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, cyanohydric 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 microorganisms provide nutrients, protection against biotic and
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 genus in the rhizosphere, and described as beneficial rhizobacteria (Pandey and Palni, 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 Palni, 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. amyloliquifaciens* 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, physiologival 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’TAAAGAGGTGATCAGCCGC3' (position 1514-1522).

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 (35 cycles, 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* DH5α (F supE44 Φ80 ΔlacZΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17 (rK-, mK+) 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-β-D-galactopyranoside (IPTG), and X-gal for 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 an automated 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 BLAST 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 performed to estimate the confidence of tree topologies (Saitou et al, 1987; Tamura et al, 2004; Kumar et al, 2018; Stecher et al, 2020).

Screening 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 with 10h 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 366 nm (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$_3$(PO$_4$)$_2$ 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° 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 measured: 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 Kloeper *et al.* (1980). Successive subcultures of the isolate were made on Nutrient agar medium supplemented with two labelling antibiotics; rifampicin 50 µg ml$^{-1}$ and ampicillin 50 µg 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).

| 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 carbon, 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 dihydrose (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)                     | +   | Melibiose (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 genus *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’-AGAGTTTGATCCTGCTGCTAG-3’
Rev_name: 1525R, Rev_seq: 5’-AAGGAGGTGATCCAGCC-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 phylogenetic 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 rhizophaerae* strain SC-NO12 (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.

![Restriction map of plasmid pB8-16S carrying the 16S rRNA gene from strain B8](image1)

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

![Evolutionary relationships of Bacillus clausii strain](image2)

**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 (Table 3, Figure 3).

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

**Table 3.** Enzyme production results of the 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).
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

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 \( \lambda = 600 \text{nm} \) and \( \lambda = 400 \text{nm} \) were measured. PD broth seems to be the best for siderophore production compared with the other broths (Table 5).

| Strain | Medium | OD (\( \lambda = 540 \text{nm} \)) | OD (\( \lambda = 600 \text{nm} \)) | OD (\( \lambda = 400 \text{nm} \)) | OD (\( \lambda = 400 \text{nm} \)) / DO (\( \lambda = 600 \text{nm} \)) | 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](image)

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 (\( P \geq 0.05 \)).

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).

| Species  | Root length | Nr. of lateral roots | Stem length | Height of shoots |
|----------|-------------|----------------------|-------------|-----------------|
|          | 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 (\( P \leq 0.01 \)) between the morphological parameters studied, as well as between the two plants (\( P \leq 0.01 \)).
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](image)

**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](image)

**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).
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 $4.5 \times 10^3$ 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 $2.7 \times 10^3$ 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).

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).
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, hydrolysis 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

![Figure 10](image.png)
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 (IAA) 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 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.

References

Abdel Latef AAH, Omer AM, Badawy AA, Osman MS, Ragae MM (2021). Strategy of salt tolerance and interactive impact of Azotobacter chroococcum and/or Alcaligenes faecalis inoculation on canola (Brassica napus L.) plants grown in saline soil. Plants 10:110. https://doi.org/10.3390/plants10010110

Akinrinlola RJ, Yuen GY, Drijber RA, Adesemoye AO (2018). Evaluation of Bacillus strains for plant growth promotion and predictability of efficacy by in vitro physiological traits. Hindawi International Journal of Microbiology V 5686874:11. https://doi.org/10.1155/2018/5686874

Ayantola KJ, Fagbohun ED (2020). Enzymatic activity of Rhizobacillus isolated from tomato rhizosphere. Asian Journal of Biochemistry, Genetics and Molecular Biology 4(3):11-19. https://doi.org/10.9734/ajbgmb/2020/v4i330106.

Berendsen RL, Pieterse CMJ, Bakker PAHM (2012). The rhizosphere microbiome and plant health. Trends in Plant Science 17(8):478-486. https://doi.org/10.1016/j.tplants.2012.04.001

Bric J, Bostock R, Silverstonet A (1991). Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. Applied and Environmental Microbiology 57(2):535-538. https://doi.org/10.1128/AEM.57.2.535-538.1991

Castiglione AM, Mannino G, Contartese V, Bertea CM, Ertani A (2021). Microbial biostimulants as response to modern agriculture needs: composition, role and application of these innovative products. Plants 10:1533. https://doi.org/10.3390/plants10081533

Cooksey DA, Azad HR, Cha JS, Lim CK (1990). Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. Applied Environmental Microbiology 56:431-435.

De la Vega LM, Barboza-Corona JE, Aguilar-Uscanga MG, Ramírez-Lepe M (2009). Purification and characterization of an exochitinase from Bacillus thuringiensis subsp. azawai and its action against phytopathogenic fungi. Canadian Journal of Microbiology 52(7). https://doi.org/10.1139/w06-019

De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (2009). Bergey’s Manual of Systematic Bacteriology, 2nd Ed. The Firmicute. Springer, New York 3:63-67.

Dommergues Y, Mangenot F (1985). Ecologie microbienne du sol. Paris, Ed. Masson, pp 769.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Filiz O, Takil E, Kayan N (2021). The role of plant growth promoting rhizobacteria (PGPR) and phosphorus fertilization in improving phenology and physiology of bean (Phaseolus vulgaris L.). Applied Ecology and Environmental Research 19(3):2507-2517. https://doi.org/10.15666/aer/1903_25072517

Goldman E, Green LH (2008). Practical handbook of microbiology. Second Edition, pp 306-326.

Guiraud JP (1998). Microbiologie alimentaire [Food microbiology]. Ed. Dunod, Paris, pp 652.

Guiraud JP (2003). Microbiologie Alimentaire [Food microbiology]. Ed. Dunod. Paris, pp 136-139.

Guo DL, Wan B, Xiao SJ, Allen S, Gu YC, Ding L, Zhou Y (2016). Cyclic lipopeptides with herbicidal and insecticidal activities produced by Bacillus clausii DTM1. Natural Product Communications 10(12):2151-2153. https://doi.org/10.1177/1934578X1501001235

Gupta A, Gopal M (2008). Siderophore production by plant growth promoting rhizobacteria. Indian Journal of Agricultural Research 42(2):153-156.

Gurtler V, Stanisich VA (1996). Microbiology 142:3-16. https://doi.org/10.1099/13500872-142-1-3
Jang JH, Kim SH, Khaine I, Kwak MJ, Lee HK, Lee TY, Woo SY (2018). Physiological changes and growth promotion induced in poplar seedlings by the plant growth-promoting rhizobacteria Bacillus subtilis JS. Photosynthetica 56(4):1188-1203. https://doi.org/10.1007/s11099-018-0801-0

Joffin JN, Leyral G (2006). Microbiologie technique [Technical microbiology]. TI-Dictionnaire des techniques. 4ème édition. Bordeaux: CRDP d’aquitaine, pp 368.

Karunga S, Huang D, Atoni E, Waruhiu C, Agwanda B, Hu X, Yuan Z (2018). Isolation, identification and evaluation of mosquito entomopathogenic Bacillus species and related genera from randomly selected sites in Kenya. African Journal of Microbiology Research 12(12):290-299. https://doi.org/10.5897/AJMR2018.8824

Kumar P, Dubey RC, Maheshwari DK (2012). Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. Microbiological Research 167(8):493-499. https://doi.org/10.1016/j.micres.2012.05.002

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.

Lemaire G, Giroud B, Bathily B, Lecomte P, Christian Corniaux C (2019). Toward integrated crop-livestock systems in West Africa: a project for dairy production along Senegal River. In: Lemaire G, De Faccio Carvalho PC, Kronberg S, Recous S (Eds). Agroecosystem Diversity. Academic Press, pp 275-285. https://doi.org/10.1016/B978-0-12-811050-8.00017-0

Li Z, Bai Z, Zhang B, Li B, Jin B, Zhang M, Lin F, Zhang H (2021). Purification and characterization of alkaline pectin lyase from a newly isolated Bacillus clausii and its application in elicitation of plant disease resistance. Applied Biochemistry and Biotechnology 167(8):2241-2256. https://doi.org/10.1007/s12010-012-9758-9

Li Y, Shao J, Xie Y, Jia L, Fu Y, Xu Z, Zhang R (2021). Volatile compounds from beneficial rhizobacteria Bacillus spp. promote periodical root development in Arabidopsis. Plant, Cell & Environment 44(5):1663-1678.

Logan NA, De Vos P (2009). Genus I. Bacillus. In: De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (Eds). Bergey’s Manual of Systematic Bacteriology, 2nd Edition. Springer, New York 3:121-128.

McGregor DI, Kimber DS (1995). Brassica oilseeds: production and utilization. CAB International, pp 394.

Mehra S, Nautiyal CS (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. Current Microbiology 43(1):51-56. https://doi.org/10.1007/s002840010259

Meyer JM, Abdellah MA (1978). The florescent pigment of Pseudomonas fluorescens biosynthesis, purification and physical-chemical properties. Journal of General Microbiology 107:319-328. https://doi.org/10.1002/00221287-107-2-319

Muhtaq Z, Nigah F (2019). In vitro antimicrobial and antioxidant activities of organic and aqueous extracts of Bacillus clausii KP10. Journal- Chemical Society of Pakistan 41(1):161-168.

Naik PR, Sakhthivel N (2009). Functional characterization of a novel hydrocarbonoclastic Pseudomonas sp. strain PUP6 with plant-growth-promoting traits and antifungal potential. Research in Microbiology 157(6):538-546. https://doi.org/10.1016/j.resmic.2005.11.009

Oulebsir-MohandKaci H, Benzina-Tihar F, AitBelkacem C, Belgrade AN (2020). Recherche de molécules bioactives d’intérêt à partir d’une collection de souches bactériennes rhizosphériques et étude de leur effet antifongique [Search for bioactive molecules of interest from a collection of rhizospheric bacterial strains and study of their antifungal effect]. Algerian Journal of Environmental Science and Technology 6(3):1663-1678.

Pandey LM, Palni LM (1997). Bacillus species: the dominant bacteria of the rhizosphere of established tea bushes. Microbiological Research 52(4):359-365. https://doi.org/10.1016/S0944-5013(97)80052-3

Prescott LM, Harley JP, Klein DA (2003). Microbiologie. Ed De Boeck, Bruxelles.

Rani MS, Madar IH, Al Saadh H, Ogu GI, Tayubi IA (2008). Biochemical and phenotypic profiling of Bacillus clausii: a potent commercial probiotic. International Journal of Scientific Innovations 5(03):099-106. https://doi.org/10.32594/IJSI_20180503
Raut LS, Hamde VS (2018). *In vitro* antagonism of resident rhizobacteria, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* against the bacterial blight pathogen of Bt cotton. International Journal of Pharm Bio Sciences 8:611-618.

Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Salazar-Ramirez MT, Saenz-Mata J, Preciado-Rangel P, Fortis-Hernandez M, Euceda-Puente EO, Yescas-Coronado P, Orozco-Vidal JA (2021). Plant growth-promoting rhizobacteria associated to *Candelilla* rhizosphere (*Euphorbia antisiphilitica*) and its effects on *Arabidopsis thaliana* seedlings. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 49(2):1229-1234. https://doi.org/10.15835/nbha49212294

Sambrook J, Fritsch E, Maniatis T (1989). *Molecular Cloning. A Laboratory Manual*. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Senthilkumar M, Swarnalakshmi K, Govindasamy V, Lee YK, Annapurna K (2009). Biocontrol potential of soybean bacterial endophytes against charcoal rot fungus, *Rhizoctonia bataticola*. Current Microbiology 58(4):288-293. https://doi.org/10.1007/s00284-008-9329-z

Singh N, Pandey P, Dubey RC, Maheshwari DK (2008). Biological control of root rot fungus *Macrophomina phaseolina* and growth enhancement of *Pinus roxburghii* (Sarg.) by rhizosphere competent *Bacillus subtilis* BN1. World Journal of Microbiology & Biotechnology 24(9):1669-1679. https://doi.org/10.1007/s11274-008-9680-z

Singleton P (2005) Bactériologie pour la médecine. La biologie et la Biotechnologie [Biology and Biotechnology]. Ed. Dunod. Paris, pp 541.

Stecher G, Tamura K, and Kumar S (2020). Molecular evolutionary genetics analysis (MEGA) for macOS. Molecular Biology and Evolution 37(4):1237-1239. https://doi.org/10.1093/molbev/msz312

Stein T (2005). *Bacillus subtilis*: antibiotics: structures, syntheses and specific functions. Molecular Microbiology 56(4):845-857. https://doi.org/10.1111/j.1365-2958.2005.04587.x

Swarnalakshmi K, Yadav V, Tyagi D, Dhar DW, Kannepalli A, Kumar S (2020). Significance of plant growth promoting rhizobacteria in grain legumes: growth promotion and crop production. Plants 9:1596. https://doi.org/10.3390/plants9111596

Tamura K, Nei M, and Kumar S (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101:11030-11035. https://doi.org/10.1073/pnas.0404206101

Verma M, Brar SK, Tyagi RD, Surampalli RY, Valéro JR (2007). Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. Biochemical Engineering Journal 37(1):1-20. https://doi.org/10.1016/j.bej.2007.05.012

Verma RK, Sachan M, Vishwakarma K, Upadhyay N, Mishra RK, Tripathi DK, Sharma S (2018). Role of PGPR in sustainable agriculture: molecular approach toward disease suppression and growth promotion. In: Role of Rhizospheric microbes in soil. Springer, Singapore, pp 259-290. https://doi.org/10.1007/978-981-13-0044-8_9

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173(2):697-703. https://doi.org/10.1128/jb.173.2.697-703.1991

Yasmin H, Naz R, Nosheen A, Hassan MN, Ilyas N, Sajjad M, Anjum S, Gao X, Geng Z (2020). Identification of new biocontrol agent against charcoal rot disease caused by *Macrophomina phaseolina* in soybean (*Glycine max* L.). Sustainability 12:6856. https://doi.org/10.3390/su12176856

Wang Z, Li Y, Zhuang L, Yu Y, Liu J, Zhang L (2019). A rhizosphere-derived consortium of *Bacillus subtilis* and *Trichoderma harzianum* suppresses common scab of potato and increases yield. Computational and Structural Biotechnology Journal 645-653. https://doi.org/10.1016/j.csbj.2019.05.003
