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Research Article

Exploring biofertilizer potential of plant growth-promoting rhizobacteria *Bacillus clausii* strain B8 (MT305787) on *Brassica napus* and *Medicago sativa*

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Abstract

Plant Growth Promoting Rhizobacteria (PGPR) are soil bacteria that can stimulate plant growth by supplying substances that are usually in limited quantities in the soil especially phosphorous, nitrogen and growth hormone such as indole acetic acid (AIA). These bacteria can also slow the growth of plant pathogens through the production of several antimicrobial metabolites. To investigate the role of rhizobacteria as a biostimulant agent a novel bacterium B8, isolated from the rhizospheric soil of medlar (*Mespilus germanica* L.- Family Rosaceae), was evaluated on *Brassica napus* and *Medicago sativa*. In addition to the classical methods of identification (physiological and biochemical tests), B8 was identified by 16S rRNA gene sequencing as *Bacillus clausii*. The ability of the strain to produce lytic enzymes such as cellulases, chitinases, pectinases, and phospholipases was studied. Furthermore, the strain B8 was tested for the capability to produce plant growth metabolites like phosphatases and phytases in order to solubilize inorganic phosphate and production of siderophores, cyanhydric acid (HCN) and indole-3-acetic acid. The strain was able to produce lytic enzymes, with an intense production of siderophores and to solubilize inorganic phosphate. Result of *in vivo* experiments indicated that the application of B8 at 10^7 CFU/mL, improved markedly the germination rate of rapeseed, whereas alfalfa seeds treated with the same strain showed a lower germination rate than the controls. The vegetative growth parameters; Roots length, lateral roots number, stem length, number of leaves, diameters of stems and plant weight were significantly improved. We also noted capacity of bacteria to colonize root systems of both plants *B. napus* and *M. sativa* in one week of inoculation. The overall results of this study showed that *B clausii* B8 has a great potential to be commercialized as a biostimulant agent and provide promising new option for sustainable agriculture.

Keywords: *Bacillus clausii; Brassica napus*; growth promoting; *Medicago sativa*; Rhizobacteria

Introduction

The rhizosphere is the part of soil where plant roots, soil and organisms interact. These interactions are often of benefit to plants. Rhizospheric microoorganisms provide nutrients, protection against biotic and

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abiotic stresses and plant growth stimulation (Lynch *et al.*, 2001; Filiz *et al.*, 2021). The importance of the rhizospheric microbiome composition on plant health and productivity has been increasingly recognized (Wang *et al.*, 2019).

Tens of thousands of species of microorganisms associated with plant roots, make an important diversity in soil. They play important roles against phytopathogens and plant insect attacks (Roeland *et al.*, 2012). The group of plant-associated, endospore-forming rhizobacteria, *Bacillus* is the most distributed bacteria genius in the rhizosphere, and described as beneficial rhizobacteria (Pandey and Palin, 1997; Li *et al.*, 2021).

In addition to bacteria from the *Pseudomonas* spp fluorescent group, *Bacillus* is the most distributed bacteria genus in the rhizosphere, and described as beneficial rhizobacteria (Pandey and Palin, 1997).

Bacillus spp. hold remarkable abilities for synthesizing a vast variety of beneficial biomolecules (Stein, 2005). They have potent plant growth promoting traits such as phosphate solubilization, phytohormones and siderophore production, hydrolytic enzymes synthesis, nitrogen fixation, and pest management (Senthilkumar *et al.*, 2009; Jang *et al.*, 2018).

Several studies have focused on the enhancement and exploitation of their agronomic potential, among the most studied species; *B. thuringiensis, Lysinibacillus sphaericus, Paenibacillus popilliae* and *B. lentimorbus* as entomopathogenic agents (Goldman and Green, 2008; Oulebsir–Mohandkaci *et al.*, 2021), *B. subtilis, B. mycoides* and *B. amyloliquefaciens* as antagonist agents (Pandey and Palni, 1997; Singh *et al.*, 2008; Raut *et al.*, 2018) and *Bacillus megaterium, B. safensis, B. simplex* and *Paenibacillus graminis* as plant growth-promoting agents (Akinrinlola *et al.*, 2018).

Moreover, many species of *Bacillus* are well known for other applications, while their PGPR and biocontrol potentialities are not much explored. For example, *Bacillus clausii* is used for treatments of many gastrointestinal infections and as probiotics (Rani *et al.*, 2018), but its agronomic interest did not attract attention. Few studies proved herbicidal, insecticidal and antagonistic activity of *Bacillus clausii* (Ghadbane *et al.*, 2013; Mushtaq and Nighat, 2019).

In this perspective, the present study was designed to characterize a new indigenous strain of *B. clausii* (MT305787) isolated from the rhizosphere of medlar in northern Algeria with evaluation of its enzymatic activity and its PGP effect *in vitro* and *in vivo* on two plants: *Brassica napus* and *Medicago sativa*.

Materials and Methods

Soil sampling and isolation technique

Soil samples were collected (in April 2018) in Boumerdes (Coastal region of Northern Algeria; latitude: 36° 76′ 75″ North, longitude: 3° 70′ 29″ East). Samples were taken between 50 and 80 cm from the rhizosphere of Medlar (*Mespilus germanica* L) belonging to the Rosaceae family native to the Mediterranean area. Partial root systems were taken with the adherent soil (Dommergues et Mangenot, 1970). Soil was heated at 80 °C during 20mn to eliminate non-spore forming bacteria (Karungu*et al.*, 2018). Finally, serial dilutions were used to isolate bacteria from soil samples using nutrient agar medium. Isolated bacteria were conserved at 4 °C.

Phenotypic characterization of the strain B8

Taxonomic characteristics were studied using microscopic examination (Gram and spore staining). Also, physiological and biochemical tests were realized. Here we note catalase, oxidase, nitrate reductase, caseinase, lecithinase, respiratory type, capacity of growth at 45 °C, 55 °C and 65 °C, degradation of sugars at the triple sugar- iron agar (TSI) and mannitol mobility test. Other biochemical tests were obtained using API gallery systems such as starch and gelatin hydrolysis, degradation of sorbitol, rhamnose and arabinose. The result is manifested by a positive (+) or negative (-) reaction (Guiraud, 1998, 2003; Prescot *et al.*, 2003; Singleton, 2005; Joffin and Leyral, 2006; De Vos *et al.*, 2009).

Molecular identification and DAN sequencing

The amplification tests are carried out with a PCR Hot Start (94 °C), using the universal primers described by Weisburg *et al.* (1991):

-16F27 (forward): (5'AGAGTTTGATCCTGGCTCAG3', position 8-27).

-16R1522 (reverse): (5'TAAGGAGGTGATCCAGGC3', position1541-1522).

These are conserved zones within the rRNA operon of *E. coli.* (Gurtler and Stanisich, 1996). The Genomic DNA of strain B8 (MT305787) was used as template for PCR amplification (35cycles, 94 °C for 30 s denaturation, 60 °C for 1 min primer annealing,and 72 °C for 1.5 min extension). The amplified approximately 1.5-kbPCR product was cloned in pGEM-T Easy vector (Promega, Madison,WI), to obtain pB8-16S plasmid.E. coli DH5(F- *supE44 Φ80 δlacZΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17* (r_k^-, m_k^+)*deoR thi-1* λ^- *gyrA96 relA1*) (Invitrogen LifeTechnologies) was used as host strain.. All recombinant clones of *E. coli* were grown in LB broth medium with the addition of ampicillin, isopropyl-thio-_-D-galactopyranoside (IPTG), and X-galfor screening. DNA electrophoresis, DNA purification, restriction, ligation, and transformation were all performed by a method previously described by Sambrook*et al.* (1989). The nucleotide sequence of the 16S rRNA gene was determined on both strands using a BigDye® Terminator v3.1 Cycle Sequencing Kit and anautomated DNA sequencer ABI Prism_ 3100-Avant Genetic Analyser (Applied Biosystems).

Phylogenetic analysis

The first step in phylogenetic analysis is to align the sequences to be identified. The sequences obtained are identified by carrying out local alignments by pair of our sequences with those found in the "nr" database (Nucleotide collection nr / nt) thanks to the BLASTN program (*http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi*) (Felsenstein, 1985). Phylogenetic and molecular evolutionary analyses were conducted by means of molecular evolutionary genetics analysis (MEGAX) software. Distances and clustering were calculated by the neighbor-joining method. Bootstrap resembling analysis for 1000 replicates was per- formed to estimate the confidence of tree topologies (Saitou *et al.*, 1987; Tamura *et al.*, 2004; Kumar *et al.*, 2018; Stecher *et al.*, 2020).

Screaning for enzymatic activities of strain B8

Phospholipases production

Nutrient agar supplemented with 10 ml of sterile egg yolk emulsion in physiological water. After 24 °C of incubation at 30 °C, the presence of an opaque halo around colonies indicates a positive result (De Vos *et al.*, 2009).

Pectinase production

Nutrient agar supplemented with 10 g of pectin was used to determine pectinase production. The appearance of a trough around colonies after 4 days indicates a positive reaction (Naik and Sakthivel, 2006). Pectinase production was also tested on disinfected potato tubers. Bacterial suspension was inoculated on potato slices and incubated at 30 °C during 4 days. The presence of a rot on potato slices with strong odour indicates a positive result (Cooksey *et al.*, 1990).

Chitinase production

Bacteria were inoculated on nutrient agar medium supplemented with10h of non-colloidal chitin and incubated at 30 °C during 72h. A white halo around colonies indicates a positive reaction (De la Vega *et al.*, 2006).

Cellulase production

Bacteria were inoculated on M9 agar supplemented with 10g of cellulose and 1.2 g of yeast extract. The appearance of a clear halo around colonies after 8 days of incubation at 30 °C indicates a positive result (Verma *et al.*, 2007).

Determination of plant growth promoting (PGP) attributes

Siderophore production

After inoculation on King B medium and incubation during 24 to 96 hours at 30 °C, the detection of florescent pigmentation with the naked eye and under ultraviolet (UV) light at wavelengths 254 and 366nm (Guiraud, 1998; Gupta and Gopal, 2008).

The evaluation of siderophore production rate was tested on three broth media: King B, Potato Dextrose (PD) and succinate (SM). Bacterial suspensions were prepared (OD=0.3 at 540 nm) and incubated at 28 °C during 48h. Optical density was recorded at 600 nm and 400 nm after centrifugation (6000 rpm) during 20 mn for each broth. Siderophore production rate was calculated using the following ratio: OD (λ= 400nm)/OD (λ= 600nm) (Meyer and Abdellah, 1978).

Phosphatase production

Phosphate solubilisation was evaluated on Pikovskaya (PVK) medium containing $Ca_3(PO4)_2$ as the only source phosphate (Nautiyal, 2001). After 72 h of inoculation and incubation at 30 °C, a clear halo around colonies indicates a positive reaction.

Hydrogen cyanide production

HCN production was estimated on TSA medium (Tryptophane Soya Agar) supplemented with 4.4 g of glycine. A filter paper grade 1 was flooded with 0.5% picric acid in 2% sodium carbonate and stuck underneath the Petri-dish lids. The presence of orange to red colour on the filter paper margins indicates a positive result for HCN product (Verma *et al.*, 2007).

The hormone Indole Acetic Acid (IAA)

IAA production was estimated on Luria Bertani broth (LB) supplemented with 0.01% of D-tryptophane. 50 ml of LB broth was used to prepare bacterial suspension (10^6 CFU ml-1) (Kumar, 2012) and incubated during 3 days at 28 °C in dark with under continuous stirring at 180 rpm. The suspension was pelleted through centrifugation at 9000 rpm for 20 min. One ml of the supernatant was incubated with 2ml of Salkowski reagent in the dark at room temperature. After 30 min, the red coloration indicates a positive result for IAA production (Bric *et al.*, 1991).

Plant Growth Promoting Potential of strain B8 on Brassica napus and Medicago sativa

Seeds of *Brassica napus* and *Medicago sativa* were used to evaluate the plant growth promoting potential (PGP) of the tested bacterial strain, including germination and vegetative growth assays.

For the germination test, the seeds were sterilized by soaking in 2% sodium hypochlorite for 3 minutes and then they were washed with sterile distilled water 5 times and then incubated in 50 ml of the bacterial suspension at the ambient air temperature for 24 hours. Then the seeds were placed in a sterilized cup containing moist cotton and they were incubated at the ambient air temperature for 10 days to calculate the germination percentage (Lwin *et al.*, 2012). For vegetative growth assays seeds were sterilized and inoculated the same as described for germination test. After 24 hours of incubation, seeds were sown in plastic bags containing sterilized soil. After 30 days, the plants will be harvested separately and vegetative growth parameters were measered: Roots length, lateral roots number, stem length, number of leaves, diameters of stems and plant weight (Lwin *et al.*, 2012).

Colonization of the rhizosphere and study of the resistance

Roots colonization ability of *Bacillus clausii* strain (MT305787) in a non-sterile substrate requires the use of labelled bacteria to facilitate their monitoring and re-isolation. The labelling of the bacterium by spontaneous resistance to antibiotics is a technique widely used by researchers in soil microbiology. It is in this context that spontaneous antibiotic resistant mutants are prepared from the B8 strain during *in vitro* biological treatment. Labelling of the isolate with antibiotic resistance was done according to the method of Kloepper *et al.* (1980). Successive subcultures of the isolate were made on Nutrient agar medium supplemented with two labelling antibiotics; rifampicin 50 $\mu g\ ml^{-1}$ and ampicillin 50 $\mu g\ ml^{-1}$. Immediately after autoclaving the Nutrient Agar medium (NA), the antibiotics sterilized beforehand by filtration are added to have the required concentrations. The culture medium, thus prepared, is then distributed in Petri dishes. After solidification, the culture medium is inoculated with the tested strain B8. Cultures were incubated at 30 °C for 24 to 48 hours. The colonies which grow were subculture, three successive times, on the same medium before being stored in 40% glycerol at -25 °C. For this experiment, rapeseed and alfalfa seedlings, grown under the same conditions as those of the biological treatments, were used. The evaluation of the colonization of the roots is carried out at different inoculation times including: T0: one week after treatment, T1: 14 days after inoculation, T2: 21 days after treatment.

The seedlings corresponding to each treatment are released from the substrate. The roots are agitated to get rid of large debris from the substrate. Two types of roots are collected: the tip 1 cm, and the end 2-6 cm from the end of the root. Each root is then cut into three equal pieces using a sterile scalpel, weighed and homogenized in a laboratory mixer for 2 minutes with 10 ml of sterile physiological water per gram of fresh root material. The root fragments are carefully spread on the NA + culture medium (NA with the addition of labelling antibiotics). The cultures were subsequently incubated at 30 °C for 48 h. Colonization of the roots by the introduced B8 is evaluated with the naked eye, looking for the development of bacterial colonies around the root fragments, and under UV at 365 nm to confirm fluorescence. The percentage of plantlets with colonized roots is also determined in order to qualitatively assess the colonization of the roots by the bacteria tested. If the bacterium is located 1 cm from the end of the roots, it is said to be colonizing, if it is 2 to 6 cm, it is therefore persistent.

Statistical analysis

The results are expressed as mean values and standard deviation (SD). The differences between the different treatments were analysed using one-way analysis of variance (ANOVA). The value $p < 0.05$ was considered significant. This treatment was carried out using SPSS v. 25.0 programs.

Results and Discussion

Phenotypic characterization

After incubation at 30 °C during 24h, B8 showed visible distinct colonies with specific morphological criteria (Table 1).

Table 1. Cultural characteristics of strain B8 on solid and liquid medium

Strain B8						
Cultural characteristics on solid medium	Shape	Diameter	Colour	Opacity	Elevation	Appearance
	Circular with Regular Contour	5 mm	Beige	Opaque	Plate	Smooth
Cultural characteristics on liquid medium	Ring	Voile	Homogeneous disorder	Heterogeneous disorder	Base	
	-	+	+	-	-	

+: positive reaction, - : negative reaction

Strain B8 presents some cultural characteristics; optimum growth temperature 28-30 °C up to 45 °C, aerobic or facultative anaerobes, rapid growth on usual media (24 h on nutrient agar). Circular creamy colonies with opaque texture and regular contour.

Microscopic observation of cells (fresh and Gram staining) showed rod-shaped and Gram-positive bacteria, positive motility, Para-central / central spores and non-deforming the vegetative cell.

The strain B8 gave positive result for starch hydrolysis, Voges-Proskaur reaction, use of citrate as source of carbone, reduction of nitrate to nitrite, mannose, sorbitol, mannitol and growth at 45 °C. Whereas negative results were detected for acid production from glucose, rhamnose production, anaerobioses and growth at 55 °C. Variable results are observed for oxidase and decomposition of casein and lecithin (Table 2).

Table 2. Biochemical and physiological characters of strain B8

Test	B8	Test	B8	Test	B8
Spore	+	Beta-galactosidase (ONPG)	+	Glucose (GLU)	+
Para-central / central spore	+	Arginine dihydrolase (ADH)	+	Mannitol (MAN)	+
Sub-terminal / terminal spore	-	Lysine decarboxylase (LDC)	+	Inositol (INO)	+
Gram	+	Ornithine decarboxylase (ODC)	+	Sorbitol (SOR)	+
Growth on usual media	+	Citrate utilization (CIT)	+	Rhamnose (RHA)	-
Motility	+	H2S	-	Saccharose (SAC)	+
Catalase	+	Urease (URE)	+	Mélibiose (MEL)	-
Nitrates reduction (NR)	+	Tryptophan Deaminase (TDA)	-	Amylase (AMY)	+
Oxidase	V	Indole (IND)	+	Arabinose (ARA)	-
Growth at 45 °C	+	Voges-Proskauer (VP)	+	Casein hydrolysis	V
Growth at 55 °C	-	Starch hydrolysis	+	Lecithin hydrolysis	V
Anaerobioses	-	Gelatin hydrolysis	+		

+: positive reaction, - : negative reaction, V: variable

Phenotypic, physiological and biochemical characteristics of the strain B8 such as spore-forming, vegetative cell shape and Gram stain made it possible to affiliate this strain to the genius *Bacillus*.

Molecular identification of strain B8 and phylogenetic study

The cloning of RNA16S. of strain B8 on the *Escherichia coli* plasmid is shown in the figure below (Figure 1):

PCR_Universal primers (Gurtler and Stanisich, 1996).

Fwd_name: 27F, Fwd_seq: 5'-AGAGTTTGATCCTGGCTCAG-3'

Rev_name: 1525R, Rev_seq: 5'-AAGGAGGTGATCCAAGCC-3'

The obtained partial gene sequence of the strain B8 was 1503 nucleotides in length (Genbank, MT305787). The comparison of the obtained 16S rRNA gene partial sequence of the strain B8 against the 16S ribosomal RNA sequences (Bacteria and Archaea) database was performed by BLASTN. The closed sequences were imported into the MEGAX software and aligned. The phylogenic analysis indicated that the strain B8 belong to the genus *Bacillus* within the family *Bacillaceae* (Figure 2). A significant similarity for possible species relatedness (96.07%) was found with the validly described species *Bacillus clausii* strain *DSM 8716(NR_026140)*. However, this strain shows a lower similarity (less then 96%) with the other species *Bacillus rhizosphaerae* strain *SC-N012 (NR_108311)* (95.55), *Bacillus lehensis* strain *MLB2 (NR_036940)* (92.67%), *Bacillus shacheensis* strain *HNA-14 (NR_133980)* (92.62%), *Bacillus murimartini* strain *LMG*

21005 (NR_042084) (92.26%), *Bacillus lindianensis* strain *12-3 (NR_146035)* (91.90%). These results obtained strongly suggested that the strain B8 ought to be identified as *Bacillus clausii* strain *B8.*

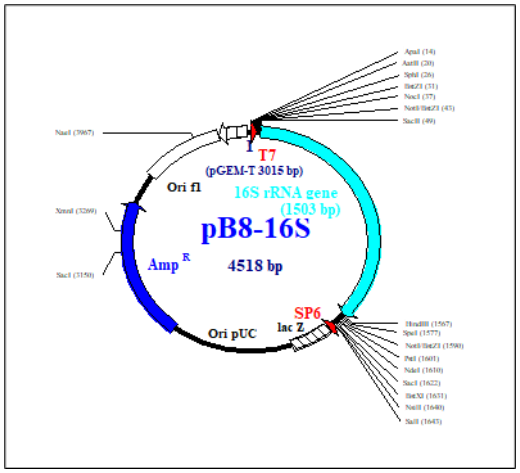


Figure 1. Restriction map of plasmid pB8-16S carrying the 16S rRNA gene from strain B8

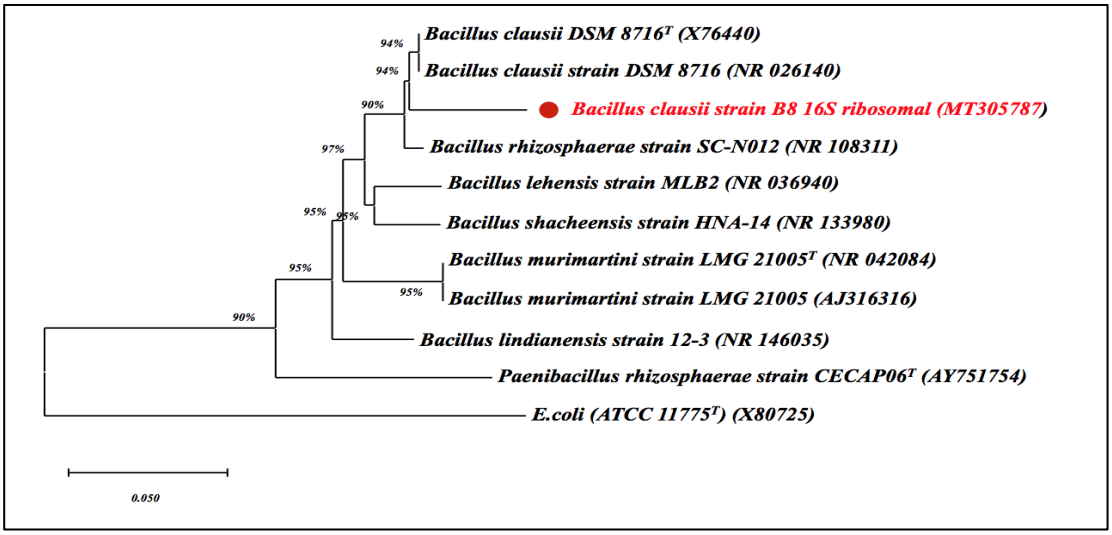


Figure 2. The evolutionary relationships of *Bacillus clausii* strain (MT305787)
The evolutionary history was inferred Neighbour-Joining method showing the phylogenetic position of *Bacillus clausii* Strain B8 and representatives of certain other related taxa based on 16S rDNA sequences. Access numbers in the EMBL/Genbank databases are given after the name of each strain. The bar represents 1 substitution per 100 nucleotides. The values at the nodes indicate the probabilities calculated by bootstrap

Enzyme production of the strain B8

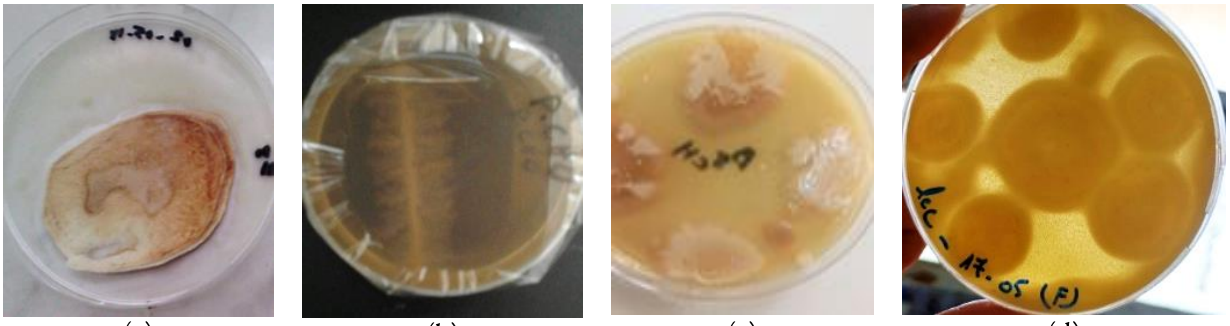
The strain B8 showed capacity to produce the majority of tested enzymes (Table3, Figure3).

Table 3. Enzyme production results of the strain B8

Enzyme production	Pectinase	Cellulase	Chitinase	Phospholipase
Strain B8	-	+	+	+

Plant growth promoting attributes of the strain B8

The studied strain showed capacity to produce different PGPR molecules (Table 4 and Figure 4).



(a) (b) (c) (d)

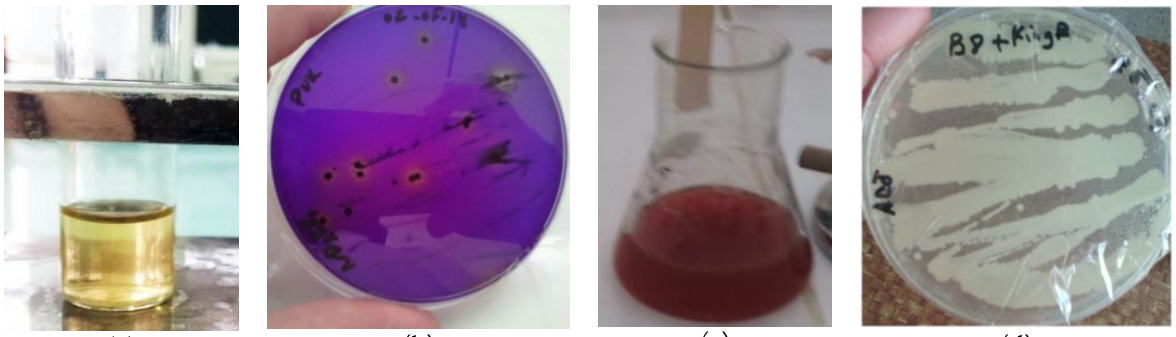
Figure 3. Strain B8 (MT305787) enzyme production results

(a) Negative result for pectinase production; (b), (c), (d) cellulose, chitinase and phospholipases production respectively, revealed by clear halo around colonies on specific media.

Table 4. PGPR traits of the strain B8

Strain	IAA	Phosphatase	HCN	Siderophores		
B8 (MT305787)	-	+	+	KB	SM	PD
				+++	++	+

- : absence of production, + : production, ++ : good production, +++ : high production



(a) (b) (c) (d)

Figure 4. PGPR molecules production.

(a) Absence of red coloration of the medium indicates negative result for IAA production; (b)Positive result of phosphate solubilisation revealed by a clear halo around colonies, (c) HCN production revealed by colour change from medium to red (d) detection of fluorescent pigments with the naked eye indicates siderophore production.

The hormone Indole Acetic Acid (IAA) production

The yellow colour of LB liquid medium indicates a negative result for IAA production by the tested strain.

Hydrogen cyanide production

Regarding the production of volatile substances (HCN), after adding the alkaline picrate solution vertically in the Erlens Meyer, we observe the colour change towards brown on Whatman paper, this result indicates a good production of HCN at strain B8.

Phosphate solubilisation

A clear halo was formed around colonies on PVK medium indication a positive production of phosphatase.

Siderophore production

The studied strain showed fluorescent pigments on the three used medium (King B, succinate and Potato Dextrose), which indicates positive production of siderophores. King B, succinate and PD broths were used to evaluate siderophore production intensity. Optical densities were at λ=600nm and λ= 400 nm were measured. PD broth seems to be the best for siderophore production compared with the other broths (Table 5).

Table 5. Evaluation of siderophore production intensity

Strain	Medium	OD (ʎ = 540 nm)	OD (ʎ = 600 nm)	OD (ʎ = 400 nm)	OD (ʎ = 400 nm) / DO (ʎ = 600 nm)	Siderophore production intensity
B8	KB	0,3	1,036	0,093	0,314	Good production
	PD	0,3	1,253	0,641	0,548	Intense production

OD: Optical Density, KB: King B broth, PD: Potato Dextrose broth, SM: succinate broth

Plant growth promoting potential of strain B8 on B. napus and M. sativa

The germination rate of seeds of *Brassica napus* (rapeseed) and *Medicago sativa* (alfalfa) was observed 10 days after inoculation of strain B8. Inoculation of strain B8 showed a positive effect on the germination rate of *B. napus*, the effect of the inoculated strain appeared from the second day. Unlike on *M. sativa* which showed negative effect compared to control (Figure 5).

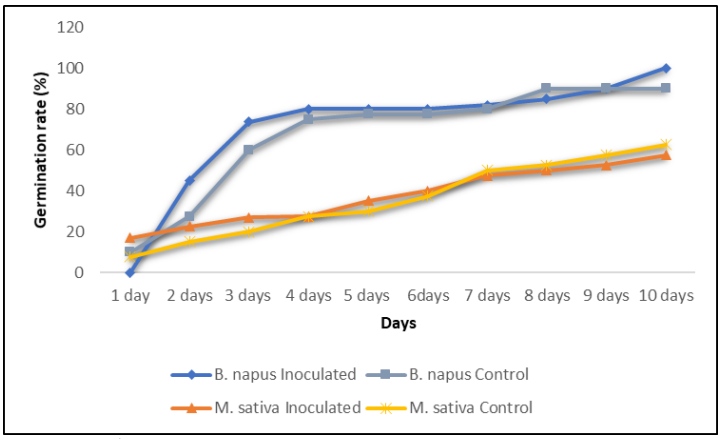


Figure 5. Germination rate (%) of *B. napus* and *M. sativa* inoculated with Strain B8 for 10 days

Analysis of variance does not reveal any significant difference for the germination rate (P ≥0.05).

After 30 days of inoculation, strain B8 showed significant effect on vegetative growth parameters. In *B. napus*, the parameters stimulated are roots length, number of lateral roots, and Length of the aerial part. In *M. sativa* strain b8 increased number of lateral roots and stem length (Table 6).

Table 6. Results of the morphological characters of *B. napus* and *M. sativa* inoculated with B8

Species	Root length		Nr. of lateral roots		Stem length		Height of shoots	
	C	B8	C	B8	C	B8	C	B8
B. napus	2.46 ± 0.011	2.91± 0.025	2.4 ± 0.021	2.6±0.044	5.61±0.4	8.33±1	0.61± 0.331	0.42± 0.24
	+		+		+		-	
M. sativa	2.9 ±0.129	2.6 ± 0.561	1.6± 0.054	2.1± 0	2.6± 0	2.86± 0.787	3.9±0.910	2.86±0.001
	-		+		+		-	

Analysis of variance ANOVA reveals a significant difference at the 5% level (P ≤ 0.01) between the morphological parameters studied, as well as between the two plants (P ≤ 0.01).

The results of the average number of leaves is shown in the table below. They show that the *B. napus* plants inoculated with the B8 strain show the best results during the four weeks compared to the controls. Very similar but less important results are obtained for *M. sativa* treated with strain B8 which also gave a better effect than the controls. The difference is significant for the first week but over the following weeks no significant difference was detected between the two plants or compared to the control (Figure 6).

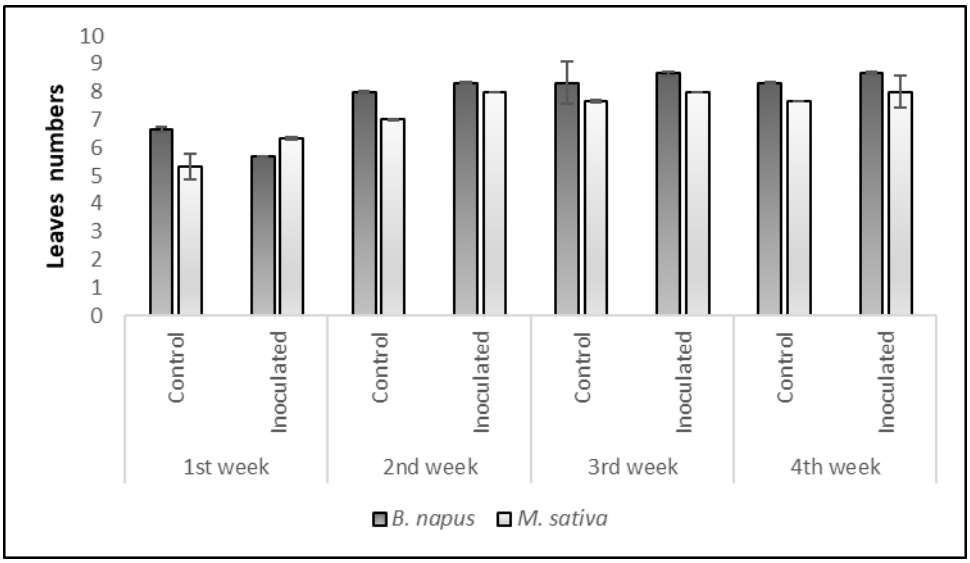


Figure 6. Average leaf numbers of *B. napus* and *M. sativa* inoculated by strain B8
Analysis of variance ANOVA denote significant differences between plants inoculated (ANOVA test, $p \leq 0.05$)

The mean diameters of the stems of *B. napus* showed a slight increase in the treated compared to the controls. On the other hand, for *M. sativa*, the effect of inoculating the strain is not apparent (Figure 7).

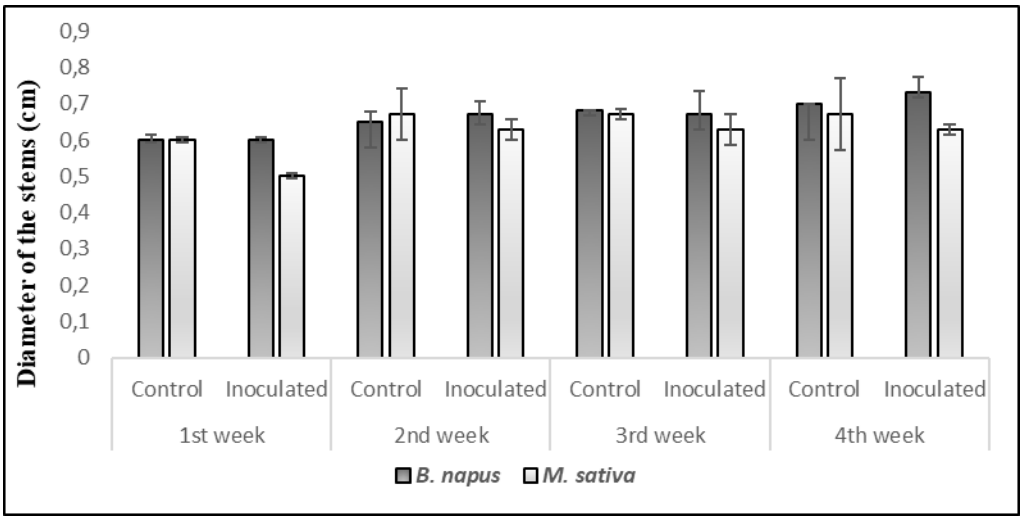


Figure 7. The average diameter of the stems of *B. napus* and *M. sativa* inoculated by strain B8
No significant difference was observed by ANOVA at $\alpha > 5\%$.

Dry weight and stem length of *M. sativa* showed remarkable difference after Inoculation compared to control, unlike inoculation with *B. napus* (Figure 8).

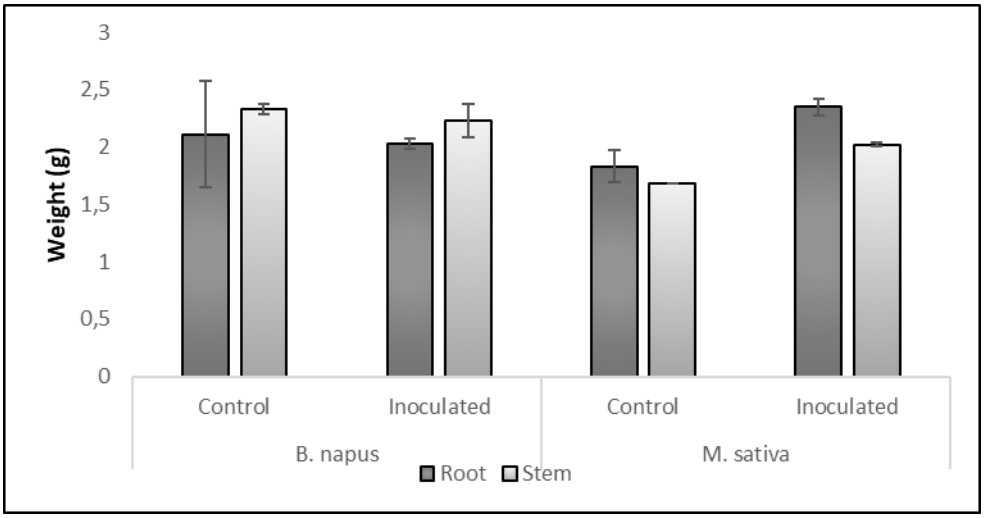


Figure 8. Effect of strain B8 on the dry weight of *B. napus* and *M. sativa* plants compared to the two controls
Analysis of variance ANOVA denote significant differences between plants inoculated (ANOVA test, $p \leq 0.05$)

Colonization test

The analysis of the results indicates that the strain B8 was able to colonize the root system of all the seedlings after one week of treatment. A maximum concentration was recorded on the third week on the rapeseed with 4.5 $x10^3$ CFU / g of root, the strain succeeded in colonize the main root and lateral roots of this plant with an increase in concentration over time. For alfalfa the concentration was lower in the third week, not exceeding 2.7$x10^3$ CFU / g of soil. Strain B8 has adapted perfectly to the root systems of both plants and can therefore be considered a good colonizer of their roots (Figure 9).

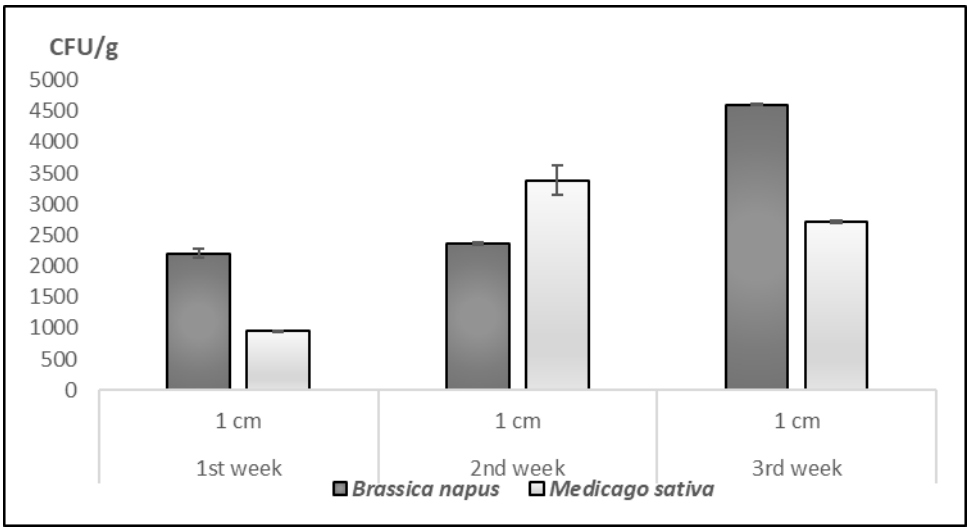


Figure 9. Root colonization by strain B8 in *B. napus* and *M. sativa* expressed in CFU / g of soil
Analysis of variance ANOVA indicate significant differences between plants inoculated (Tuky test, $p \leq 0.05$)

Persistence test

A good biological control agent is one, which exhibits colonizing power in all parts of the root system while persisting for several weeks. Our results reported that strain B8 is able to colonize and persist effectively in the rhizosphere from the 1cm end to the 2-6 cm part of the root and persist for three weeks. So, we can conclude that the B8 strain exhibits better colonization for in *B. napus* compared to *M. sativa* (Figure 10).

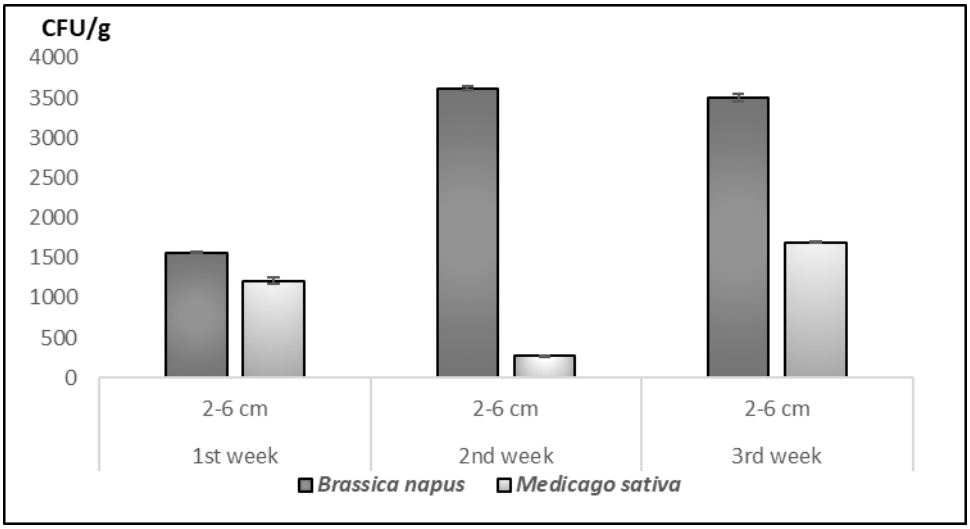


Figure 10. The persistence of strain B8 at 2-6 cm from the main root of *B. napus* sand *M. sativa* expressed in CFU / g of soil
A significant difference between plants inoculated was indicated by Analysis of variance ANOVA ($p \leq 0.05$)

Discussion

The rhizosphere is an important habitat area for bacteria beneficial to plants, called rhizobacteria. Among them bacteria of the genus *Bacillus* and related constitutes the majority.

Our study allowed to isolate the strain *Bacillus clausii* strain B8 (MT305787) from the rhizosphere of medlar (*Mespilus germanica* L.- family Rosaceae). This strain presents very interesting agronomic traits according to previous studies (Li *et al.*, 2012; Yasmin *et al.*, 2020).

According to Logan and De Vos (2009), *Bacillus clausii* is a rhizosphere bacterium that grow at pH7-8 with growth temperature of 15-50 °C, it presents positive results for hydrolysis of gelatin, nitrate reduction, hydrolisis of casein and hydrolysis of starch. This is in accordance with our results.

Three commercially available *Bacillus clausii* spore suspensions were collected by Rani *et al.* (2018) for the phenotypic characterization and biochemical analysis, the results obtained are similar to ours.

Sequence alignment of the studied strain was performed to check the evolutionary relationship with organisms of different taxa and thus a phylogenetic tree was constructed. Species selected from BLAST program showed *Bacillus rizosphareae* to be the closest neighbour in speciation to our strain. These results also agree with those of Rani *et al.* (2018).

The profile of the extracellular hydrolytic activity of the bacterial strain B8 isolated from of the medlar soil has shown that the latter have the capacity to produce a diverse range of enzymes which can thus degrade numerous substrates such as lipid (phospholipase) and complex sugars (cellulose and chitin), these enzymes are very important for the biocontrol of phytopathogens and pests.

In a previous study, an enzymatic assay was carried out on ten strains of *Bacillus* isolated from the rhizospheric soil of a tomato plant, in order to examine their capacity to produce hydrolytic enzymes necessary for biological control. All isolates demonstrated the ability to produce the hydrolytic enzymes with the highest activity recorded in *Bacillus macquariensis* BM2 (60.28 µmol) for chitinase, *Bacillus macerans* BC9 (11.14 µmol) for protease, *Bacillus macquariensis* BM2 (150.00 µmol) for glucanase and *Bacillus circulans* BC1 (46.45 µmol) for cellulase respectively (Ayantola*et al.*, 2020). *Bacillus clausii* SM3 isolated from soil showed a high level of protease and amylase production (Hema and Shiny, 2012).

In another context, the current study aimed to evaluate PGPR traits and biocontrol efficacy of the studied strain. The strain showed ability to produce phosphatases, indole, HCN and siderophores. Those

molecules are a well-known plant growth promoting molecules (Verma *et al.*, 2018). The study of the production of PGP molecules in a collection of rhizobacteria from the genus *Bacillus*; shows a high production of indole acetic acid (AIA) and cyanidhydrogeniecis (HCN) (Oulebsir-Mohandkaci *et al.*, 2020).

On the other hand, *Brassica* oil seeds are one of the few edible oil crops that can be cultivated in the temperate zones of the world (McGregor and Kimber (1995). Alfalfa (*Medicago sativa* L.) is the more important cultivated forage in the world (Lemaire *et al.*, 2019). So these two culture occupies considerable economic importance hence the importance of promoting their growth.

For the evaluation of the PGP effect of strain B8 on *Brassica napus* and *Medicago sativa*, the vegetative growth parameters studied (Roots length, lateral roots number, stem length, number of leaves, diameters of stems and plant weight) were significantly improved and the bacterium strain B8 was able to colonize the root system all the seedlings of both plants after one week of inoculation.

In fact, 21 PGPR strains isolated from the rhizosphere of the desert of Mexico from *Euphorbia antisyphilitica* Zucc., gave the best results in the variables of the number of secondary roots and fresh weight as well as their salinity tolerance. In addition, the bacterial strains showed the presence of Indole-3-acetic acid, siderophores and ACC deaminase enzyme (Salazar-Ramirez *et al.*, 2021). The co-inoculation of two plant growth-promoting rhizobacteria *Azotobacter chroococcum* and/or *Alcaligenes faecalis* in pot has a positive effect on growth criteria and physio-biochemical attributes of *Brassica napus* L. grown in saline soil. At the same time, antioxidant enzymes production and minerals' uptake (N, K, Ca, Mg) were augmented due to the inoculation with the bacteria (Abdel Latef *et al.*, 2021). *B. subtilis* and *B. megaterium* stimulated plant height, canopy diameter, fresh and dry herbage and leaf yield with in essential oil content, and oil yield in oregano plants (Kutlu *et al.*, 2019). Several microorganisms form natural colonization with plant roots have the ability to help the plants in nutrient acquisition and disease protection. The inoculation of a single PGPR strain appears to be effective as a plant biostimulant and biofertilisant, however, the mixtures can exert synergistic effects (Castiglione *et al.*, 2021). Therefore, the identification of novel PGPRs using high throughput sequencing methods will allow to improve their needs (Swarnalakshmi *et al.*, 2020).

In the present study, it was shown that there is relationship and correlation between the PGPR traits of strain B8 and growth promotion efficacy in *B. napus* and *M. sativa.*

Conclusions

Bacillus clausii strain B8 (MT305787) successfully enhanced growth of *Brassica napus* and *Medicago sativa* and produce important enzymes and PGPRs molecules. Knowledge of mode of action could be studied to better exploit this strain as a biofertilizer for sustainable agriculture. The strain may have an important place in industrial enzyme production, and as biological input, aiming to reduce the excessive use of chemical fertilizers and pesticides which will allow to ensure better agricultural yield with high nutritional values, while overcoming the environmental negative effects.

Authors' Contributions

Conceptualization (OMH); Formal analysis (OMH, HR, BTF); Funding acquisition (OMH, BTF); Methodology (OMH, BTF, HR); Resources (OMH, BTF); Software (OMH); Supervision (OMH); Validation (OMH); Writing - original draft (OMH); Writing - review and editing (OMH, HR, BTF). All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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