https://doi.org/10.1080/09583157.2017.1334257

Development of formulations based on *Streptomyces rochei* strain PTL2 spores for biocontrol of *Rhizoctonia solani* damping-off of tomato seedlings

Miyada Zamoum[a,b], Yacine Goudjal[a,b], Nasserdine Sabaou[a], Florence Mathieu[c] and Abdelghani Zitouni[a]

[a]Laboratoire de Biologie des Systèmes Microbiens (LBSM), Ecole Normale Supérieure de Kouba, Alger, Algeria; [b]Département d'Agronomie, Faculté des Sciences, Université Amar Telidji, Laghouat, Algeria; [c]Laboratoire de Génie Chimique, LGC, Université de Toulouse, Toulouse, France

ABSTRACT

Rhizoctonia solani is one of the most problematic soil-borne pathogenic fungi for several crop cultures worldwide. This study highlights the effectiveness of high-antagonistic *Streptomyces rochei* strain PTL2, isolated from root tissues of *Panicum turgidum*, in controlling the *R. solani* damping-off and growth promotion of tomato (cv. Marmande) seedlings. The isolate PTL2 was characterised for *in vitro* biocontrol and plant growth-promoting traits. It exhibited remarkable positive results in all trials, including production of hydrogen cyanide, siderophores, 1-aminocyclopropane-1-carboxylate deaminase and phytohormones, chitinolytic activity and inorganic phosphate solubilisation. PTL2 spores were formulated as wettable talcum powder, sodium alginate pellets and sodium alginate-clay pellets. Their abilities in the biocontrol of *R. solani* and plant growth promotion were investigated in autoclaved and non-autoclaved soils. Talcum powder and sodium alginate pellets significantly reduced the damping-off severity index compared to a positive control. The talcum powder exhibited the highest protective activity, reducing the disease incidence from 89.3% to 14.1%, whereas chemical seed treatment with Thiram® provided a disease incidence of 16.7%. Furthermore, the talc-based powder formulation resulted in greatest increases in the root length, shoot length and dry weight of seedlings. The interesting biocontrol potential and growth enhancement of tomato seedlings open up promising perspectives for the possible application of talcum powder formulation based on PTL2 spores in crop improvement.

KEYWORDS *Streptomyces rochei* strain PTL2; powder and pellets formulations; biocontrol; *Rhizoctonia solani*; tomato

Introduction

Rhizoctonia solani is considered to be among the most important ubiquitous phytopathogenic fungi. It lives both in cropped and non-cropped soils, where it persists in the form of sclerotia (Huang, Zhang, Yong, Yang, & Shen, 2012). Damping-off of seedlings is a common disease caused by *R. solani* (Sadeghi, Hessan, Askari, Aghighi, & Shahidi

CONTACT Yacine Goudjal goudjal_y@yahoo.fr Département d'Agronomie, Faculté des Sciences, Université Amar Telidji, BP. 37G, Laghouat 03000, Algeria

Bonjar, 2006) in a variety of crop plants, such as tomato, cucumber (Patil, Srivastava, Kumar, Chaudhari, & Arora, 2010) and sugar beet (Sadeghi et al. 2006).

Chemical treatments are commonly used to prevent crop losses from *R. solani* but the intensive use of chemical fungicides may have an impact on the environment (De-Oliveira, Da Silva, & Van Der Sand, 2010). Chemical fungicides are not ideally effective on *R. solani*, and the disease remains a persistent problem (Huang et al., 2012). Due to the increased consumer concern regarding chemical pesticides residues in foods and environmental safety, there is an increasing demand for developing alternative methods, such as biocontrol (Spadaro & Gullino, 2005).

Actinobacteria are considered as potential biocontrol agents of plant diseases and the literature offers several reports on the isolation of actinobacteria of interest from the soil, the rhizosphere and also from the roots of various healthy plants (Goudjal et al., 2014; Liu et al., 2014; Patil et al., 2010; Petrolini, Quaroni, Saracchi, & Sardi, 1996; Sadeghi et al., 2012; Sardi et al., 1992). In addition, the potential uses of plant-associated actinobacteria as agents to manage various soil-borne pathogenic fungi and/or to stimulate plant growth have been reported (El-Tarabily, Nassar, Hardy, & Sivasithamparam, 2009). Suggested modes of action are the production of antibiotic compounds, siderophores, hydrogen cyanide (HCN) and hydrolytic enzymes such as chitinases and glucanases (De-Oliveira et al., 2010; Passari et al., 2015). Also, actinobacteria can promote the growth of host plants by solubilising inorganic phosphate and enhancing the uptake of phosphorus by the plant when its bioavailability is low in the soil (Hamdali, Hafidi, Virolle, & Ouhdouch, 2008). Some of them are also known to develop symbiotic associations with crop plants, colonising their internal tissues without causing disease symptoms and producing plant growth regulators such as gibberellic acid (GA3) and indole-3-acetic acid (IAA) (El-Tarabily et al., 2009; Goudjal et al., 2013; Khamna, Yokota, Peberdy, & Lumyong, 2010; Ruanpanun, Tangchitsomkid, Hyde, & Lumyong, 2010).

The use of fresh microbial cultures is not suitable in the agricultural sector. For this reason, several forms of solid, liquid and powder formulations based on bacterial agent spores have been developed (Tamreihao et al., 2016). The formulation process plays an important role in the efficacy of products by improving the survival and efficacy of the microbial agent (Martinez-Alvarez, Castro-Martiez, Sanchez-Pena, Gutierrez-Dorado, & Maldonado-Mendoza, 2016). Dry formulations, such as powders or granules, are preferred over liquid ones because of easiness in storage and transportation, and extended shelf life. Moreover, most dry formulations can be transformed into liquid- or water-based suspensions as required for spray, drench or root-soak applications (Lumsden, Lewis, & Fravel, 1995).

In this context, we focused on the study of *Streptomyces rochei* strain PTL2, a strong antagonistic actinobacterium isolated in our research laboratory from root tissues of a Saharan native plant, *Panicum turgidum*, in Algeria. We aimed to highlight its *in vitro* biocontrol and plant growth-promoting traits and to develop powder and pellets formulations for agricultural use. The shelf life of PTL2 spores and the efficacy of formulations for the *in vivo* biocontrol of *R. solani* damping-off of tomato seedlings were investigated.

Materials and methods

Bacterial strain

The endophytic strain PTL2 was isolated by our research team from the inner root tissues of *P. turgidum*, a Saharan native plant in Algeria (32°56′ N 3°17′ E) (Goudjal et al., 2013). Based on the morphological characteristics and phylogenetic analysis of the 16S rRNA gene sequences (accession number in the GenBank data Library: KC414013), the strain PTL2 was closely related to *S. rochei* NBRC 12908T with 99.52% similarity.

Antifungal activity

The cross-streak method (Toumatia et al., 2014) was used to evaluate the antagonistic activities of strain PTL2 against 5 soil-borne phytopathogenic fungi (collection of the LBSM, ENS de Kouba, Alger, Algeria) as test microorganisms: *R. solani* (LAG3), *Fusarium solani* (LF22), *F. oxysporum* f. sp. *radicis-lycopersici* (LF30), and *F. oxysporum* f. sp. *lycopersici* (LF31) and *Botrytis cinerea* (LB11). The strain PTL2 was first cultivated in straight lines on yeast extract-malt extract (ISP2) (Shirling & Gottlieb, 1966) plates (90 mm diameter), and incubated for 8 days at 30°C. After that, target fungi were seeded in streaks perpendicular to those of actinobacterial strain and incubated at 25° C for 5 days. Three independent replicates were conducted for each test. The antifungal activity was evaluated by measuring the distance of inhibition between target fungus and actinobacterial colony margins. Compared to control cultures, the percentage of inhibition was calculated.

In vitro *biocontrol and plant growth promotion traits*

HCN production

HCN production was highlighted by growing PTL2 strain in Bennett agar amended with 4.4 gl^{-1} of glycine. A Whatman filter paper was flooded with 0.5% picric acid in 2% sodium carbonate for a minute and stuck underneath the Petri dish lids. The plates were sealed with parafilm and incubated at 30°C. After 7 days, an orange to red colour on the filter paper was indicative of positive HCN production (Passari et al., 2015).

Siderophore production

The production of siderophores was investigated on chrome azurol S plates (Sadeghi et al., 2012). Six-mm plugs from the strain PTL2 culture were placed on the medium and incubated at 30°C for 7 days. The development of orange halo was considered as positive for siderophore production.

Chitinolytic activity

The chitinolytic activity was performed using colloidal chitin medium (Gonzalez-Franco, Deobald, Spivak, & Crawford, 2003). The strain PTL2 was spot inoculated onto the medium and incubated at 30°C for 5 days. Chitinolytic activity was determined by evaluating the size of the hydrolytic halo surrounding the bacterial colony.

Inorganic phosphate solubilisation

The experiments were performed in 500-ml Erlenmeyer flasks containing 100 ml of liquid Pikovskaya (PVK) medium containing $Ca_3(PO_4)_2$, $AlPO_4$ or $FePO_4$ as insoluble phosphate sources at a concentration of 5 gl^{-1}. Flasks were inoculated by adding a 1 ml aliquot of the spore suspension ($\approx 10^6$ CFU ml^{-1}) of the strain PTL2 and cultured on a rotary shaker (200 rpm, 30°C) for 7 days. The supernatant culture was harvested by centrifugation at 10,000*g* for 10 min and was used to assess the phosphate released into the solution. Soluble phosphate was determined by the molybdenum blue colorimetric method (Liu et al., 2014).

Phytohormones' production

The strain PTL2 was investigated for its indole-3-acetic acid (IAA) and GA3 production abilities using the methods of Berríos, Illanes, and Aroca (2004) and Khamna et al. (2010). Aliquots of 1 ml of the spore suspension of strain PTL2 ($\approx 10^6$ CFU ml^{-1}) were transferred into 250-ml Erlenmeyer flasks containing 50 ml of yeast extract-tryptone broth supplemented with 5 gl^{-1} of L-tryptophan (Khamna et al., 2010). The same quantity of ISP2 broth was used for GA3 production. Flasks were cultured on a rotary shaker (200 rpm, 30°C) for 5 days and supernatant cultures were harvested by centrifugation at 10,000*g* for 30 min.

IAA production was revealed by mixing 2 ml of supernatant culture with 4 ml of Salkowski reagent. The appearance of a pink colour after 30 min in a dark room indicated positive IAA production. Optical density was read at 530 nm using a spectrophotometer (JANWAY-6405) and the level of IAA produced was determined from a standard IAA graph (Acros Organics) (Passari et al., 2015).

For GA3 investigation, the supernatant culture was recovered and the cell mass was re-extracted with phosphate buffer (pH 8.0) and centrifuged again (10,000*g* for 3 min). Both supernatants were pooled, acidified to pH 2.5 by 5 N hydrochloric acid and partitioned with equal volumes of ethyl acetate five times. Two ml of zinc acetate solution (10% w/v) and 2 ml of potassium ferrocyanide (1% w/v) were added to 15 ml of ethyl acetate fractions and the mixture was centrifuged at 10,000*g* for 10 min. Five ml of supernatant were added to 5 ml of hydrochloric acid (30% v/v) and the mixture was incubated at 20°C for 75 min. The absorbance was measured at 254 nm and the amount of GA3 produced was calculated from the standard graph using standard GA3 (Sigma-Aldrich) solution (Berríos et al., 2004).

1-aminocyclopropane-1-carboxylate deaminase production

Production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase was done using the nitrogen-free Dworkin and Foster's salts minimal agar medium (Dworkin & Foster, 1958). The medium was supplemented with ACC (3 mM l^{-1}) as a sole nitrogen source. The strain PTL2 was streak-inoculated on the medium and incubated at 30°C for 7 days. Growth of strain PTL2 indicated positive result for ACC-deaminase production (Tamreihao et al., 2016).

All the *in vitro* biocontrol and plant growth-promoting traits were performed with three independent replicates.

Formulation processes

Spores of the strain PTL2 were formulated as wettable talcum powder, sodium alginate pellets and sodium alginate-clay pellets. All of the formulation processes were performed under sterile laboratory conditions.

Preparation of the spore suspension

The strain PTL2 was cultivated on ISP2 plates and incubated at 30°C for 10 days. Spores were recovered in Tween-20 solution (0.05% v/v) and adjusted to $\approx 10^6$ CFU ml^{-1} using the Thoma cell counting method.

Talcum powder formulation

A modified method of Sabaratnam and Traquair (2002) was used for the preparation of wettable talcum powder formulation. A 25 ml spore suspension was thoroughly mixed with 100 g of autoclaved talc powder (Fisher Scientific), 1.5 g of calcium carbonate and 10 g of carboxymethyl cellulose. After drying the formulation overnight under a laminar flow hood, the talcum powder formulation was weighed and stored at room temperature in the dark. Using the plate count method, PTL2 spores' density was evaluated at $\approx 2.1 \times 10^5$ CFU g^{-1}.

Pellet formulations

Sodium alginate pellets were prepared using a modified method of Zacky and Ting (2015). The sodium alginate solution (Sigma-Aldrich; 2%) was prepared with sterile distilled water and heated at 45°C under agitation, until alginate was completely dissolved. Then, the alginate solution was boiled for 5 min to reduce contamination. Alginate stock solution was cooled to room temperature before use. A 25 ml spore suspension was mixed with 200 ml alginate solution. This mixture was pumped using a peristaltic pump through two hypodermic syringes (45 mm length × 0.6 mm diameter) and dropped into sterile $CaCl_2$ solution (2.5%) to solidify the alginate and form the pellets (0.3 mm average diameter). Alginate pellets were then dried overnight under a laminar flow hood, weighted and stored at room temperature. The density of PTL2 spores was evaluated at $\approx 2 \times 10^6$ CFU g^{-1}.

Sodium alginate-clay pellets were prepared using the same process as described above. A 200 ml alginate stock solution was thoroughly mixed with 50 g autoclaved clay (0.2 mm in particle diameter). The mixture was dropped through hypodermic syringes (45 mm length × 1.2 mm diameter) into the sterile $CaCl_2$ solution to form pellets 3 mm in diameter. Dried alginate-clay pellets yielded $\approx 4.5 \times 10^5$ CFU g^{-1}.

Purity of formulations and viability of PTL2 spores

The purity of the three formulations based on PTL2 spores was verified after the formulation processes. A 0.2 g of each formulation was mixed for 1 min (15 min for alginate and alginate-clay pellets) in 4 ml of sterile distilled water and 0.1 ml of the spore suspension was inoculated to ISP2 plates and incubated at 30°C. After 7 days' incubation, PTL2 cultures were checked for microbial contamination. The viability of formulated PTL2 spores was determined every 2-month intervals for one year storage period. Viable spores of strain PTL2 grown on ISP2 medium were determined by counting CFU g^{-1} using the

counting plate method. Two independent subsamples were analysed twice, with three replicates per treatment (Martinez-Alvarez et al., 2016).

In vivo *biocontrol of* Rhizoctonia solani

The potential of PTL2 spore formulations in the *in vivo* biocontrol of *R. solani* (LAG3) and their ability to promote the growth of tomato (cv. Marmande) seedlings were tested in a sandy soil sampled from a tomato field in the Algerian Sahara (33°62′ N, 2°91′ E). Trials were performed both in autoclaved and non-autoclaved soils. Sterilisation was carried out by autoclaving soil at 120°C for 60 min as used by Goudjal et al. (2014).

Surface-sterilisation of tomato seeds was performed by sequential dipping in ethanol solution (70% v/v, 3 min), NaClO solution (0.9% w/v, 4 min) followed by washing three times in sterile distilled water (Goudjal et al., 2013).

Autoclaved and non-autoclaved soils were infested with the *R. solani* using a modified method of Sadeghi et al. (2009). Plastic pots (12 cm high × 10 cm in diameter) filled with soil were infested with 5 g of the pathogen inoculum previously prepared by growing on autoclaved wheat semolina (5 g autoclaved wheat semolina for non-infested soil). Pots were then watered with 100 ml sterile distilled water and incubated at room temperature for 7 days to enhance the growth of the pathogen. Using the plate count method, the density of *R. solani* in the infested soil was evaluated at ≈ 1.2×10^3 CFU g^{-1}.

Five treatments were conducted in this assay: Surface-sterilised seeds were sown in non-infested soils as a negative control. Surface-sterilised seeds were sown in infested soils to evaluate the varietal sensitivity as a positive control. Surface-sterilised seeds were sown in infested soils to evaluate the biocontrol potential of each formulated product. For this, the biocontrol agents were used in infested pots by adding 20 ml of talcum powder suspension (10 g talcum powder watered in 100 ml sterile distilled water), thoroughly mixing 0.14 g alginate pellets or 0.9 g alginate-clay pellets with infested soil. Quantities of the formulated biocontrol agents were calculated to reach similar spore densities in the soil (≈2×10^3 CFU g^{-1}). A chemical fungicide: Thiram® 75WP was used to appreciate the biocontrol efficacy of the three formulated products. For this, sterilised seeds were treated with the Thiram® 75WP as recommended by the manufacturer (thorough mixing, for 3 min, of 10 g of the surface-sterilised tomato seeds in 0.08 g of chemical control agent wetted with 10 ml of distilled water and drying for 2 h under a laminar flow hood), before being cultivated in infested soils.

Five seeds were sown per pot with 10 replicates for each treatment. Pots were then placed in a greenhouse (24–27°C, 15 h light and 9 h dark) in a fully randomised complete block design. Cultures were watered daily with tap water (10 ml per pot) for 6 weeks.

Pathogenicity of *R. solani* in tomato seedlings was confirmed as previously described by Barnes, Csinos, and Branch (1990) and Ithurrart, Büttner, and Petersen (2004). The fungal strains re-isolated from disease lesions developed on inoculated plants showed the same characteristics of inoculated potential pathogens ones fulfilling Koch's postulates.

The *R. solani* symptoms were noted using a 5-class scale (Dhanasekaran et al., 2005): 0 = no symptom, 1 = 0–25% of root browning, 2 = 26–50% of root browning, 3 = 51–75% of root browning, 4 = 76–100% of root browning and 5 = plant death. The readings

were converted to a disease severity index (DSI) using the following equation:

$$\mathrm{DSI}(\%) = \left[\sum (R \times N)\right] \times 100/H \times T$$

where R is the disease rating, N is the number of plants with this rating, H is the highest rating category and T is the total number of plants counted.

In the aim to evaluate the effect of the three formulated products on the plant growth, the shoot and root lengths, and the dry weight were measured for healthy seedlings. All *in vivo* experiments were conducted twice to ensure reproducibility.

Statistical analysis

Data were subjected to ANOVA analysis and results represent the mean ± standard deviation. Significant differences between means were compared using Duncan's multiple range test at $P = .05$.

Results

Antagonistic activity

The strain PTL2 showed positive antagonistic activities against all targeted fungi and the percentages of inhibition were greater than 30% (Table 1). The strongest antagonistic activity (percentage of inhibition > 50%) were observed against *Botrytis cinerea* (LB11), *F. oxysporum* f. sp. *radicis-lycopersici* (LF22) and *R. solani* (LAG3).

HCN and siderophore production, and chitinolytic activity

The strain PTL2 was studied for HCN and siderophore production abilities, and chitinolytic activity (Table 2). A positive production of HCN was detected for this strain, which gave an orange colour on the filter paper. This strain was found able to produce siderophores on CAS medium, forming yellowish orange halos around colonies. Results also showed that strain PTL2 grow well on colloidal chitin medium and had positive chitinolytic activity.

Phosphate solubilisation ability and production of phytohormones and ACC-deaminase

The strain PTL2 grew well and dissolved phosphorus in PVK media containing $Ca_3(PO_4)_2$ or $Ca_3(PO_4)_2$ as sole phosphorus sources. The amounts of dissolved phosphorus were

Table 1. Antifungal activity of *S. rochei* strain PTL2 towards phytopathogenic fungi.

Targeted fungi	Zone of inhibition (mm)[a]	Percentage of inhibition (%)[a]
Rhizoctonia solani (LAG3)	32 ± 0.9	64 ± 1.3
Fusarium solani (LF22)	18 ± 1.9	35 ± 1.6
F. oxysporum f. sp. *radicis-lycopersici* (LF30)	26 ± 1.5	51 ± 1.6
F. oxysporum f. sp. *lycopersici* (LF31)	16 ± 1,6	31 ± 2.1
Botrytis cinerea (LB11)	30 ± 1.6	54 ± 1.9

[a] The data shown are the mean of three independent replicates ± standard deviation.

Table 2. Production of HCN and siderophores, and chitinolytic activity.

Strain	Hydrogen cyanide	Siderophores Halo diameter (mm)[a]	Chitinolytic activity Halo diameter (mm)[a]
PTL2	+	20 ± 0.9	18 ± 1.2

[a] The data shown are the mean of three independent replicates ± standard deviation.
+ Positive production of HCN.

278 mg × 100 ml^{-1} from $Ca_3(PO_4)_2$ and 149 mg × 100 ml^{-1} from $AlPO_4$. However, the strain PTL2 failed to grow and to dissolve phosphorus in the PVK medium containing $FePO_4$.

The results showed also that strain PTL2 was positive for both IAA and GA3 production (100.3 µg ml^{-1} and 86.6 µg ml^{-1}, respectively). Furthermore, it was qualified by positive production of ACC-deaminase.

Purity and viability of formulated PTL2 spores

The microbial purity of the three formulated products was verified on ISP2 plates. The actinobacterial spore suspensions obtained from the talcum powder, alginate pellets and alginate-clay pellets showed pure cultures of the strain PTL2 and no microbial contamination has been noted.

Kinetics of viable spore counts evaluated at 2-months intervals for one year are given in Figure 1. The amount of PTL2 spores in the alginate pellets and talcum powder formulations remained relatively stable throughout the 12 months after the formulation process. Spore's counts in the alginate pellets decreased from 2×10^6 CFU g^{-1} on the day of preparation to 1.7×10^6 CFU g^{-1} after one year of storage at room temperature,

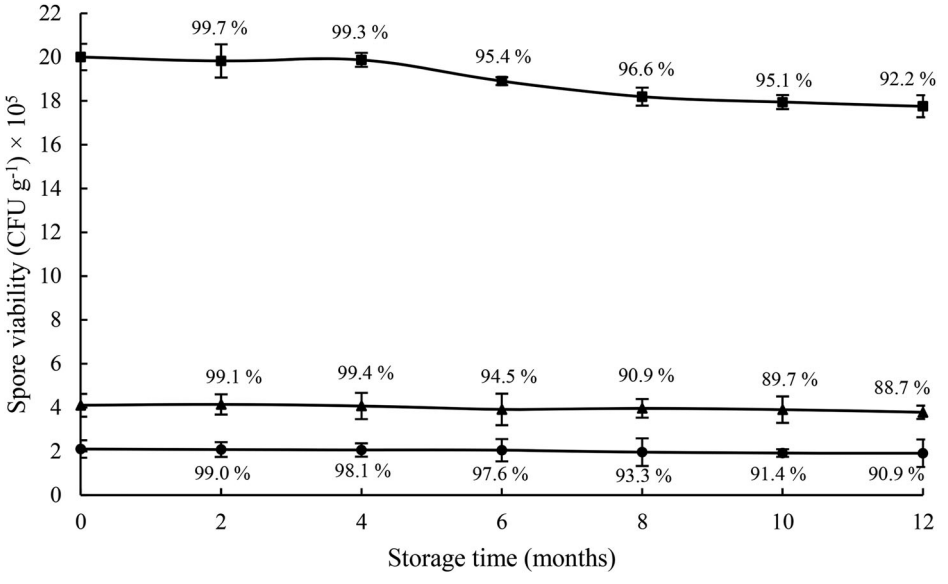

Figure 1. Spore viability of *S. rochei* strain PTL2 in the talcum powder (–●–), alginate pellets (–■–) and alginate-clay pellets (–▲–) formulations stored at room temperature and evaluated at 2-month intervals post-formulation. The CFU values for each sampling date represent the mean of three independent replicates ± standard deviation. The percentage values of each point represent the viability percentage.

which corresponded to 7.8% dead spores. The spore's counts in the talcum formulation decreased from 2.1×10^5 CFU g^{-1} to 1.9×10^5 CFU g^{-1} (9.1% dead spores). However, density of viable spores after storage (3.7×10^5 CFU g^{-1}) in the alginate-clay pellets showed the highest death rate, which reached 11.3% dead spores.

Biocontrol of R. solani

Results of the biocontrol potential and plant growth-promoting effect of the three formulation products are given in Figure 2. High DSI of the *R. solani* damping-off of tomato seedlings in both autoclaved (90.3%) and non-autoclaved (89.3%) soils were obtained for the positive control. Damping-off symptoms were observed in germinated seeds and young seedlings, leading to desiccation of the roots and damping-off of the seedlings.

Chemical treatment with Thiram® and biocontrol with the talcum powder and alginate pellets significantly ($P < .05$) reduced the DSI, which was relatively more marked in non-autoclaved soil than in autoclaved soil. However, the biocontrol effect of alginate-clay pellets showed high DSI (70.7%) (Figure 2(A)). Chemical control showed a strong protective effect against *R. solani* damping-off. In both autoclaved and non-autoclaved soils, non-significant differences were observed between the chemical treatment and the biocontrol effect assessed with the talcum powder formulation, which reached the highest biocontrol potential in non-autoclaved soil (14.1% DSI). The rate of healthy seedlings obtained in the negative control was 97.5%. With the Thiram®, talcum powder, alginate pellets and alginate-clay pellets, the rates were 83.3%, 85.9%, 75.2% and 29.3%, respectively.

Compared to the negative control, the treatment with talcum powder formulation enhanced the seedling dry weight from 1.41 g to 1.52 g (Figure 2(B)) and the seedling shoot length from 9.8 cm to 11.5 cm (Figure 2(C)). Furthermore, it significantly increased ($P < .05$) the seedling root length from 7.8 cm to 9.0 cm (Figure 1(D)) in non-autoclaved soil. The treatment with alginate pellets and alginate-clay pellets showed seedling dry weight varied from 1.2 g to 1.5 g, seedling shoot length from 7.3 cm to 10.3 cm and seedling root length from 7.7 cm to 8.4 cm (Figure 1(B–D)).

Discussion

Several actinobacteria have been reported as potential agents for the biocontrol of various soil-borne phytopathogenic fungi and as stimulators of plant growth (De-Oliveira et al., 2010; Goudjal et al., 2014; Zamoum et al., 2015). Patil et al. (2010) and Sadeghi et al. (2006) highlighted the efficacy of actinobacterial isolates in the biocontrol of the *R. solani* damping-off disease. Actually, various species of actinobacteria, such as *Streptomyces lydicus* strain WYEC108 and *S. griseoviridis* strain K61, are formulated for exploration in the biocontrol of plant diseases and crop improvement (Lahdenperä, Simon, & Uoti, 1991).

In the present study, the endophytic *S. rochei* strain PTL2 isolated in our research laboratory (Goudjal et al., 2013) was selected on the basis of its broad spectrum of antifungal activities. The strain PTL2 was firstly *in vitro* studied for biocontrol and plant growth promotion properties and then formulated as the forms of wettable talcum powder, alginate pellets or alginate-clay pellets. The three formulated products based on PTL2 spores were

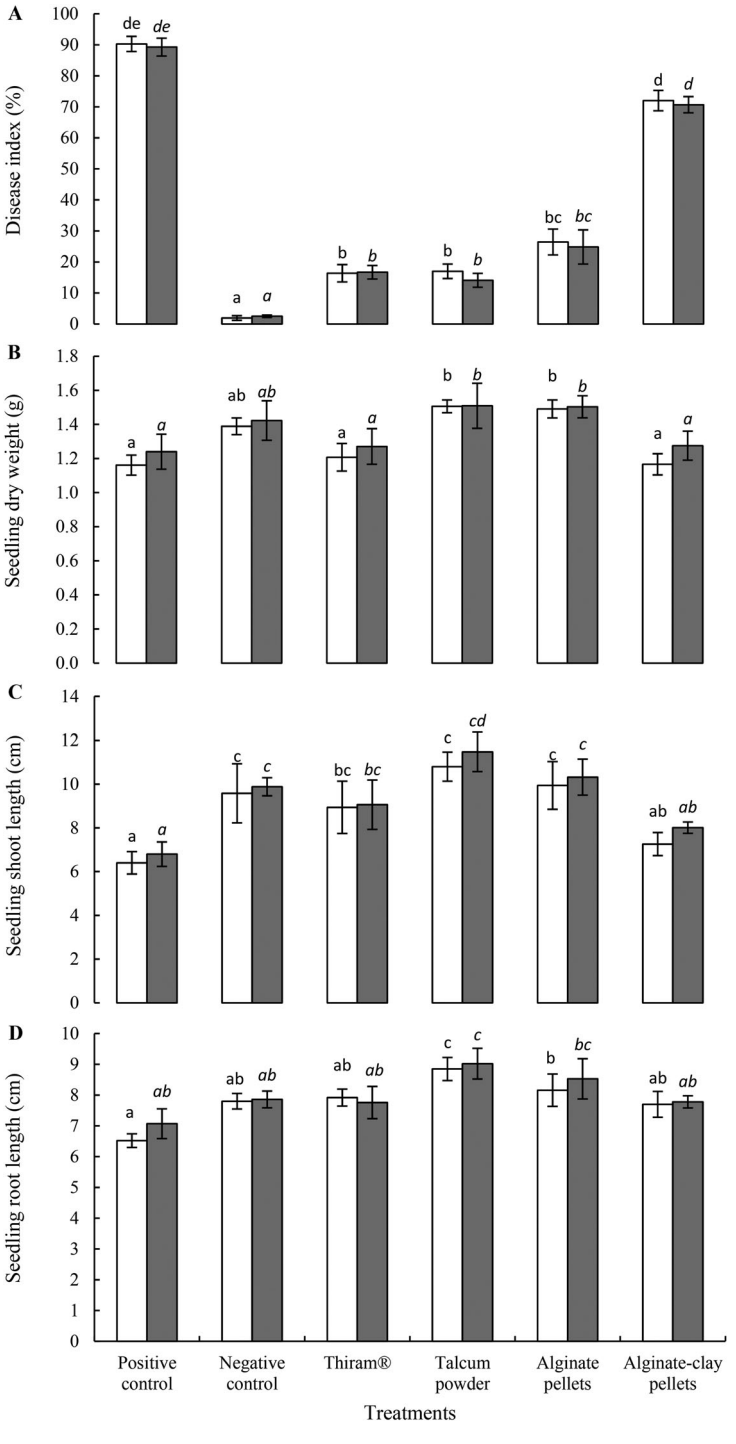

Figure 2. Effect of Thiram® and talcum powder, alginate pellets and alginate-clay pellets formulations based on *S. rochei* strain PTL2 spores on the *R. solani* damping-off disease index (A), seedling dry weight (B), seedling shoot length (C) and seedling root length (D) in autoclaved (*white bars*) and non-autoclaved (*grey bars*) soils. Evaluation was made 6 weeks after planting of tomato (cv. Marmande) seeds. Bars labelled with the same letters are not significantly different according to Fisher's protected LSD test at $P = .05$.

then investigated for *in vivo* biocontrol of *R. solani* damping-off of tomato (cv. Marmande) seedlings.

The strain PTL2 showed positive antifungal activity against all targeted fungi. The strongest activity was observed against the fungus *R. solani*. Several *Streptomyces* species have already been reported as active against *R. solani* and have been explored (or proposed) as biocontrol agents against soil-borne phytopathogenic fungi, especially *R. solani* (Patil et al., 2010; Sadeghi et al., 2006). They can diffuse in the soil and therefore direct contact between the biocontrol agent and fungi is not necessary (Shimizu, 2011).

Streptomyces sp. PTL2 produced HCN, known to act as a volatile antifungal compound (Passari et al., 2015), might help *R. solani* damping-off suppression. This result is in agreement with those reported for antagonistic *Streptomyces* sp. (Passari et al., 2015) and *Pseudomonas fluorescens* (Shimizu, 2011) producing HCN as a biocontrol mechanism against *Rhizoctonia* and *Pythium* phytopathogenic species.

The strain PTL2 has been found to produce siderophores, which is an important factor for antagonism activities. Siderophores are low molecular weight compounds secreted by many *Streptomyces* species that permit the acquisition of ferric ion, which can inhibit phytopathogen growth by competing for iron in rhizosphere soils (Sadeghi et al., 2012). In addition, Verma, Singh, and Prakash (2011) reported that actinobacteria producing siderophores may help the plant to take up iron from the soil. Our results are in agreement with those of Passari et al. (2015) who reported the role of endophytic *Streptomyces* producing siderophores in the antagonistic effect on *R. solani*.

Chitinase-producing *Streptomyces* spp. are commonly implicated in the biocontrol processes and are formulated as active biofungicides (Zacky & Ting, 2015). Chitinase from antagonistic *Streptomyces* acts on the cell wall of phytopathogenic fungi. As reported for *Streptomyces* spp. producing chitinase, which were active in the biocontrol of *R. solani* damping-off (Passari et al., 2015; Sadeghi et al., 2012), the strain PTL2 showed positive chitinolytic activity.

Inorganic phosphate-solubilising actinobacteria play an important role in the plant growth enhancement (Hamdali et al., 2008). In our results, the strain PTL2 grew in the PVK broth and dissolved phosphorus from both tricalcium phosphate and aluminium phosphate. However, it failed to grow using iron phosphate. These results are supported by those of Goudjal, Zamoum, Sabaou, Mathieu, and Zitouni (2016) and Liu et al. (2014), who reported low activities of *Streptomyces* species in the solubilisation of iron phosphate. Hamdali et al. (2008) studied the capacity of phosphate-solubilising actinobacteria to promote the growth of wheat plants. They reported that the isolate exhibiting the best solubilisation activity also gave the best enhancement of plant growth.

Production of phytohormones is another mechanism by which actinobacteria can improve the germination of seeds and elongation of roots (Goudjal et al., 2013; Khamna et al., 2010). In our findings, the strain PTL2 was found to produce both IAA and GA3. The amounts of phytohormones were higher than those reported for *S. caeruleatus* strain ZL2 (Zamoum et al., 2015) and *S. viridis* strain CMU-H09 (Ruanpanun et al., 2010).

The strain PTL2 showed positive results for ACC-deaminase production. This result is supported by those of Glick (2014), who reported ACC-deaminase production by several actinobacterial species. Bacteria producing ACC-deaminase are able to promote root elongation and plant growth by lowering ethylene levels in the roots (Suarez et al., 2015).

Formulated products based on spore-producing bacteria have been proposed in the agricultural sector as alternatives to the chemical compounds used in disease control and plant growth promotion (Martinez-Alvarez et al., 2016; Soe & De Costa, 2012). Several works reported the screening of microorganisms that possess biocontrol activity. However, very few of these reports describe information regarding the formulation of microorganisms due to secrecy issues imposed by commercial companies (Herrmann & Lesueur, 2013). The formulation of a biocontrol agent is the primary step towards its use for crop improvement, having the aim of preserving the microorganism and its biocontrol and plant growth-promoting traits. Attractive biopesticides are characterised with microorganisms without danger to the consumer, to the applicator and to the ecological system. It should also be easy for application in the production system, with microbial purity and ensure a long shelf life (Sadeghi et al., 2009; Soe & De Costa, 2012). In our study, three variants of formulated products based on *S. rochei* strain PTL2 spores were performed. Their microbial purity has been highlighted and pure cultures of *S. rochei* strain PTL2 have been obtained on ISP2 plates. Consequently, the three formulated products cannot be a source of propagation of unknown or suspect microorganisms.

For an increased shelf life, formulation carriers should support the viability of the biocontrol agent, taking into account a desirable storage period of 6–12 months for industrialisation. Furthermore, carriers and other components should not affect the viability and activity of the biocontrol agent over time (Soe & De Costa, 2012; Zacky & Ting, 2015). In our study, the talcum powder, alginate pellets and alginate-clay pellets were evaluated for their shelf life for one year of storage at room temperature. Globally, the three formulated products showed high levels of spore viability. This result can be explained by the strong resistance of bacterial spores from the genus *Streptomyces* (Tamreihao et al., 2016). Consequently, this notable feature opened promising possibilities for their investigation in crop improvement.

The three formulated biofungicides were screened for the *in vivo* biocontrol of *R. solani* damping-off and growth promotion of tomato seedlings. A high DSI has been showed for the positive control due to the sensitivity of tomato (cv. Marmande) to the pathogen. Compared to the positive control results, treatment with Thiram® significantly ($P < .05$) decreased the DSI by over 83%. These results are supported by those of Goudjal et al. (2014) and Pereira da Silva, Alves de Freitas, and Nascimento (2013), who reported the efficacy of Thiram® in the control of *R. solani* diseases. Nevertheless, seed treatment with this chemical control agent has been proven to be an effective method (Pereira da Silva et al., 2013). In contrast, intensive uses of chemical compounds to ensure crop production may destabilise the ecosystem and pollute the environment, which are major problems in the agricultural sector (De-Oliveira et al., 2010; Zamoum et al., 2015). As an alternative, biopesticides are proposed for the biocontrol of plant diseases and plant growth promotion and several biofungicide forms have been marketed (Martinez-Alvarez et al., 2016; Tamreihao et al., 2016). Lumsden et al. (1995) reported the efficacy of powder and granule formulations compared to liquid ones. Furthermore, preliminary biocontrol trials carried out in our research laboratory showed that the coating of tomato seeds with PTL2 spores has been a moderate biocontrol effect on *R. solani* compared to soil treatment with talcum powder and pellet formulations.

Talcum powder and alginate pellets showed an interesting effect in the biocontrol of *R. solani*. They reduced the DSI by over 85% and 75%, respectively. Although alginate-clay pellets showed high amount of viable spores, they have been found ineffective to suppress the phytopathogen. The biocontrol effectiveness of the talcum powder and alginate pellets, with a small pellet diameter, can be explained by their good and homogeny repartition in the soil, a good germination of *S. rochei* strain PTL2 spores and sufficient diffusion of antagonistic compounds produced by this biocontrol agent. However, the repartition of alginate-clay pellets, with a large diameter, can be heterogeneous. Moreover, the clay fraction can absorb the produced antagonistic compounds and limit their diffusion in the soil. Our results are in agreement with those of Sabaratnam and Traquair (2002), who reported the efficacy of talcum powder formulation based on *Streptomyces* sp. Di-944 in the biocontrol of *R. solani* damping-off.

In non-autoclaved soil, the native microorganisms showed a synergic effect in the biocontrol of *R. solani*. These findings are in agreement with those of Goudjal et al. (2014, 2016) in the biocontrol soil-borne phytopathogenic fungi, who suggest the presence of indigenous antagonistic microbes.

The talcum powder formulation significantly ($P < .05$) improved the seedling root length, and clearly enhanced the shoot length and dry weigh of seedlings. These results are in agreement with several reports that have highlighted the promotion of plant growth with formulated biopesticides based on spore-forming bacteria (Martinez-Alvarez et al., 2016).

S. rochei strain PTL2 showed interesting traits for all the biocontrol and plant growth-promoting mechanisms determined. Formulation of PTL2 spores as wettable talcum powder showed a high rate of viable spores after one year storage at room temperature. Furthermore, this formulation product exhibited the highest effect in the biocontrol of *R. solani* damping-off and growth promotion of tomato (cv. Marmande) seedlings. Consequently, talcum powder formulations based on spores of *S. rochei* strain PTL2 open up promising perspectives for possible application in the biocontrol of *R. solani* diseases.

Acknowledgements

We thank Susan Becker, translator and native English speaker, for her kind contribution in revising the English of this article.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Amar Telidji University Laghouat, Algeria and by the LBSM laboratory, ENS de Kouba, Alger, Algeria.

References

Barnes, J. S., Csinos, A. S., & Branch, W. D. (1990). Sensitivity of *Rhizoctonia solani* isolates to fungicides and evaluation of peanut cultivars to *Rhizoctonia* limb rot. *Peanut Science*, *17*, 62–65.

Berríos, J., Illanes, A., & Aroca, G. (2004). Spectrophotometric method for determining gibberellic acid in fermentation broths. *Biotechnology Letters*, *26*, 67–70.
De-Oliveira, M. F., Da Silva, M. G., & Van Der Sand, S. T. (2010). Anti-phytopathogen potential of endophytic actinobacteria isolated from tomato plants (*Lycopersicon esculentum*) in southern Brazil, and characterization of *Streptomyces* sp. R18, a potential biocontrol agent. *Research in Microbiology*, *161*, 565–572.
Dhanasekaran, D., Sivamani, P., Panneerselvam, A., Thajuddin, N., Rajakumar, G., & Selvamani, S. (2005). Biological control of tomato seedling damping off with *Streptomyces* sp. *Plant Pathology Journal*, *4*, 91–95.
Dworkin, M., & Foster, J. (1958). Experiments with some microorganisms which utilize ethane and hydrogen. *Journal of Bacteriology*, *75*, 592–601.
El-Tarabily, K. A., Nassar, A. H., Hardy, G. E. S. J., & Sivasithamparam, K. (2009). Plant growth promotion and biological control of *Pythium aphanidermatum* a pathogen of cucumber, by endophytic actinomycetes. *Journal of Applied Microbiology*, *106*, 13–26.
Glick, B. R. (2014). Bacteria with ACC-deaminase can promote plant growth and help to feed the world. *Microbiological Research*, *169*, 30–39.
Gonzalez-Franco, A. C., Deobald, L. A., Spivak, A., & Crawford, D. L. (2003). Actinobacterial chitinase-like enzymes: Profiles of rhizosphere versus non-rhizosphere isolates. *Canadian Journal of Microbiology*, *49*, 683–698.
Goudjal, Y., Toumatia, O., Sabaou, N., Barakate, M., Mathieu, F., & Zitouni, A. (2013). Endophytic actinomycetes from spontaneous plants of Algerian Sahara: Indole-3-acetic acid production and tomato plants-growth-promoting activity. *World Journal of Microbiology and Biotechnology*, *29*, 1821–1829.
Goudjal, Y., Toumatia, O., Yekkour, A., Sabaou, N., Mathieu, F., & Zitouni, A. (2014). Biocontrol of *Rhizoctonia solani* damping-off and promotion of tomato plant growth by endophytic actinomycetes isolated from native plants of Algerian Sahara. *Microbiological Research*, *169*, 59–65.
Goudjal, Y., Zamoum, M., Sabaou, N., Mathieu, F., & Zitouni, A. (2016). Potential of endophytic *Streptomyces* spp. for biocontrol of *Fusarium* root rot disease and growth promotion of tomato seedlings. *Biocontrol Science and Technology*, *26*, 1691–1705.
Hamdali, H., Hafidi, M., Virolle, M. J., & Ouhdouch, Y. (2008). Growth promotion and protection against damping-off of wheat by two rock phosphate solubilizing actinomycetes in a P-deficient soil under greenhouse conditions. *Applied Soil Ecology*, *40*, 510–517.
Herrmann, L., & Lesueur, D. (2013). Challenges of formulation and quality of biofertilizers for successful inoculation. *Applied Microbiology and Biotechnology*, *97*, 8859–8873.
Huang, X., Zhang, N., Yong, X., Yang, X., & Shen, O. (2012). Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. *Microbiological Research*, *167*, 135–143.
Ithurrart, M. E. F., Büttner, G., & Petersen, J. (2004). *Rhizoctonia* root rot in sugar beet (*Beta vulgaris* ssp. *altissima*) – epidemiological aspects in relation to maize (*Zea mays*) as a host plant. *Journal of Plant Diseases and Protection*, *111*, 302–312.
Khamna, S., Yokota, A., Peberdy, J. F., & Lumyong, S. (2010). Indole-3-acetic acid production by *Streptomyces* sp. isolated from some Thai medicinal plant rhizosphere soils. *EurAsian Journal of BioSciences*, *4*, 23–32.
Lahdenperä, M. L., Simon, E., & Uoti, J. (1991). Mycostop – a novel biofungicide based on *Streptomyces* bacteria. In A. B. R. Beemster, G. J. Bollen, M. Gerlagh, M. A. Ruissen, B. Schippers, & A. Temple (Eds.), *Biotic interactions and soil-borne disease* (pp. 258–263). Amsterdam: Elsevier.
Liu, F., Liu, H., Zhou, H., Dong, Z., Bai, X., Bai, P., & Qioa, J. (2014). Isolation and characterization of phosphate-solubilizing bacteria from betel nut (*Areca catechu*) and their effects on plant growth and phosphorus mobilization in tropical soils. *Biology and Fertility of Soils*, *50*, 927–937.
Lumsden, R. D., Lewis, J. A., & Fravel, D. R. (1995). Formulation and delivery of biocontrol agents for use against soil-borne plant pathogens. In F. R. Hall & J. W. Barry (Eds.), *Biorational pest control agents*. Washington, DC: American Chemical Society.

Martinez-Alvarez, J. C., Castro-Martiez, C., Sanchez-Pena, P., Gutierrez-Dorado, R., & Maldonado-Mendoza, I. E. (2016). Development of a powder formulation based on *Bacillus cereus* sensu lato strain B25 spores for biological control of *Fusarium verticillioides* in maize plants. *World Journal of Micorbiology and Biotechnology*, doi:10.1007/s11274-015-2000-5

Passari, A. K., Mishra, V. K., Gupta, V. K., Yadav, M. K., Saikia, R., Singh, B. P., & Virolle, M.-J. (2015). In vitro and in vivo plant-growth-promoting activities and DNA fingerprinting of antagonistic endophytic actinomycetes associates with medicinal plants. *PloS ONE*, *10*, e0139468.

Patil, H. J., Srivastava, A. K., Kumar, S., Chaudhari, B. L., & Arora, D. K. (2010). Selective isolation, evaluation and characterization of antagonistic actinomycetes against *Rhizoctonia solani*. *World Journal of Microbiology and Biotechnology*, *26*, 2163–2170.

Pereira da Silva, P., Alves de Freitas, R., & Nascimento, W. M. (2013). Pea seed treatment for *Rhizoctonia solani* control. *Journal of Seed Science*, *35*, 17–20.

Petrolini, B., Quaroni, S., Saracchi, M., & Sardi, P. (1996). Studies on the streptomycete population inhabiting plant roots. *Actinomycetes*, *7*, 66–78.

Ruanpanun, P., Tangchitsomkid, N., Hyde, K. D., & Lumyong, S. (2010). Actinomycetes and fungi isolated from plant-parasitic nematode infested soils: Screening of the effective biocontrol potential, indole-3-acetic acid and siderophore production. *World Journal of Microbiology and Biotechnology*, *26*, 1569–1578.

Sabaratnam, S., & Traquair, J. A. (2002). Formulation of a *Streptomyces* biocontrol agent for the suppression of *Rhizoctonia* damping-off in tomato transplants. *Biological Control*, *23*, 245–253.

Sadeghi, A., Hessan, A. R., Askari, H., Aghighi, S., & Shahidi Bonjar, G. H. (2006). Biological control potential of two *Streptomyces* isolates on *Rhizoctonia solani*, the causal agent of damping-off of sugar beet. *Pakistan Journal of Biological Sciences*, *9*, 904–910.

Sadeghi, A., Hesan, A., Askari, H., Qomi, D. N., Farsi, M., & Hervan, E. M. (2009). Biocontrol of *Rhizoctonia solani* damping-off of sugar beet with native *Streptomyces* strains under field conditions. *Biocontrol Science and Technology*, *19*, 985–991.

Sadeghi, A., Karimi, E., Dahaji, P. A., Javid, M. G., Dalvand, Y., & Askari, H. (2012). Plant-growth-promoting activity of an auxin and siderophore producing isolate of *Streptomyces* under saline soil conditions. *World Journal of Microbiology and Biotechnology*, *28*, 1503–1509.

Sardi, P., Saracchi, M., Quaroni, S., Petroloni, B., Borgonovi, G. E., & Merli, S. (1992). Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. *Applied and Environmental Microbiology*, *58*, 2691–2693.

Shimizu, M. (2011). Endophytic actinomycetes: Biocontrol agents and growth promoters. In D. K. Maheshwari (Ed.), *Bacteria in agrobiology: Plant growth responses* (pp. 201–220). Heidelberg: Springer.

Shirling, E. B., & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology*, *16*, 313–340.

Soe, K. T., & De Costa, D. M. (2012). Development of a spore-based formulation of microbial pesticides for control of rice sheath blight. *Biocontrol Science and Technology*, *22*, 633–657.

Spadaro, D., & Gullino, M. L. (2005). Improving the efficacy of biocontrol agents against soil borne pathogens. *Crop Protection*, *24*, 601–613.

Suarez, C., Cardinale, M., Ratering, S., Steffens, D., Jung, S., Zapata-Montoya, A. M., … Schnell, S. (2015). Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare* L.) under salt stress. *Applied Soil Ecology*, *95*, 23–30.

Tamreihao, K., Ningthoujam, D. S., Nimaichand, S., Singh, E. S., Reena, P., Singh, S. H., & Nongthomba, U. (2016). Biocontrol and plant growth promoting activities of a *Streptomyces corchorusii* strain UCR3-16 and preparation of powder formulation for application as biofertilizer agents for rice plant. *Microbiological Research*, *192*, 260–270.

Toumatia, O., Yekkour, A., Goudjal, Y., Riba, A., Coppel, Y., Mathieu, F., … Zitouni, A. (2014). Antifungal properties of an actinomycin D-producing strain, *Streptomyces* sp. IA1, isolated from a Saharan soil. *Journal of Basic Microbiology*, *54*, 1–8.

Verma, V. C., Singh, S. K., & Prakash, S. (2011). Biocontrol and plant growth promotion potential of siderophore producing endophytic *Streptomyces* from *Azadirachta indica* A Juss. *Journal of Basic Microbiology*, *51*, 550–556.

Zacky, F. M., & Ting, A. S. Y. (2015). Biocontrol of *Fusarium oxysporum* f.sp. *cubense* tropical race 4 by formulated cells and cell-free extracts of *Streptomyces griseus* in sterile soil environment. *Biocontrol Science and Technology*, *25*, 685–696.

Zamoum, M., Goudjal, Y., Sabaou, N., Barakate, M., Mathieu, F., & Zitouni, A. (2015). Biocontrol capacities and plant growth-promoting traits of endophytic actinobacteria isolated from native plants of Algerian Sahara. *Journal of Plant Diseases and Protection*, *122*, 215–223.