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The potential of antagonistic moroccan *Streptomyces* isolates for the biological control of damping-off disease of pea (*Pisum sativum* L.) caused by *Aphanomyces euteiches*

Brahim Oubaha[1,2] | **Ahmed Nafis**[1] | **Mohamed Baz**[1] | **Felix Mauch**[2] | **Mustapha Barakate**[1,3]

[1]Laboratory of Biology and Biotechnology of Microorganisms, Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco

[2]Laboratory of Interaction Plant-Microorganisms, Department of Biology, University of Fribourg, Fribourg, Switzerland

[3]Agrobiosciences & Fertilizers Program, University Mohammed IV Polytechnic (UM6P), Benguerir, Morocco

Correspondence
Mustapha Barakate, Laboratory of Biology and Biotechnology of Microorganisms (LBBM), Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco.
Emails: mbarakate@uca.ma; mbarakate@hotmail.com

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Abstract

Three hundred and fifty-nine isolates of actinobacteria collected from different Moroccan soils were evaluated for their in vitro antimicrobial activity against the oomycete pathogen *Aphanomyces euteiches*, the causal agent of damping-off of pea and other legumes. Eighty-seven isolates (24%) had an inhibitory in vitro effect against *A. euteiches*. Fourteen bioactive isolates with the greatest inhibitory effect against *A. euteiches* and no inhibitory effect on plant beneficial rhizobia were tested for their ability to protect pea seeds and seedlings against the damping-off disease using culture supernatants or spore suspensions as treatments. The two most protective isolates, OB21 and BA15, significantly reduced, compared to untreated control plants, damping-off by 33% and 47%, respectively. The two bioactive isolates were classified as species of the genus *Streptomyces* based on 16S rDNA analysis and morphological and chemical characteristics.

KEYWORDS
16S rDNA, actinobacteria, *Aphanomyces euteiches*, biocontrol, damping-off, pea, *Streptomyces*

1 | INTRODUCTION

Legumes are very important to agriculture as they fix nitrogen in association with rhizobia, thereby reducing the need for fertilizer inputs. Economically, they are an important source of protein and other nutrients for human needs (Iqbal, Khalil, Ateeq, & Sayyar, 2006; Maphosa & Jideani, 2017). However, many pathogens cause major yield losses in legumes. Soilborne oomycete pathogens attack roots of legumes, limit nutrient uptake and cause the damping-off and/or root rot disease complex resulting in the death of plants (Attard et al., 2014; Black, 2000). The soilborne pathogenic oomycete, *Aphanomyces euteiches*, has been reported in most pea growing areas of North America, northern Europe, Australia, New Zealand and Japan (Heyman, 2008; Wakelin, McCarthy, Stewart, & Walter, 1998).

Damping-off and root rot are caused in saturated soil conditions when *A. euteiches* infects seeds or the root of pea plants via zoospores. After an early attack, the plants often collapse and die. The intensity of symptoms of root rot varies with the environmental conditions (Gaulin et al., 2008; Wakelin, Walter, Jaspers, & Stewart, 2002). There are currently no efficient fungicides or other treatments available for the control of this pathogen. Some chemicals effectively suppress *A. euteiches* under controlled conditions but have limited protective effects in field trials (Wu et al., 2018). Moreover, the oospores can

persist in soil for 10 years excluding crop rotation as mean of control (Sauvage et al., 2007). Alternative strategies have been developed to fight against this pathogen, but only the biological control strategies are promising for sustainable protection. *Bacillus*, *Paenibacillus* and *Pseudomonas* strains (*P. fluorescens* and *P. cepacia*) were reported to control *A. euteiches* (Parke, Joy, & King, 1991; Wakelin et al., 2002). However, no previous study had tested the potential of actinobacteria to control diseases caused by *A. euteiches*.

Actinobacteria are well known to produce several kinds of bioactive secondary metabolites that are used in biological control of several plant pathogens (Elbendary et al., 2018; Ganesan et al., 2017; Yu et al., 2015). Approximately 80% of the known commercial antibiotics used in medicine and agriculture have initially been isolated from actinobacteria, and especially from strains of genus *Streptomyces* (Sanglier, Haag, Huck, & Fehr, 1996), that proved to be a rich source of antibiotics. Furthermore, *Streptomyces* have been reported in several studies as biological control and showed potential to control a wide range of plant pathogenic bacteria, fungi and oomycetes by producing diverse bioactive substances (Baz, Lahbabi, et al., 2012; Baz, Tran, et al., 2012; Errakhi, Lebrihi, & Barakate, 2009; Goudjal, Zamoum, Sabaou, Mathieu, & Zitouni, 2016; Samri et al., 2015). These bacteria like many other microorganisms can also promote plant growth by producing phytohormones (Goudjal et al., 2013), by improved phosphate mobilization (Hamdali, Hafidi, Virolle, & Ouhdouch, 2008) and by production of siderophores for iron acquisition (Macagnan, Romeiro, Pomella, & Souza, 2008). The aim of the present study was to analyse the potential of actinobacteria isolates to protect legumes against *A. euteiches*. To this end, a Moroccan collection of actinobacteria was screened for antagonistic properties against *A. euteiches* and no inhibitory effect on rhizobial bacteria. The ability of selected antagonistic strains to reduce damping-off symptoms was further analysed on pea seeds, and their taxonomic affiliation was determined.

2 | MATERIALS AND METHODS

2.1 | Actinobacteria isolates

The 359 actinobacteria isolates used in this study were from the collection of the Laboratory of Biology and Biotechnology of Microorganisms (LBBM), Cadi Ayyad University, Marrakesh, Morocco. They were isolated from different Moroccan habitats including rhizospheric soils (including rhizospheric soils of legumes), endophytic environments of aromatic and medicinal plants and from extreme ecosystems (Saharan oasis) by the protocol described by Barakate, Ouhdouch, Oufdou, and Beaulieu (2002). After purification, all isolates were maintained as glycerol stocks (20% v/v at −20°C).

2.2 | Strains of rhizobia

Four rhizobia strains (Rh1, Rh2, Rh3 and Rh5) of our laboratory collection (Prof. Khalid Oufdou, Laboratory of Biology and Biotechnology of Microorganisms, Department of Biology, UCAM, Morocco) were used in the present study in order to avoid selected bioactive actinobacteria that could negatively affect the symbiotic relationship between legumes and rhizobia.

2.3 | *Aphanomyces euteiches*

The *A. euteiches* strain *ATCC 201684* was kindly provided by Prof. Elodie Gaulin (Laboratoire de recherche en sciences végétales, University of Toulouse III Paul Sabtier, France). The pathogen was stored in corn meal agar at 4°C.

2.4 | Screening of actinobacteria for inhibitory activity against *A. euteiches*

Actinobacteria isolates were tested for their effects on mycelial growth of *A. euteiches* using the dual culture technique as in vitro confrontation tests (Dennis & Webster, 1971). Actinobacteria isolates were first grown on Bennett medium (beef extract 1 g/L; yeast extract 1 g/L; peptone 2 g/L; glucose 10 g/L; agar 15 g/L) for 14 days at 30°C. Agar discs of 9 mm diameter were cut from these plates and placed at a distance of 2 cm from *A. euteiches* plugs placed on PGA (peptone 20 g/L; glucose 5 g/L; agar 15 g/L). Plates were first kept in a refrigerator (4°C) for at least 4 hr to allow the diffusion of secreted compounds and were then incubated at 24°C in the dark. Inhibition zones caused by bioactive actinobacteria isolates were determined 5 days postinoculation.

2.5 | Screening of selected bioactive actinobacteria isolates for inhibition of rhizobia

To avoid that selected bioactive actinobacteria isolates would negatively affect the symbiotic relationship between legumes and rhizobia, the active isolates were screened against four different strains of rhizobia, using the method of agar disc-diffusion technique as described by Bauer, Kirby, Sherris, and Turck (1966). Agar plugs of active actinobacteria isolates (9 mm in diameter) were placed on solid yeast extract mannitol medium (K_2HPO_4 0.5 g/L; $MgSO_4$ $7H_2O$ 0.2 g/L; NaCl 0.1 g/L; yeast extract 0.6 g/L; mannitol 10 g/L; agar 15 g/L) previously seeded with a 10 CFU/ml suspension of individual rhizobia strains. Plates were kept in a refrigerator (4°C) for at least 4 hr and were then incubated at 28°C in the dark for 3–4 days.

2.6 | Preparation of selected actinobacteria inoculum

Actinobacteria isolates that inhibited mycelia growth of *A. euteiches* and did not have any activity against rhizobia strains were tested for their ability to control damping-off of pea seeds caused by *A. euteiches* using spore suspensions or culture filtrates. For spore production, actinobacteria isolates were grown on Bennett agar medium at 28°C, and after 14 days of incubation, spores were harvested in distilled water and counted with Malassez haemocytometer and the density was adjusted to 10^8 spores/ml. Culture filtrates were obtained by

culturing agar plug of purified selected actinobacteria isolates in 50 ml of Bennett liquid medium and after incubation at 28°C for 7 days. The supernatants were harvested after centrifugation (15 min at 25,200 *g*) and kept at 4°C until use.

2.7 | Zoospore production of *A. euteiches*

Zoospores of *A. euteiches ATCC 201684* were produced by the modified method of Deacon and Saxena (1998). Agar discs of *A. euteiches* grown on PGA medium were cultured in 30 ml PG broth for 10 days at 24°C in the dark. The mycelium was harvested and washed three times with autoclaved Volvic water (VW) and incubated for 2 hr at 24°C in 20 ml of autoclaved VW after each wash. The production of zoospores was initiated by incubation of the mycelium washed in 20 ml VW for 19 hr at 24°C in the dark. Zoospores were harvested in autoclaved VW, and the density was adjusted to 10^5 zoospores/ml using a Malassez haemocytometer.

2.8 | Effect of selected actinobacteria isolates on *A. euteiches* damping-off of pea seeds and seedlings

Seeds of *Pisum sativum* L. were surface-sterilized with 4% sodium hypochlorite for 5 min, washed three times with sterile distilled water and air-dried (Benidire et al., 2015). Biological control studies were performed as described by Sadeghipour and Abbasi (2012) with some modifications. Disinfected pea seeds were immersed in flasks containing culture supernatant or spore suspension of each selected actinobacteria isolate for 30 min with a slow shaking. Petri dishes were covered by Whatman paper Number 1, and pea seeds were submerged in 5 ml of 10^5 zoospore/ml of *A. euteiches* for 5 min. Ten seeds per dish were incubated at 24°C and 75% of humidity in the dark. Seed germination and seedling stand were determined after 1 week (inhibition of germination, disease severity index, seedling fresh weight and seedling root length). Tests were repeated three times and included four replicate dishes for each treatment.

2.9 | Morphological, physiological and chemotaxonomic characterizations of selected antagonistic actinobacteria isolates

The taxonomic classification of the two selected antagonistic actinobacteria isolates was evaluated as described in the *International Streptomyces Project* (ISP) (Shirling & Gottlieb, 1968). All cultures were incubated at 28°C for 7 days, and the colour of the aerial spore mass of colonies and the colour of the growth medium were observed according to the scale adopted by Nonomura (1974). The production of melanoid pigments was tested on peptone yeast extract iron agar (ISP6) and tyrosine agar (ISP7) (Shirling & Gottlieb, 1968). The assimilation of carbohydrates as sole carbon sources at concentration 1% (w/v) was studied using the ISP9 medium (Shirling & Gottlieb, 1968). The chemical analyses of the diaminopimelic acid isomer were performed as adopted by Becker, Lechevalier, Gordon, and Lechevalier (1964).

2.10 | DNA preparation, amplification and sequencing of the 16S rDNA

The DNA of the two selected actinobacteria isolates was extracted as described by Hopwood et al. (1985). The 16S rDNA was PCR-amplified using the primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTT GTTACGACTT-3′) (Lane, 1991). Reaction conditions were as follows: initial denaturation of the target DNA at 97°C for 4 min, followed by 35 cycles (94°C for 60 s, 52°C 60 s and 72 for 5 min). Amplification products were separated on a 0.8% (w/v) agarose gel and stained with ethidium bromide. The primers used for sequencing were the same as mentioned above. Sequences were compared by blast search for similarity with sequences present in genomic database at NCBI (http://www.ncbi.nih.gov/). A phylogenetic tree based on 16S rDNA sequences was constructed with the neighbour-joining algorithm in MEGA version 6.0. The nucleotide sequence of the two isolates OB21 and BA 15 has provided GenBank Accession numbers as SUB4319770 *Streptomyces* MH820126 and SUB4488354 *Streptomyces* MH828291, respectively.

2.11 | Statistical analyses

All collected quantitative data were analysed statistically by variance analysis test (ANOVA), and all numeric differences in the data were considered significantly different at the probability level of $p \leq 0.05$.

3 | RESULTS

3.1 | Screening of actinobacteria isolates for inhibition of *A. euteiches* but no activity towards rhizobial bacteria

Three hundred and fifty-nine isolates of actinobacteria were tested for their antagonistic activity against *A. euteiches*. Figure 1a shows that 87 out of the tested 359 isolates (24%) had an inhibitory activity towards *A. euteiches*. Active isolates caused a clear zone of growth inhibition of *A. euteiches* around the discs as shown in Figure 2a. The 87 bioactive isolates were also tested for growth inhibition of four strains of rhizobia (Rh1, Rh2, Rh3 and Rh5). An example of such a test is shown in Figure 2b. Fourteen of the 87 bioactive isolates did not cause any harm to the tested rhizobial strains (Figure 1b). Hence, 3.8% (14/359) of all tested actinobacteria fulfilled the selection criteria. These active isolates were selected for further experiments.

3.2 | Biocontrol of *A. euteiches* damping-off on pea seedlings

Pea seeds were treated with two types of treatment: culture supernatant or spore suspension of selected actinobacteria isolates. From the 14 tested isolates, only two isolates, BA15 and OB21, significantly reduced the damping-off severity of pea plants (Figure 3). Treating seeds with either of the two selected actinobacteria isolates

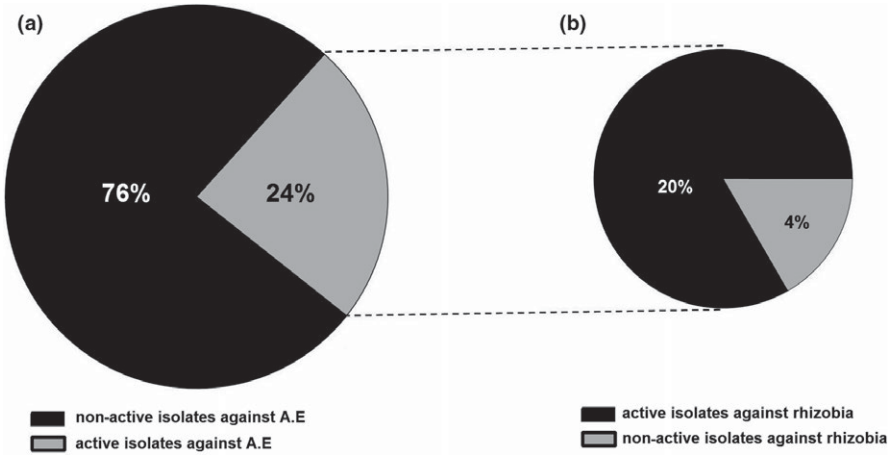

FIGURE 1 (a) Percentage of actinobacteria isolates able to inhibit the growth of *Aphanomyces euteiches* mycelium in Petri dish test, (b) percentage of active actinobacteria isolates against *A. euteiches* that not able to cause any damage to rhizobia in Petri dish test by using the confrontation method

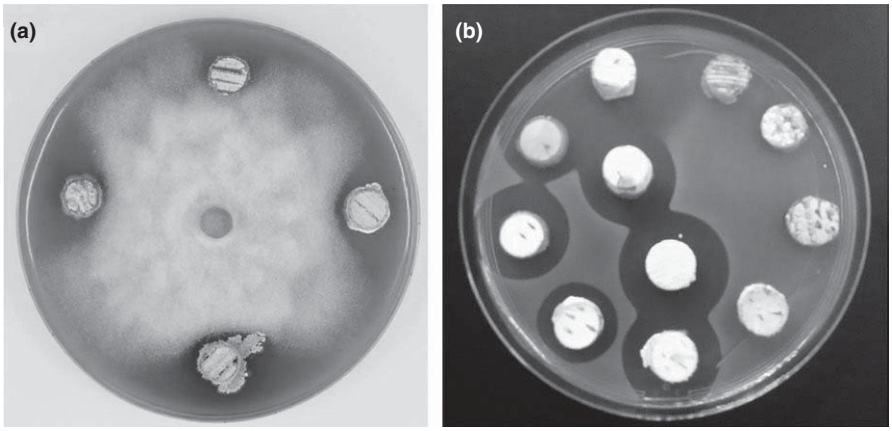

FIGURE 2 Screening method to isolate: (a) isolates able to inhibit the growth of *Aphanomyces euteiches* in PGA medium, (b) actinobacteria isolates with an in vitro inhibitory activity against *A. euteiches* but without any activity against rhizobia

resulted in a germination rate of about 92% (culture supernatant) and 88% (spore treatment) as compared to 6% in the untreated control (Table 1). The results of the diseases severity index (DSI) show that OB21 was more efficient than BA15 in either type of treatment (Figure 3a). The DSI of untreated control seedlings was 92.5%. The inoculation of pea seeds with culture supernatant of OB21 decreased significantly the DSI to 25.4%, whereas treatment of seeds with OB21 spores led to a DSI of 37.9%. Similarly, the culture supernatant of the BA15 was more efficient in decreasing the DSI (37.8%) than the application of BA15 spores (DSI 53.6%). The application of different treatments of the two tested actinobacteria isolates had a significant effect on the seedling fresh weight compared to the inoculated control (Figure 3b). The average fresh weight of OB21-treated seedlings was 7.7 g (culture supernatant treatment) and 6.9 g (spore treatment) compared to 4.0 g of untreated control seedlings. The BA15 isolate was less efficient with average seedling weights of 6.5 and 5.7 g, respectively. As shown in Figure 3c, treatments with OB21 or BA15 greatly improved the seedling root length compared to controls infected by *A. euteiches* alone. Treatment with culture filtrates of OB21 and BA15 led to an average root length of 6.3 and 5.1 cm, respectively, compared to a length of 0.5 cm in untreated inoculated controls. Together, the results presented in Figure 3 support the conclusion that both, the OB21 and the BA15 isolates, protect pea seedlings against *A. euteiches*.

3.3 | Taxonomic characterization of the selected isolates

The two antagonistic promising isolates OB21 and BA15 were tested for taxonomical identification using morphological, cultural, physiological and biochemical criteria as well as other features (Table 2). The morphology of colonies and the mycelium colour (aerial and substrate) were determined on ISP media. The analysis showed that the two strains, OB21 and BA15, have different profiles in different test media. In ISP3 medium, the colour of the aerial mycelium of OB21 was greenish, but the BA15 colour of the aerial mycelium was white. The substrate mycelium colour for OB21 in ISP2 was brown, whereas it was yellow for BA15 (Table 2). The results showed also

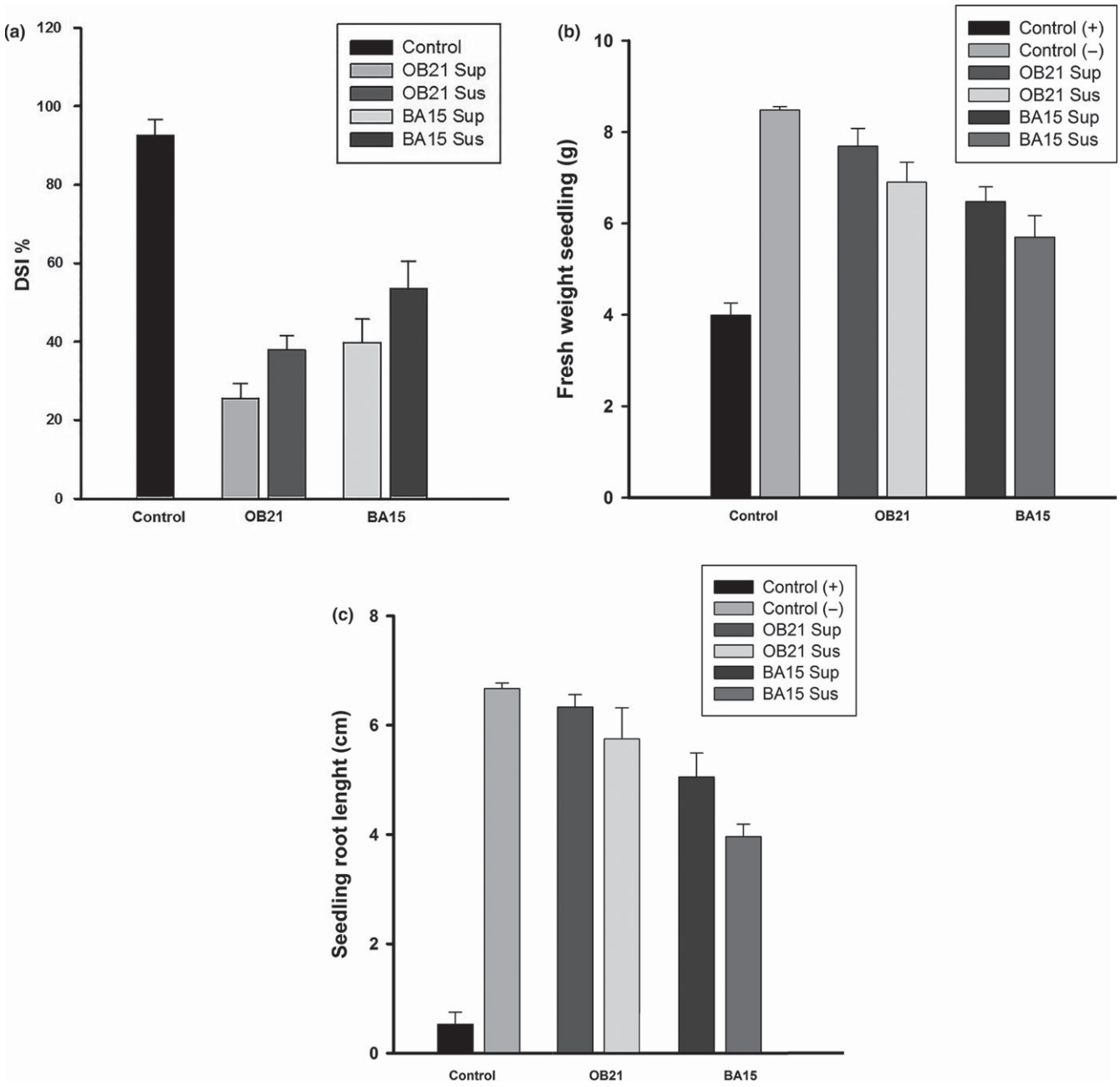

FIGURE 3 Protective effect of the application of supernatant liquid culture and spore suspensions of antagonistic actinomycetes OB21 and BA15 on pea seeds prior to infection with 10^5 spores/ml of *Aphanomyces euteiches* zoospores. (a) Effect of the treatment on disease severity index (DSI), (b) effect of the treatment on seedlings fresh weight, (c) effect of treatment on seedling root length. OB21 Sup: supernatant liquid culture of OB21; OB21 Sus: spore suspension of OB21; BA15 Sup: supernatant liquid culture of BA15; BA15 Sus: spore suspension of BA15

Treatment type	Supernatant liquid culture	Spore treatment	Control
Isolate			
OB21	92.5% ± 3.3	87.5% ± 3.2	6.3% ± 2.6
BA15	92.5% ± 1.8	87.5% ± 2.5	

TABLE 1 The protective effect of inoculation with OB21 and BA15 treatment on the germination rate of pea seeds infected with 10^5 spore/ml of *Aphanomyces euteiches*

that OB21 and BA15 do not produce melanin pigment on ISP6 and ISP7 media. Both selected strains were able to use sucrose, galactose, lactose, mannose, mannitol, arabinose, fructose, dextrin and maltose as sole carbon sources. However, they were not able to use inositol, raffinose and rhamnose (Table 2).

TABLE 2 Growth performance and some phenotypic characteristics of strain OB21 and BA15 on different media

	Growth		Aerial mycelium		Substrate hyphae		Soluble pigment	
Medium	OB21	BA15	OB21	BA15	OB21	BA15	OB21	BA15
ISP 2	+++	+++	White/brown	White	Brown	Yellow	None	None
ISP 3	+++	+++	Greenish	White	Brown	Brown	None	None
ISP 4	+++	+++	White	White	Greenish	Yellow	None	None
ISP 6	+++	+++	White/brown	White/yellow	Brown	Brown	None	Brown
ISP 7	+++	+++	Greenish	White/pink	Brown	Yellow/brown	None	Brown
BENNETT	+++	+++	White/yellow	White	White	White-grey	None	None
GYEA	+++	+++	White/greenish	White	Brown	White	Brown	Brown

Note. (+++) Good growth.

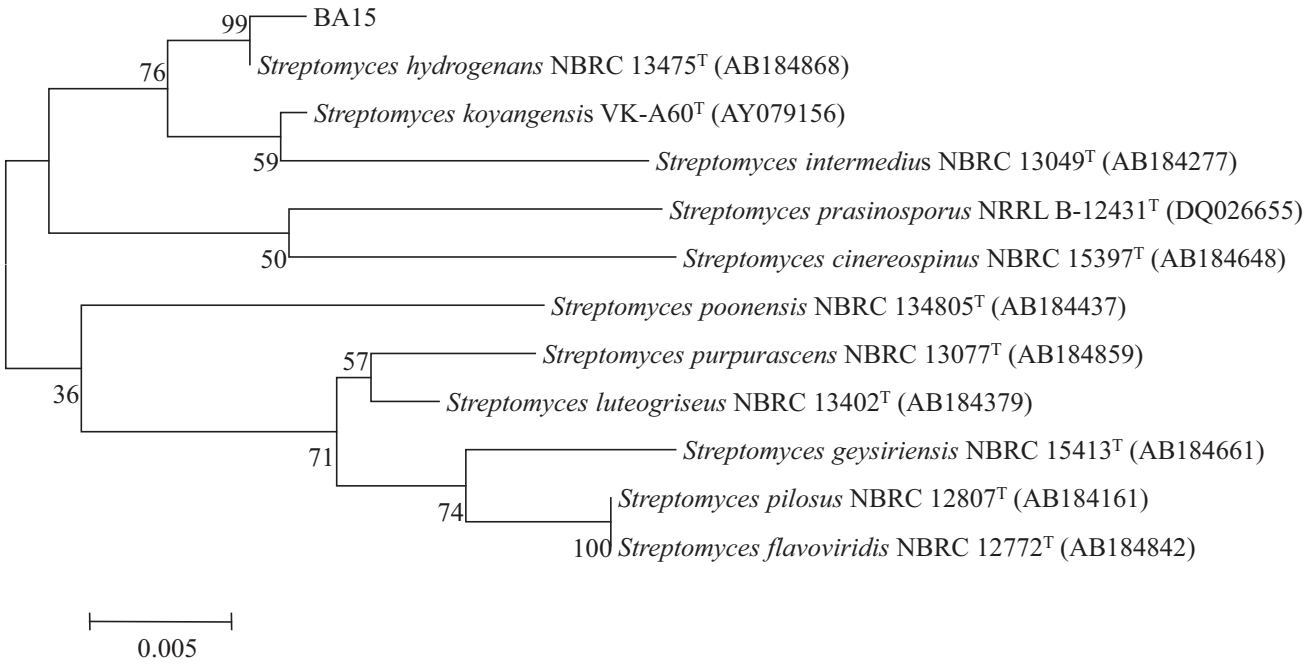

FIGURE 4 Neighbour-joining phylogenetic tree based on 16S rDNA sequences showing the relationships between strains BA15 and related bacteria from the GenBank database. The numbers in parentheses represent the sequence accession numbers in GenBank. The scale bar represents 0.02 substitutions/nucleotide position

The cell wall peptidoglycan of both isolates OB21 and BA15 contained LL-diaminopimelic acid and glycine, indicating that they had a chemotype I cell wall (Lechevalier & Lechevalier, 1970), which is characteristic of the genus *Streptomyces*. The amplification and sequence analysis of 16S rDNA confirmed the assignment of both isolates to the genus *Streptomyces* (Figures 4 and 5). BA15 and OB21 isolates exhibited 99.79% and 99.35% sequence identity, respectively, to *Streptomyces hydrogenans NBRC 13475*^{T}. The comparison of cultural, morphological and biochemical criteria, however, showed several differences. *Streptomyces hydrogenans NBRC 13475*^{T} in contrast to *Streptomyces* strains OB21 and BA15 were able to use rhamnose but not sucrose, fructose or mannitol as a sole carbon source. The mycelium colour of OB21 in the ISP3 medium (greenish) was different from the colour obtained of the mycelia of BA15 and *S. hydrogenans NBRC 13475*^{T} in the same medium (Table 3).

4 | DISCUSSION

There is a growing interest in endophytic and soil microorganisms that can protect plants against pathogens and thus may contribute to sustainable agriculture. The protective effect of such biocontrol microorganisms is often based on the production of compounds that directly interfere with spore germination and pathogen growth (Compant, Duffy, Nowak, Cle, & Barka, 2005; Freitas, Germida, & De, 1991). Biocontrol strains can also have positive indirect effects on disease resistance by stimulation of plant defence mechanisms (Lehr, Schrey, Hampp, & Tarkka, 2008; Prévost, Couture, Shipley, Brzezinski, & Beaulieu, 2006). Actinobacteria are widespread soil microorganisms known to produce a large number of bioactive compounds including antibiotics used in medicine. Bioactive actinobacteria from indigenous Moroccan habitats were reported before

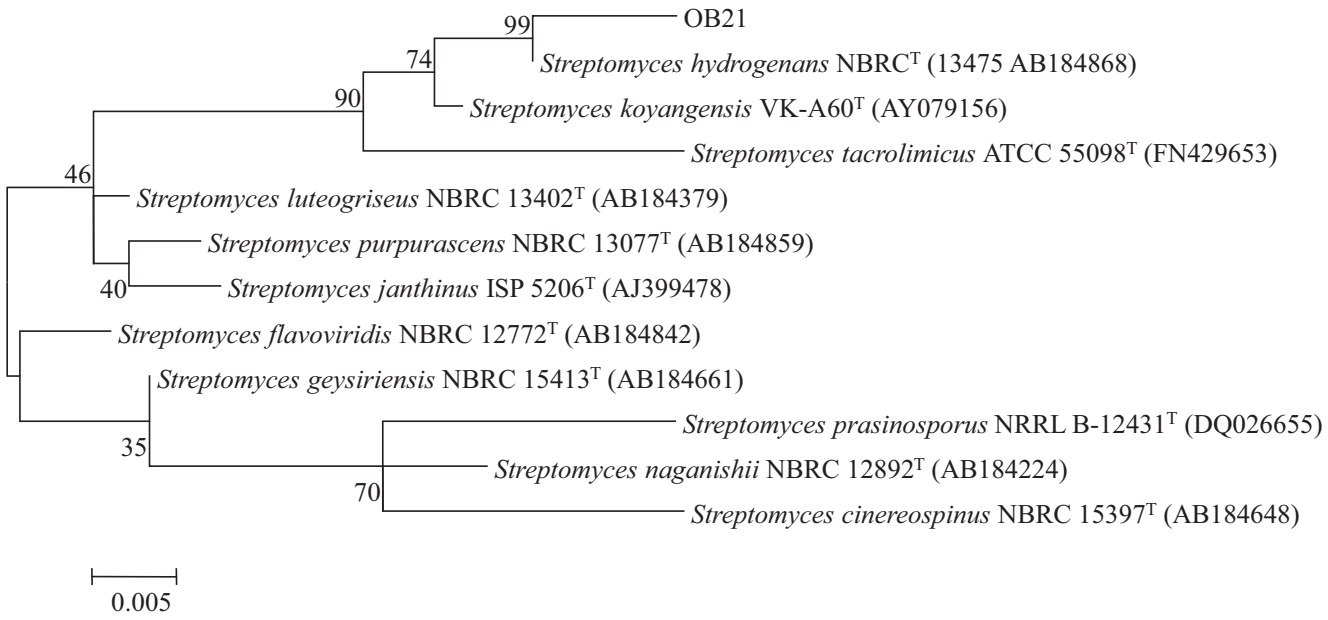

FIGURE 5 Neighbour-joining phylogenetic tree based on 16S rDNA sequences showing the relationships between strains OB21 and related bacteria from GenBank database. The numbers in parentheses represent the sequence accession numbers in GenBank. The scale bar represents 0.02 substitutions/nucleotide position

TABLE 3 Phenotypic properties that separate strain OB21 and BA15 from related *Streptomyces* species

Characteristics	OB21	BA15	*Streptomyces hydrogenans NBRC 13475^T*
ISP3 medium			
Colour of aerial mycelium	Greenish	White	White
Colour of soluble pigment	Brown	Brown	
Use of			
Sucrose	+	+	–
D-Fructose	+	+	–
Mannitol	+	+	–
Rhamnose	–	–	+

Note. (+) Positive reaction; (–) negative reaction.

(Barakate et al., 2002; Baz, Lahbabi, et al., 2012; Baz, Tran, et al., 2012; Hamdali et al., 2008; Samri et al., 2015). Many studies focused on the role of actinobacteria as biocontrol agents against a large number of phytopathogens (Costa, Zucchi, & Melo, 2013; Goudjal et al., 2016; Shrivastava, Kumar, & Yandigeri, 2017). However, no previous studies reported the use of actinobacteria against legume diseases caused by *A. euteiches*. Therefore, we investigated in this study the potential of actinobacteria isolated from Moroccan ecosystems for biological control of the damping-off disease of pea caused by *A. euteiches*. Only 3.8% of the screened actinobacteria isolates were antagonistic towards *A. euteiches* without causing damage to rhizobia. Isolates with specific antioomycete activity were reported previously (Lee et al., 2005). This finding confirms that actinobacteria, especially the genus of *Streptomyces*, have an important potential to produce bioactive compounds such as aminoglycosides, which are selectively active against several oomycete pathogens. Seed treatment with supernatants of liquid cultures and spore suspensions used in the present study dramatically increased pea seed germination rates in the presence of *A. euteiches* from 6% in the untreated control to 92.5% and 87.5%, respectively. Similar effects were reported in previous studies (80.5%, Handelsman, Raffel, Mester, Wunderlich, & Grau, 1990; 92%, Wakelin et al., 2002; 25%, Sadeghi, Hessan, Askari, Aghighi, & Shahidi Bonjar, 2006). Our results showed that the application of OB21 or BA15 caused a considerable decrease in the diseases severity index (DSI) compared to untreated controls. Seed treatment decreased the DSI to values between 25% and 54% depending on the type of treatment. Comparable results were reported by Hamid, Bhat, Sofi, Bhat, and Asif (2013) who showed that *Trichoderma* and *Pseudomonas fluorescens* strains can decrease the DSI to 12% and 15%, respectively. It remains unknown whether the decrease in DSI is only caused by the production of antimicrobial compounds or by an additional effect on the accumulation of plant-derived defence compounds. Treatment with OB21 and BA15 resulted in enhanced growth of pea seedlings compared to unprotected controls. Treatment with the two selected actinobacteria clearly increased both the pea seedling weight and root length. Wakelin et al. (2002) reported a significant promotion of plant and root weight by inoculation of pea seed with spore-forming bacteria after infection with *A. euteiches*. Sadeghi et al. (2006) reported enhanced plant weight by using *Streptomyces* strains as biocontrol agents against damping-off and root rot caused by *Rhizoctonia solani*.

Other studies demonstrated the ability of *Streptomyces* species to be effective agents for biocontrol of soilborne pathogens such as *Rhizoctonia solani* (Chung, Huang, & Huang, 2005), *Sclerotium rolfsii* (Errakhi et al., 2009), *Pectobacterium carotovorum* and *P. atrosepticum* (Baz, Lahbabi, et al., 2012; Baz, Tran, et al., 2012). *Streptomyces* species were also reported to control diseases caused by oomycete pathogens (Bibi, Yasir, Song, Lee, & Chung, 2012; Rodríguez-Villarreal et al., 2014).

The taxonomical results proved that both selected actinobacteria isolates belong to the genus *Streptomyces*. Sequencing of 16S rDNA revealed highest sequence identity (99%) with *S. hydrogenans NBRC 13475*^{T} (Lindner, Junk, Nesmann, & Schmidt-Thomé, 1958). However, cultural, morphological and biochemical studies revealed several differences indicating that OB21 and BA15 represent new strains of *Streptomyces*.

The present study demonstrates that the two selected *Streptomyces* isolates isolated from Moroccan soils showed a good potential to protect pea seedling against the damping-off disease caused by *A. euteiches*. It remains to be tested how effective this protection is under field conditions.

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ORCID

Mustapha Barakate https://orcid.org/0000-0001-7949-5592

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