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Biocontrol and Plant Growth Promotion Characterization of *Bacillus* Species Isolated from *Calendula officinalis* Rhizosphere

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Abstract The phenotypic and genotypic diversity of the plant growth promoting *Bacillus* genus have been widely investigated in the rhizosphere of various agricultural crops. However, to our knowledge this is the first report on the *Bacillus* species isolated from the rhizosphere of *Calendula officinalis*. 15 % of the isolated bacteria were screened for their important antifungal activity against *Fusarium oxysporum*, *Botrytis cinerea*, *Aspergillus niger*, *Cladosporium cucumerinum* and *Alternaria alternata*. The bacteria identification based on *16S r*-RNA and *gyrase-A* genes analysis, revealed strains closely related to *Bacillus amyloliquefaciens*, *B. velezensis*, *B. subtilis* sub sp *spizezenii* and *Paenibacillus polymyxa* species. The electro-spray

mass spectrometry coupled to liquid chromatography (ESI-LC MS) analysis showed that most of the *Bacillus* isolates produced the three lipopeptides families. However, the *P. polymyxa* (18SRTS) didn't produce any type of lipopeptides. All the tested *Bacillus* isolates produced cellulase but the protease activity was observed only in the *B. amyloliquefaciens* species (9SRTS). The Salkowsky colorimetric test showed that the screened bacteria synthesized 6–52 µg/ml of indole 3 acetic acid. These bacteria produced siderophores with more than 10 mm wide orange zones on chromazurol S. The greenhouse experiment using a naturally infested soil with *Sclerotonia sclerotiorum* showed that the *B. amyloliquefaciens* (9SRTS) had no significant ($P > 0.05$) effect on the pre-germination of the chickpea seeds. However, it increased the size of the chickpea plants and reduced the stem rot disease ($P < 0.05$). These results suggested that the *Bacillus* strains isolated in this work may be further used as bioinoculants to improve the production of *C. officinalis* and other crop systems.

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Siderophores

Introduction

The demand for medicinal plants is increasing worldwide due to the growing recognition of biological products, being non-toxic, having no side effects and affordable prices [23]. *Calendula officinalis* is an important medicinal herb used in Europe, China, US and India. *C. officinalis* has numerous medicinal properties among which it is worth mentioning the anti-inflammatory and antioedematous activities, the antibacterial and antifungal activities,

the anticancer and lymphocyte activation dual activities, the anti-HIV activity, the immunostimulant activity, the antioxidant activity, the hepatoprotective activity, the wound healing activity and the antiviral activity [17]. Several phytopathogenic fungi were detected on *C. officinalis* seeds such *Alternaria alternata*, *Alternaria porri*, *Botrytis cinerea*, *Drechslera* (*Cochliobolus*) *hawaiiensis*, *Fusarium avenaceum*, *Fusarium culmorum* and *Sclerotinia sclerotiorum*. These pathogens lead to significant yield losses of *C. officinalis* crops [21]. The application of agrochemicals is necessary to increase crop yields but these products have several negative side effects [27]. The plant growth-promoting rhizobacteria (PGPR) can be used as an attractive alternative to the use of such xenobiotic compounds [6]. It has been noted by many workers that the bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* belongs to PGPR, showed synergistic effects on plant growth [9]. The PGPRs mediate biological control indirectly by the production of antimicrobial molecules [19, 20], siderophores and eliciting induced systemic resistance against a number of plant diseases. One of the most important direct ways that those bacteria affect growth and development is by producing Indole-3-acetic acid (IAA) that this hormone is led to plant root system development and subsequently nutritional uptake increase by plant [26].

The *Bacillus* species offer several advantages over the other genera because of their capacity to produce spores in unfavorable environmental conditions. This characteristic facilitates the conversion of spore suspensions to powder formulations without killing bacteria [16]. Thus, a number of *Bacillus* and *Paenibacillus* spp. have been commercially developed as biological fungicides, insecticide and nematicides or generic plant growth promoters, and their use in agriculture has recently been reviewed. The well studied and applied organisms are members of *Bacillus subtilis* spp. group [3, 7]. Such organisms have almost identical 16S rDNA sequences (99.2–99.6 % sequence similarity). Several molecular techniques were applied to assess the bacterial diversity and to analyze the genetic relationships between *Bacillus* species, i.e., DNA–DNA re-association studies, Rep-PCR, protein coding genes and internal transcribed spacers (ITS) sequences analysis, restriction fragment length polymorphism (RFLP) and *Bacillus* species-specific signature [22].

To our knowledge, this is the first study to characterize *Bacillus* isolates from the rhizosphere of *C. officinalis*. The main objectives were to: (I) isolate the predominant antifungal *Bacillus* species; (II) study their phylogenetic diversity based on 16S r-RNA and *gyr-A* genes analysis; and (III) evaluate their in vitro and in vivo biocontrol and

plant growth-promoting traits, in order to further use them as bio-inoculant strains.

Materials and Methods

Bacillus Isolation and Antagonism Test

Bacillus strains were isolated from the rhizosphere of *C. officinalis*, using a procedure involving a heat treatment at 80 °C during 12 min. The antifungal activity of the *Bacillus* isolates was investigated on PDA Petri dishes by the dual culture technique. The tested phytopathogenic fungi were *Fusarium oxysporium*, *B. cinerea*, *Aspergillus niger*, *C. cucumerinum* and *A. alternata*. Mycelia growth inhibition was calculated as the reduction percentage of mycelia expansion compared with control plates without bacteria [25]. Mean values and standard errors were calculated from three replicates used for each fungal strain.

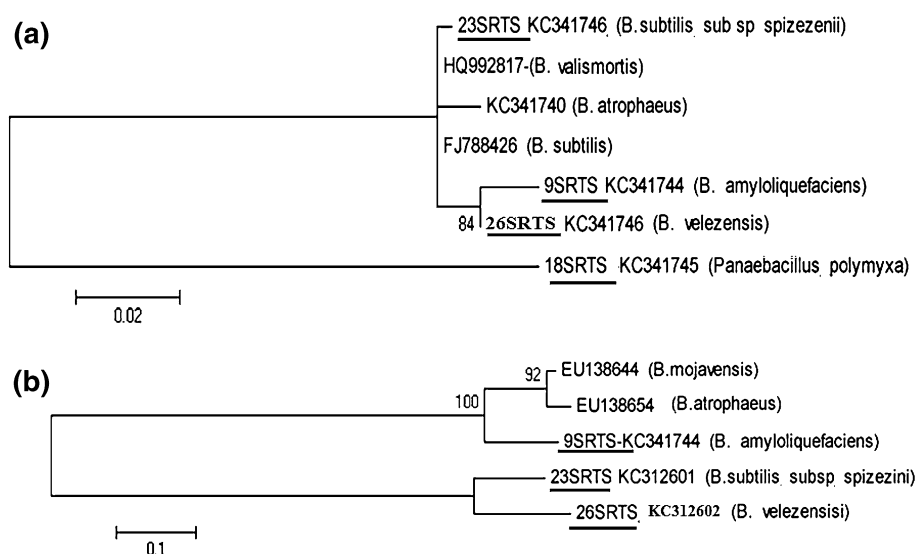
Spore Yields Determination

The *Bacillus* isolates were grown in the opt liquid medium described by Jacques et al. [12], for 72 h at 30 °C and agitated flasks (180 rpm). Spores concentration in *Bacillus* cultures was investigated using a thermal chock technique [8]. All experiments were performed as three replicates.

Identification of the *Bacillus* Strains and Phylogenetic Analysis

The total DNA was extracted from *Bacillus* liquid cultures by the wizard genomic DNA purification kit (Promega), using the manufacturer's instructions. The primers used for the PCR amplification were the universal primers 16SP0 and 16SP6 for the 16S r-RNA gene [2] and *gyr-A.f* and *gyr-A.r* for the *gyr-A* gene [11]. The purification of the PCR products was achieved using the GFX PCR DNA and Gel Band Purification Kit. The amplified genes were sequencing using the same primers sited above and the obtained sequences were corrected by the Bio-edit program. The obtained sequences were deposited in Genbank database and the accession numbers were obtained (Fig. 1). To identify the *Bacillus* isolates, the DNA sequences were compared to those previously published in Genbank using the BLASTN program. The taxonomic position of the *Bacillus* isolates studied in this work was investigated by analyzing the 16S r-RNA and *gyr-A* genes sequences. The phylogenetic trees were constructed by the MEGA 5 program, using maximum-likelihood (ML) method based on the Jukes-Cantor model.

Fig. 1 Phylogenetic trees of the *Bacillus* strains isolated from *C. officinalis* rhizosphere, based on **a** 16S r-RNA and **b** *gyr-A* genes sequences analysis. The bacteria isolated in this work were underlined and followed by the accession number provided by Genbank



Production of Cell-Wall Degrading Enzymes and Lipopeptides

The enzymatic activities were assessed in a qualitative way through a halo formation on solid media containing colloidal chitin, milk powder and carboxymethyl cellulose substrates to reveal chitinase, protease and cellulase activities respectively [4]. The lipopeptides were analyzed by mass spectrometry coupled to HPLC. The *Bacillus* strains were grown in agitated flasks (180 rpm) containing the opt medium at 30 °C for 72 h. Cultures were centrifuged at 15,000×g for 20 min. The supernatant samples were loaded on C18 solid-phase extraction cartridges (900 mg, Alltech) and lipopeptides were desorbed with 100 % ACN. The resulting samples were analyzed by reverse phase HPLC coupled with single quad mass spectrometer (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass analyzer) on a X-terra MS (Waters) 150 × 2.1 mm, 3.5 µm column as previously described by Nihorimbere et al. [18]. In this work, a single elution gradient allowing the simultaneous measurement of all three lipopeptides families was used. The water acidified with 0.1 % formic acid (A) and acetonitril (ACN) acidified with 0.1 % formic acid (B) were used as a mobile phase. The flow rate was maintained at 0.5 ml min⁻¹ and the column temperature at 40 °C, with a gradient of 35 min (43–80 %, vol/vol ACN in 18 min; 100 %, vol/vol ACN for 9 min, and 43 %, vol/vol ACN in 8 min). Compounds were identified on the basis of their retention times compared to purified standards. The identity of each homologue was confirmed on the basis of the masses detected in the SQD by setting electrospray ionization conditions in the MS as source temp., 130 °C; desolvation temp., 250 °C; nitrogen flow, 500 l/h; cone voltage, 70 V. The positive ion mode was used for analysis of all three families because a

higher signal/background ration was obtained compared to negative ion recording.

Production of Indole 3 Acetic Acid (IAA)

The indole acetic acid production was assayed calorimetrically by using the Salkowski reagent (0.01 M FeCl₃ in 36 % H₂SO₄) as described by Benduzi et al. [5]. The test was achieved in duplicate.

Production of Siderophores

The *Bacillus* isolates were streaked on azurol S medium (CAS-medium) as described by Husen [10] and siderophores production was indicated by the formation of yellow-orange halos around the colonies after incubation. This test was achieved in three replicates.

Effects of the Soil Treatment with the Biocontrol Agent (9SRTS) on Chickpea Plant Size; Damping-Off and Stem Rot Diseases Under Greenhouse Conditions

The *B. amyloliquefaciens* (9SRTS) was produced under optimized industrial conditions in a 500 L bioreactor in the society Artechno S.A (Belgium). The fermentation was stopped at the time of almost full sporulation, centrifuged and lyophilized to yield a highly concentrated stable powder. This product was resuspended in sterile distilled water to obtain the final desired spore concentration (10⁷ spores/ml). The chickpea seeds (CV. Flipe 13 90) were sown in a naturally infested soil with *S. sclerotiorum*. The treatment was carried out by spraying the bacterial suspension on the soil. Two replicates were used; each replicate consisted of three pots (4 seeds/pot). Data were recorded for damping-off; size and stem rot rating disease

(percentage of discoloration leaves per plant) after 30 days of sowing. The SAS software (SAS Institute 2000) was used for all statistical analysis. The soil treatment effect on the studied parameters was assessed by a general linear model (GLM). Least square means (LSM) and standard errors were calculated, allowing ranking of treated and control lots according to Duncan's procedure (Fig. 2).

Results and Discussion

Twenty-six *Bacillus* strains were isolated from the rhizosphere of *C. officinalis*. Four isolates (15 %) were screened for their ability to inhibit growth of some phytopathogenic fungi such as *F. oxysporium*, *B. cinerea*, *A. niger*, *C. cucumerinum* and *A. alternata* (Table 2). These bacteria were identified based on 16S r-RNA and *gyr-A* gene sequences analysis as *B. amyloliquefaciens*, *B. subtilis* sub sp *spizezenii*, *B. velezensis* and *P. polymyxa*. Approximate results were found by Martina Köberl et al. [14] where the *Bacillus* strains isolated from the rhizosphere and endorhiza of the medicinal plants (*Matricaria chamomilla*, *C. officinalis* and *Solanum distichum*) had an antagonistic effect against *Rhizoctonia solani*, *Verticillium dahliae* and *F. culmorum*. The isolated bacteria in this same study were identified based on 16S r-RNA sequences analysis as *B. subtilis*, *B. vallismortis*, *B. amyloliquefaciens* and *B. atrophaeus* [13]. In this study, the average similarity values of the 16S r-RNA sequences from *Bacillus* spp. was 99.1 % and the isolates were indistinguishable from one another.

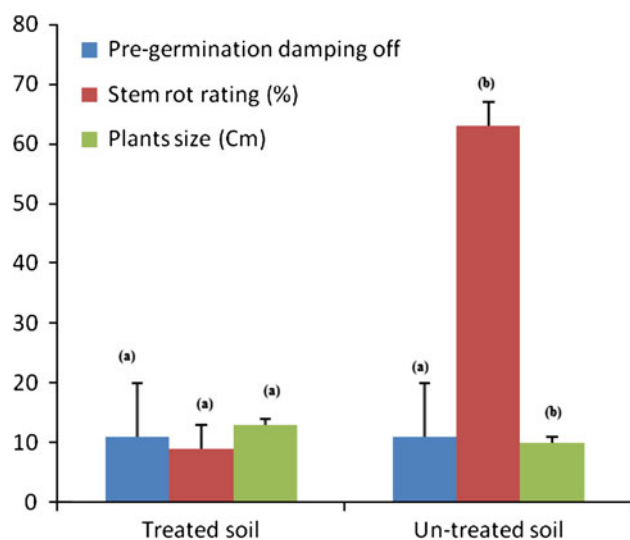


Fig. 2 The effect of the *B. amyloliquefaciens* (9SRTS) on the pre-germination damping-off, the size of chickpea plants and the stem rot disease rating after 1 month of seeds sowing. Different letters above histograms corresponding to the same parameter mention that the control pots are significantly different from the treated ones ($P < 0.05$)

However, the *gyr-A* gene sequences analysis clarified further the identification of the *Bacillus* spp. Isolates. The *gyr-A* based tree clearly delineated three distinct clusters, cluster 1 contained *B. atrophaeus* and *B. mojavensis*, cluster 2 contained *B. amyloliquefaciens* and cluster 3 contained *B. velezensis* and *B. subtilis* sub sp *spizezenii* (Fig. 1b). Comparatively, the 16S r-RNA gene-based tree yielded three clusters, cluster 1 contained strains of *B. subtilis*, *B. vallismortis*, *B. atrophaeus* and *B. subtilis* sub sp *spizezenii*, cluster 2 contained *B. amyloliquefaciens* and *B. velezensis* and cluster 3 contained *P. polymyxa*. These results were similar to that previously found by Jongsik and Kyung [13]. The *P. polymyxa* (18SRTS) strain had a very low sporulation yields. However, the other *Bacillus* isolates (9SRTS, 23SRTS and 26SRTS) had high sporulation levels which varied between 0.8×10^9 and 2.5×10^9 spores/ml (Table 1). Previously, the spore yields in submerged optimized cultivation were lower and estimated at 8.35×10^8 spores/ml [15]. In this work, the *Bacillus* isolates showed important growth inhibition percentages against *F. oxysporium* and *B. Cinerea* i.e., 39–83 % (Table 2). The detected antifungal activity can be explained by the capacity of the *Bacillus* isolates to produce the cyclic lipopeptides (cLPs) and the cell-wall degrading enzymes as previously sowed [19, 25]. Indeed, all screened bacteria here produced cellulase but the protease activity was found only in the *B. amyloliquefaciens* species (9SRTS) and non strain produced chitinase (Table 2). The LC-MS analysis showed that most of *Bacillus* isolates produced surfactin and iturin. Two types of iturin were produced, the iturin (A) and the Bcillomycin D. However, the *P. polymyxa* (18SRTS) didn't produce any type of lipopeptides. The *B. velezensis* (26SRTS) was the only strain producing fengycins. In previous works, it has been mentioned that a very limited number of strains are reported to co-produce fengycin homologues [19]. The production of the phytohormone (IAA) and siderophores by *Bacillus* species has been investigated in many studies. The IAA stimulates the plant growth and siderophores chelate iron (Fe) and deprive the phytopathogenic fungi of it [5–24]. Here, the *Bacillus* spp. isolates (9SRTS, 23SRTS and 26SRTS) produced low concentrations of IAA (7–14 $\mu\text{g/ml}$) and high levels of siderophores (more than 10 mm yellow-orange zone diameter). However, the *P. polymyxa* (18SRTS) didn't produce siderophores and produced higher concentrations of IAA which reached 53 $\mu\text{g/ml}$ (Table 3). The in vivo test carried here showed that the *B. amyloliquefaciens* (9SRTS) had no significant effect on the pre-germination of chickpea seeds ($P > 0.05$). However, it increased the size of the chickpea plants and reduced the stem rating disease ($P < 0.05$). The *B. subtilis* and *B. megaterium* species decreased the pre-germination damping-off and the stem rot rating disease of Giza variety of chickpea in the study

Table 1 Geographical origin, identification and spore yields in flasks cultivation of *Bacillus* strains isolated from the rhizosphere of *C. officinalis*

Sampled site	Antagonistic <i>Bacillus</i> strains	Spore yields ($\times 10^8$ spores/ml)
<i>C. officinalis</i> rhizosphere (greenhouse, Setif-Eastern Algeria)	(9SRTS) <i>B. amyloliquefaciens</i>	25 \pm 1
	(18SRTS) <i>P. polymyxa</i>	Not determined
	(23SRTS) <i>B. subtilis</i> sub sp <i>spizezenii</i>	8 \pm 2
	(26SRTS) <i>B. velezensis</i>	20 \pm 1

Table 2 Antifungal activity, lipopeptides and cell-wall degrading enzymes production

<i>Bacillus</i> isolates	Fungal growth inhibition (%)		Lipopeptide homologues production			Cell-wall degrading enzymes production	
	<i>F. oxysporium</i>	<i>B. cinerea</i>	Iturin	Fengycin	Surfactin	Protease activity ^a	Cellulase activity ^b
9SRTS	83 \pm 2	65 \pm 2	It A+	–	+	+	++
18SRTS	66 \pm 3	66 \pm 4	–	–	–	–	++
23SRTS	39 \pm 2	48 \pm 2	It A+	–	+	–	+
26SRTS	60 \pm 2	61 \pm 2	It B.D+	+	+	–	++

^a In vitro protease activity (plate assay): +represents hydrolysis; – represents no hydrolysis

^b In vitro cellulase activity: + represents 10–15 mm wide clear zone; ++ represents 15–20 mm wide clear zone; +++ represents >20 mm wide clear zone

Table 3 The production of the phytohormone (IAA) and siderophores

<i>Bacillus</i> isolates	IAA (μ g/ml)	Siderophores production ^a
9SRTS	7 \pm 2	+++
18SRTS	53 \pm 2	–
23SRTS	14 \pm 1	+++
26SRTS	6 \pm 1	+++

^a In vitro siderophores production: – represents the absence of siderophores production, +++ represents >10 mm wide yellow-orange zone

carried out by Abel-Monaim [1]. To conclude, the *Bacillus* strains isolated from the rhizosphere of *C. officinalis* have interesting in vitro and in vivo biocontrol and plant growth promotion characteristics and high spore yields which enable them to be a feasible product that can be further used to improve the production of *C. officinalis* and other crop systems.

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