of production; +, indicated presence of production.

Discussion

In this work, we demonstrated that the three parts of olive rhizosphere (rhizosphere, rhizoplane and endorhizoplane) from a Mediterranean region (north of Algeria) are rich in rhizobacteria with high capacity for producing plant growth molecules (auxins, siderophores and phosphatase). The findings concerning the ability of rhizobacterial strains producing these molecules, indicate that they can be used to enhance crop growth. This concurs with Muñoz *et al.* (2020) in which rhizobacteria originating from cultivated crops from extreme environments were used.

Phytohormones have regulatory and signalling functions in growth and development. They are additionally produced by rhizobacteria supplementing cell division, cell elongation, and differentiation. Auxins are represented by IAA and analogues, which increase the surface area, length of the root and root exudation, providing the plant with better access to soil nutrients (Dastager *et al.* 2011; Park *et al.* 2013; Cecagno *et al.* 2015; Koua *et al.* 2020). Phosphate is the second most important macronutrient after nitrogen, effecting plant growth. Even in soil enriched with phosphate, only 0.1% is soluble and assimilated by plants. Paucity of phosphate severely limits global crop production (Mezaache-Aichour *et al.* 2012; Anzuay *et al.* 2013). Microbial solubilisation of phosphate is a significant factor in the conversion of insoluble phosphate to soluble phosphate (Koua *et al.* 2020).

Low molecular weight molecules, siderophores have high affinity to Fe^{3+} ions. Siderophores therefore facilitate iron availability, and are secreted under conditions of iron deficiency. On formation of the siderophore ion Fe^{3+} complex, a microbial cell's external membrane produces siderophores catalysing internalisation. Under conditions of iron deficiency, a siderophore-Fe^{3+} complex is formed, and the external membrane of the microbial cell producing siderophores catalyses the internalisation of these complexes (Gupta and Gopal 2008). The results of IAA production on LB supplemented with L-tryptophan showed that 47.72% of the tested bacteria produced IAA. 68.18% of

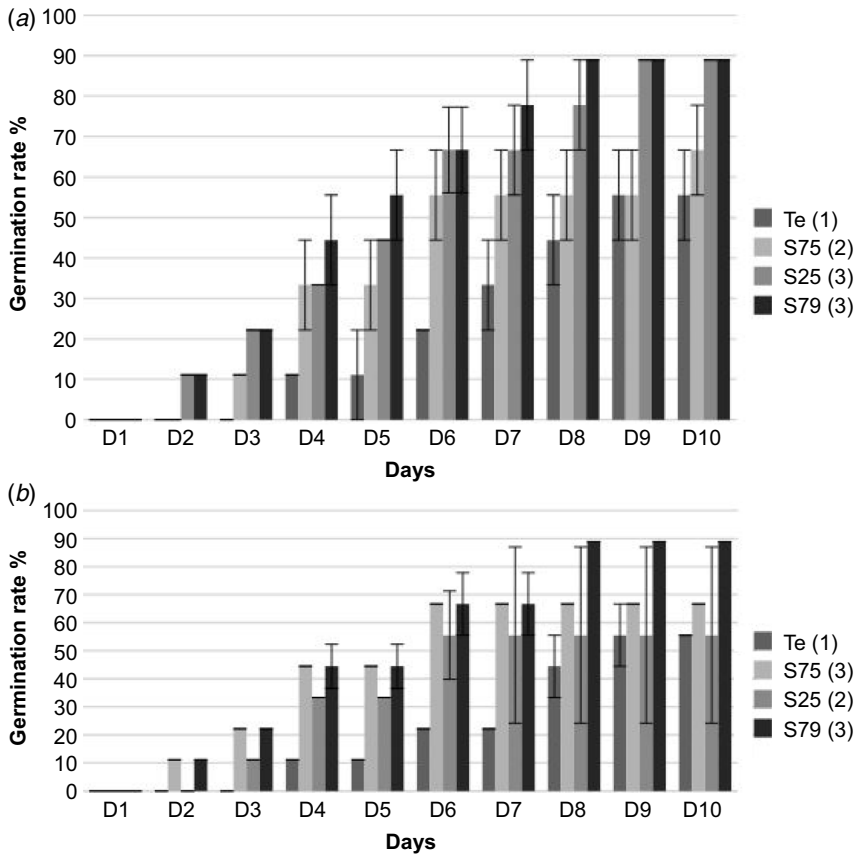

Fig. 5. Bacterial isolates effect on germination rate of zucchini (*a*) and common bean seeds (*b*). Strains with the same number (1, 2, or 3) are not significantly different using Tukey's test at $P < 0.001$. Te, control; S, strain; D, day.

isolates solubilised phosphate on PVK solid medium. 79.54% of the tested isolates were able to produce siderophores. These PGPR molecules have been reported in different rhizobacteria (Bhattacharyya and Jha 2012; Nabti *et al.* 2013; Zennouhi *et al.* 2018; Mokrani *et al.* 2019; Qessaoui *et al.* 2019; Oulebsir-Mohandkaci *et al.* 2021).

Three strains of the best producers of PGPR molecules (S25, S75 and S79) were selected and tested for their capacity of producing extracellular enzymes: chitinase, protease, glucanase, lipase, phospholipase and cellulase. Enzyme activities in soil are of increasing interest, breaching soil microbiology and biochemistry research. Bacterial and fungal microorganisms secrete extracellular enzymes with a major role in biogeochemical cycles (Bonnet *et al.* 2017). PGPR enzyme production insures sustainable plant disease management. The aforementioned enzymes break down the cell wall of fungal phytopathogens causing cell death; additionally they are lethal to nematodes and insects (Gow *et al.* 2017; Jadhav *et al.* 2017; O'Brien 2017). S25, S75 and S79 produced the mentioned enzymes apart from S25 and S75, which did not produce glucanase.

Chitin is the major component of fungi and insects exteriors. Previous research shows the chitinase from *Chitinophaga* spp. to have antifungal and nematicidal activity against *Fusarium oxysporum*, *Alternaria alternate*, *Cladosporium* spp. and root knot nematode, *Meloidogyne incognita*, a major pest responsible for economic losses in agriculture (Sharma *et al.* 2020). Chitinase from a biocontrol fungus, *Trichoderma asperellum* prevent anthracnose caused by *Colletotrichum* spp. on both mango (*Mangifera indica* L.) and chilli (*Capsicum frutescens* L.) fruits up to 72 h after enzyme pre-treatment at 40 U/mL (Loc *et al.* 2020). The study conducted by (Arora *et al.* 2007) showed inhibition of *Rhizoctonia solani* peaks when the synthesis of chitinase and glucanase is at maximum by a fluorescent pseudomonad.

The relationship between *Beauveria bassiana* producing enzymes, including proteases, against *Helicoverpa armigera* has been demonstrated (Kaur and Padmaja 2009). Protease purified from *Streptomyces flavogriseus* in the study conducted by Mostafa *et al.* (2019) showed inhibition against different phytopathogenic fungi, especially *F. oxysporium* and *R. solani*.

It is also important to note that cellulase acts against phytopathogenic fungi, *Phytophtora* and *Pythium*, whose cellulose content in cell walls is between 17% and 35% (Minotto *et al.* 2014). Sheetal *et al.* (2019) reported phospholipase from entomopathogenic *Xenorhabdus* spp. has a proven efficacy against filarial vector *Culex quinquefasciatus*.

Bacterial chemotaxonomy of S25, S75 and S79 using MALDI-TOF MS revealed S25 and S79 to be *B. cereus* and *B. thuringiensis,* respectively, while S75 could not be identified. MALDI-TOF MS profiling enables the identification of bacteria by the detection of proteins profiles, given that its

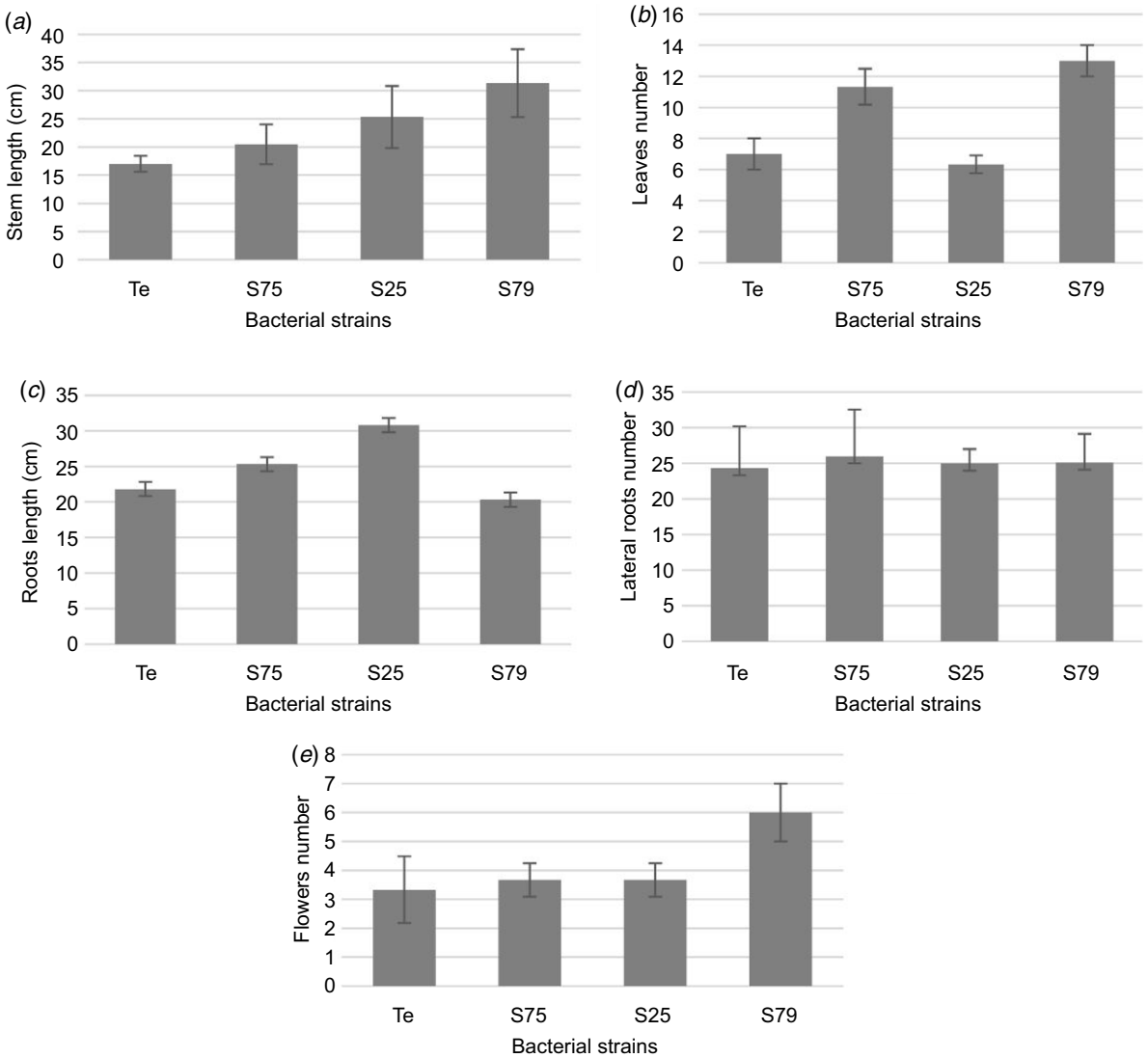

Fig. 6. Vegetative growth assays on zucchini culture after 40 days on (*a*) stem length, (*b*) leaf number, (*c*) root length, (*d*) number of lateral roots, (*e*) number of flowers. Te, control; S, strain.

Table 3. Identification results of the three selected bacteria using MALDI-TOF MS.

Strain	Organism (best match)	Logarithmic score value	Significance of results
S25	*Bacillus cereus*	2.012	Secure genus identification, probable species identification
S75	–	1.648	Unidentified
S79	*Bacillus thuringiensis*	2.044	Secure genus identification, probable species identification

spectrum is available in the mass spectrometer's database; giving the same result as *16S* rRNA and rDNA gene sequence analysis but at a rapid rate and a lower cost (Rahi *et al.* 2016; Grégory *et al.* 2018). However, its application on environmental bacteria is limited due to a lack of data on non-clinical microorganisms (Rahi *et al.* 2016; Kostrzewa and Maier 2017). Additionally, the culture medium effects the mass spectra, notably when the former does not sustain optimal growth. Growth medium compounds impede with the ionisation of the bacterial biomolecules, as the bacteria have a tendency to adhere to the culture medium surface (Wieme *et al.* 2014). Bacterial identification is related to cell concentration, various methods determine the minimum concentration of cell material needed for identification of bacteria using MALDI-TOF MS. A study showed *Escherichia coli* could be identified at species level at 8×10^4 viable

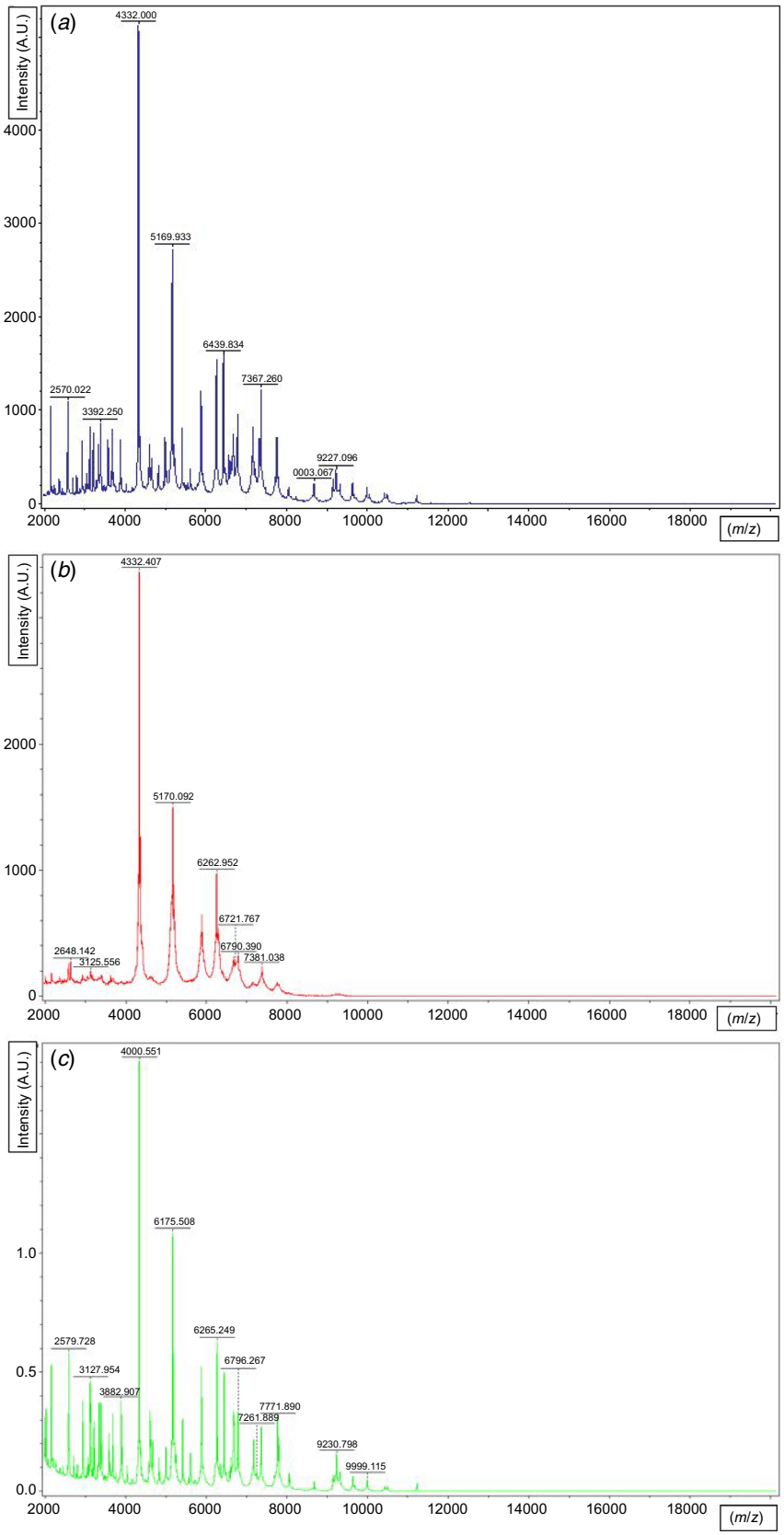

Fig. 7. Mass spectrometry profiles of the three strains. (*a*) S25, (*b*) S75, (*c*) S79.

cell count (VCC) per mL using a Bruker Autoflex, whereas *Enterococcus faecalis* did not reach consistently high identification scores even at 5×10^5 VCC per mL. Further, the detection limit may optimally be identified using diafiltration and specific extraction methods, combined with improved algorithms for spectral analyses (Mörtelmaier *et al.* 2019). As an example, the creation of a custom reference library make it possible to distinguish between *E. coli* and *Shigella* species, which were difficult to discriminate. Similarly, ClinProTools make it possible to identify *Streptococcus pneumoniae* and *Streptococcus mitis/oralis* despite the fact that these species are frequently confused (Grégory *et al.* 2018). Precedent issues, call for specific data analysis such as machine learning algorithms which have been leveraged to maximally exploit the information contained in MALDI-TOF MS, with the ultimate goal of refining species identification (Weis *et al.* 2020), otherwise, the use of machine learning techniques for microbial species identification purposes remains limited and is dominated by the application of adaptive artificial neural networks (ANNs). Research on ANN analysis of MALDI-TOF MS for bacterial identification is limited due to lack of resolution/reporting into the respective knowledge bases used. The evaluation of two other popular machine learning techniques: random forests and support vector machines by De Bruyne *et al.* (2011) proved to be very successful. Combinatorial of ANN and Fuzzy Logic Systems enables the representation of real-world. Hybrids of these methods increase their advantages and decrease their shortcomings. For example, in fuzzy neural systems, the fuzzy system can provide an input vector to a multi-layer neural network as a response to linguistic statements. Subsequently, the neural network is trained to generate required outputs or decisions (Vlamou and Papadopoulos 2019). Fuzzy logic is extremely useful for many people involved in research and development including environmental engineers, natural scientists (biology and agriculture), and medical researchers (Singh *et al.* 2013). Recently, Neuro-Fuzzy systems have gained more attention from research communities than other types of fuzzy expert systems since it combines the advantages of the learning ability of neural network and the reasoning ability of fuzzy logic to solve many non-linear and complex real world problems with high accuracy (Salleh *et al.* 2018). The concept of the fuzzy set operats via intelligent controllers formed through Mamdani and Takagi-Sugeno-Kang (TSK) systems. The TSK-type fuzzy model has advantages over the Mamdani-type in terms of computational efficiency. The defined input of TSK enables precise output ensuring accurate prevision of otimazation of adaptive techniques. The TSK system rationale enables further higher methods such as searching algorithm techniques, which can theoretically identify anything (optimisation by Hybrid Genetic Algorithm; optimisation by particle swarm, artificial bee colony. These searching optimisation techniques can then lead to functional distributes and expressions (for expansion of taxonomic information) (Furze *et al.* 2017; Yazid *et al.* 2019; Furze and Mayad 2021, 2022) .

In vivo tests were useful to ensure experimental conditions similar to the conditions of definitive application of rhizobacteria. PGPR can use different mechanisms to improve seed germination, root development or to improve mineral nutrition and water use (Dobbelaere *et al.* 2003; Mitter *et al.* 2013). Most *Bacillus* spp. have PGPR characteristics (Jin *et al.* 2019). Germination tests on common bean and zucchini seeds showed both significant effects at $P < 0.001$. Concerning vegetative growth, several studies have shown that rhizobacteria stimulate root development, such as research done by Cassán and Diaz-Zorita (2016); Agapit *et al.* (2020). Other studies have shown that rhizobacteria stimulate flowering, increase leaf and stem length compared to plants, which were not inoculated with rhizobacteria (El Habbasha *et al.* 2013; Mouradi *et al.* 2016) in agreement with our results on zucchini crops but with variable enhancement of growth. In spite of the latter, there are still no commercial *B. thuringiensis*-based PGPR products on the biofertiliser market (Azizoglu 2019), though *B. thuringiensis*-based commercial biopesticides are available. It should be noted that the resistance of *Bacillus* spores enables them to enhance plant growth in extreme environments, an advantageous feature compared to non-sporulating PGPR.

Conclusion

Rhizobacterial isolates of *B. cereus* and *B. thuringiensis* (S25, S75 and S79) can find their place in biotechnological applications such as crop production enhancement and environment protection. The strains provide a base for biofertilisers and biostimulants, in production of industrially important enzymes, and to produce pesticides.

Further work should study the biocontrol activity of the strains against different phytopathogens to evaluate their activity. It is also preferable to study the efficacy of beneficial strains combined to find the best matching mixture and investigate the mechanism of actions using biochemistry and molecular biology such as in gene expression analysis, amino acid, protein analysis and gene knockout studies.

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Author affiliations

[A]Laboratoire de Valorisation et Conservation des Ressources Biologiques (VALCOR), Faculté des Sciences, Département de Biologie, Université M'hamed Bougara de Boumerdes, BP35000 Boumerdes, Algeria.

[B]Royal Geographical Society (with the Institute of British Geographers), 1 Kensington Gore, SW7 2AR, London, UK.

[C]Laboratory of Biotechnology and Valorization of Natural Resources, Faculty of Sciences of Agadir, Department of Biology, Ibn Zohr University, BP 8106, 80000 Agadir, Morocco.

[D]Control and Systems Engineering Department, University of Technology, Alsinaah Street, P.O. Box: 19006, 10066 Baghdad, Iraq.