Identification, pathogenicity, fungicide sensitivity and biological control of *Rhizoctonia solani* associated with damping-off disease of sugar beet in Morocco

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Abstract

The fungus *Rhizoctonia solani* Kühn is an economically important pathogen involved in root rot and seedling damping-off disease of sugar beet (*Beta vulgaris* L.). In Morocco, this is the first detailed report of *R. solani* as the causative agent of sugar beet damping-off. The identification of the pathogen was based on combining the morphological characteristics and molecular characterization using the ribosomal DNA internal transcribed spacer (rDNA ITS) region. Antifungal activities of four commercial fungicides; azoxystrobin, difenoconazole, tebuconazole, and trifloxystrobin were assessed *in vitro*, displaying effective inhibition of the mycelial growth of the pathogen strains using PDA medium. A commercially available *Bacillus subtilis* Y1336 was evaluated as a potential biological control agent (BCA) against seedling damping-off caused by *R. solani.* The biological control activities of *B. subtilis* Y1336 was demonstrated by dual culture bioassays on PDA medium, *in vitro* seed inoculation with *R. solani* and *B. subtilis*, and by applying the BCA as seed coat under greenhouse conditions. This is the first time that the commercially available *B. subtilis* Y1336 has been proved as an effective BCA against the damping-off disease of sugar beet. This commercial antagonist should be incorporated into the integrated disease management startegy for sugar beet seedling mortality together with effective tested fungicides.

Introduction

Sugar beet (*Beta vulgaris* L.) is considered one of the most important sugar crops in many countries worldwide. The sugar production from this crop is the second in the world after that from sugar cane (Abd El Lateef et al. 2019). In Morocco, sugar beet is cultivated in about 57,171 hectares with a yield of 3.7 million tonnes during 2019 (FAOSTAT. 2021). The Beni Mellal-Khenifra region in central Morocco is recognized for its vast agricultural plain, with a part is reserved for industrial crops. In this region, sugar beet contributes, respectively, 20.5% and 21.2% to the national area and the national production of sugar beets in Morocco (Redani 2015). However, In many regions of Morocco, including the Beni Mellal-Khenifra region, the appearance of numerous soil-borne pathogens and outbreaks has led to economically significant losses of sugar beet yields (Snaiki et al. 2005; Chenaoui et al. 2017; Farhaoui et al. 2022). Among them, *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* [Frank] Donk) is one of the most destructive pathogens affecting sugar beet in Morocco (Rhmiza 1991; Chenaoui et al. 2017). This pathogen causes root rot and damping-off in sugar beet (Ruppel 1972; Abada 1994; El-Tarabily 2004). According to the hyphal compatibility reaction, anastomosis groups (AGs) are used to identify and classify *R. solani* isolates. Moreover, Some AGs can be classified into subgroups based on criteria like biochemical attributes and host range (Carling et al. 2002; Liu et al. 2021). *R. solani* AG 2–2 is a pathogen that causes both post-emergent damping-off and root rot of sugar beet. Whereas *R. solani* AG 4 is more often involved in damping-off (Kirk et al. 2008; Liu et al. 2019). Worldwide, *R. solani* is one of the most aggressive soil-borne pathogens that grow in soil, generating sclerotia and does not produce asexual spores. Sclerotia produced by this pathogen can persist in the soil for many years. Moreover, *R. solani* has a wide host range. Thereby, it can persist in the soil for long periods, making the control of the

disease using traditional means very difficult (Rush and Winter 1990; Anees et al. 2010; Gonzalez et al. 2011). The most important aspect of *R. solani* management in the European Union is the use of resistant cultivars (Buddemeyer & Märländer, 2005). The use of resistant cultivars is thus the most important aspect of *R. solani* management in the European Union (Buddemeyer and Märländer 2005). However, the quantitative resistance of the available cultivars does not result in complete disease management and is associated with a yield penalty in uninfected fields (Buddemeyer and Märländer 2005). Many fungicides have been used in the management of *R. solani*. Since 2000, Azoxystrobin was labeled for foliar and in-furrow application to manage *R. solani* in sugar beet (Kiewnick et al. 2001). Currently, azoxystrobin is considered the standard fungicide in the management of *R. solani* in sugar beet in several countries, including the United States (Liu and Khan 2016a). Several studies have shown the effectiveness of this fungicide in controlling sugar beet diseases caused by *R. solani* (Kiewnick et al. 2001; Stump et al. 2002). Other active ingredients such as prothioconazole and pyraclostrobin have shown their ability to reduce crown and root rots caused by *R. solani* in sugar beet, but the dose required to achieve protection similar to that achieved by Azoxystrobin is significantly higher (Liu and Khan 2016b). Besides, penthiopyrad can be used as a seed treatment in sugar beet to control seedling mortality caused by *R. solani* (Liu et al. 2021). No isolate resistant to azoxystrobin has been described in sugar beet. However, the emergence of azoxystrobin resistant isolates derived from other plants such as rice and potato (Olaya et al. 2012; Djébali et al. 2014), highlights the need for alternative fungicides for durable fungicidal management of *R. solani* in sugar beet. For this reason, an alternative approach is required that minimizes the risk of resistance development while providing a level of protection comparable to that obtained by azoxystrobin alone.

Overuse of the chemicals could cause negative impacts on human health and environmental problems. Biological control using beneficial microorganisms has also been considered an important component of plant disease management (Gardener and Fravel 2002; Mahmoud 2016a). In fields with severe infection with *R. solani, Bacillus subtilis* strain MSU-127 was able to reduce root rot index by 28.11% and increase significantly sucrose yield (Kiewnick et al. 2001). Numerous bacterial species such as *B. subtilis* and *Pseudomonas fluorescens* have demonstrated their ability to effectively control damping-off diseases (Kondoh et al. 2001; Pastor et al. 2010; Mahmoud 2016a; Al-Fadhal et al. 2019). Few trials have been conducted to evaluate the management of sugar beet damping-off induced by *R. solani* using bacterial antagonists (Nielsen et al. 1998; Moussa 2002; Jorjani et al. 2012; Afify et al. 2018). The commercially available *B. subtilis* strain Y1336 is a biological fungicide used to control various pathogens evolving on many crops (vegetable crops, fruit trees, and vines) while leaving no toxic residue. This bacteria was proved to be an efficient control agent for rose powdery mildew and to improve the yield and quality of rose-cut flowers (Wang et al. 2018a). In addition, this biological agent can act as antagonistic bacteria for suppression of balloon flower stem diseases caused by *R. solani* (Lee et al. 2012). There is a lack of research on the effectiveness of adding this beneficial bacterium to the soil to protect sugar beet against damping-off caused by *R. solani*.

The objective of this study was to evaluate *in vitro* and *planta* antifungal activity of the commercially available *B.* subtilis strain Y1336 and the antifungal effects of four commercial fungicides against

damping-off diseases caused by *R. solani.*

Materials And Methods

Isolation and morphological identification of *R. solani*

Throughout 2020 growing seasons, sugar beet seedlings showing symptoms of post-emergence damping-off with brown to black lesions on the hypocotyl and the roots (Fig 1), were collected from two Moroccan towns : Souk Essebt Oulad Nemma (32°15'35.9°N, 6°48'54.4°W) of Fquih Ben Salah Province and Taghzirt (32°26'04.6"N 6°12'04.6"W) of Beni Mellal province, in which, the damping-off of sugar beet results in a significant reduction in yield each growing season. The causative pathogen *R. solani* was isolated from infected roots using Potato Dextrose Agar medium (PDA). Briefly, infected hypocotyls were cut into 5 mm pieces which were then surface sterilized by immersion in 0.5 % sodium hypochlorite solution for 3 minutes, rinsed three times with sterile distilled water (SDW), and left dried under laminar flow. Afterward, disinfected fragments were deposited on PDA medium amended with 50 mg/l of streptomycin and incubated for 4 days in darkness at 25°C. Colonies showing characteristics of *R. solani* were then purified by selecting a single hyphal tip. Two isolates of this pathogen were obtained. Each isolate represents a region. Both isolates were maintained by periodic subculturing and stored long-term on sorghum seeds. Morphological characteristics of the pathogen (colony color and shape of mycelium and sclerotia) were examined. Measurements of sclerotia were made using 20 days old cultures.

DNA extraction, PCR, and sequencing

Both *R. solani* isolates were grown in 8 cm diameter Petri dishes containing PDA and incubated for 3 days at 25 °C in the dark. Afterward, fungal DNA extraction was performed following the protocol previously described by Doyle (1990). The amplification of the Internal Transcribed Spacer (ITS) region of the extracted DNA was done using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3 ') (White et al. 1990). Amplified PCR products were visualized by agarose gel electrophoresis 1.5 % using tris borate EDTA buffer (TBE) (0.5×). The gels were then stained using ethidium bromide and visualized by a UV transilluminator to evaluate the presence of amplicons and their size. Sequencing was carried out at the STAB Vida Lda laboratory in Portugal. The primers used in the sequencing were the same as those used in the amplification reaction. Sequences obtained were edited and aligned using the DNAMAN sequence analysis software (version 6.0, Lynnon Biosoft, Canada). Afterward, sequences obtained were verified using Blast search to find homologous sequences in GenBank databases (National Center for Biotechnology Information) and then deposited in GenBank. A phylogenetic tree was generated from obtained sequences. The two isolates were rearranged to create major clusters using MEGA 5.0 software. The *Sclerotium rolfsii* (AY684917) sequence was chosen as an out-group. Phylogenetic relationships were estimated using the maximum likelihood (ML) method. Support for each branch in the inferred tree was assessed operating 1000 bootstrap replications.

Effect of temperature on mycelial growth of *R. solani* isolates

The effect of temperature on the mycelial growth of *R. solani* isolates was evaluated on a PDA medium. From actively growing 3-day-old cultures of each isolate, mycelial discs 5 mm in diameter were cut out and placed in the center of Petri dishes, 9 cm in diameter, containing the PDA medium, then the dishes were incubated at different temperatures (5 °C, 10 °C, 20°C, 25 °C, 30 °C, 35°C, and 40 °C). Each treatment was repeated 3 times. Colony size was measured every day. **Colony diameters** were **measured every** 24 h for 20 days, then radial growth of colonies, corresponding to each temperature, was calculated.

In vitro antifungal activities of chemical fungicides

Four fungicides were evaluated in the *in vitro* tests (Table 1): trifloxystrobin (Flint), azoxystrobin (Ortiva 25 SC), tebuconazole (Supreme Super), and difenoconazole (Score 250 EC). Their effect on the radial growth rate of both isolates of *R. solani* was tested using PDA media amended with a range of seven concentrations (0, 0.1, 1, 10, 50, 100, and 500 µg/ml). To evaluate radial mycelial growth, a 5 mm mycelial disc cut from the growing edge of 3 days old PDA cultures was placed upside down in the center of the Petri plate. Dishes were incubated at 25 ° C in the dark. Colony diameter was measured 72 h after inoculation. Each treatment was repeated 3 times, and the experiment was carried out twice. The rate of mycelial growth reduction for each isolate and concentration was determined according to the following formula:

$$\text{Reduction rate } (\%) = \frac{R-r}{R}\ x\ 100$$

Where R is the radial growth of fungi in amended media and r is the radial growth of fungi in non-amended media.

For each isolate, the EC50 (effective concentration that reduced mycelial growth by 50%) was calculated by regressing the percent of mycelial growth inhibition against the log10 transformation of the seven concentrations of fungicide with the XLSTAT PRO software (Addinsoft, www.xlstat.com).

In vitro study of the antagonistic activity of the commercially available *Bacillus subtilis* strain Y1336

Dual culture assay

The capacity of inhibiting the growth of *R. solani* by the commercially available *Bacillus subtilis* strain Y1336 was evaluated under *in vivo* conditions. A dual culture bioassay was performed using a PDA medium by confrontation with 5 replications. The antagonist bacteria were streaked in four equidistant streaks along the border of the Petri plate. Then, from the edge of a 3-day-old-colony of *R. solani*, a mycelial plug of 5 mm in diameter was removed and placed in the center of the Petri plate containing PDA medium between the 4 streaks of the bacterial strain. Culture media containing only the pathogen plug were used as a negative control. All plates were parafilm-sealed and incubated in the dark at 25 °C. Each treatment was repeated 3 times, and the test was performed twice over time. After 3 days of

incubation, the inhibition rate of mycelial growth (IR) in each Petri plate was calculated using the following formula (Lahlali et al. 2020):

$$IR(\%) = \frac{C-T}{C} \times 100$$

Where : IR is the inhibition rate; C: is the diameter of the pathogen colony in the control plates; T: Diameter of the pathogen colony in dual culture bioassays.

In vitro Inoculation of Seeds Using R. solani and B. subtilis Y1336

Preparation of bacterial suspension

Bacterial isolate was cultured on Luria–Bertani (LB) medium for 3 days at 25°C, then several loopfulls were transferred to test tubes containing sterile physiological water (NaCl, 9 g/l). Bacterial cell density was determined using a spectrophotometer (OD = 600nm) and then, adjusted to 1×10^9 CFU/ml, in sterile carboxymethyl cellulose (CMC) 1% solution (Karimi et al. 2016). The bacterial suspension can be used immediately or stored at 4°C for a short time.

In vitro biological control of damping-off

B. subtilis Y1336 was tested for its ability in reducing the sugar beet seedling damping-off caused by *R. solani* under laboratory conditions using the procedure described by Haque *et al.* (2021) with slight modifications. Seeds of sugar beet (cv**.** Barrosa) were initially surface-sterilized with 70% ethanol for 1 min and washed 3 times with SDW. Then, Seeds were placed on sterile filter paper and allowed to dry in a laminar flow hood for 2 h. The seeds were soaked in 50 ml of the bacterial suspension (CMC 1%), shaken at 150 rpm for three hours. Then, they are removed from the bacterial suspension, dried under a laminar flow hood for 4 hours (Karimi *et al.*, 2016). Non-bacterized seeds served as a negative control, while seeds treated with a fungicide (Difenoconazol at5 µg g/ml) were served as a positive control. The test was performed using 50% PDA plates. Four seeds were planted employing sterile forceps at 1 cm apart on each culture dish. The mycelial plug of *R. solani* was deposited 2 cm from each seed. Seven treatments were involved : (1) Non-inoculated seeds alone; (2) Mycelial plugs of *R. solani* Rs1 and non bacterized seeds; (3) Mycelial plugs of *R. solani* RsM and non-bacterized seeds; (4) Bacterized seeds alone; (5) Mycelial plugs of *R. solani* (Rs1) and Bacterized seeds ; (6) Mycelial plugs of *R. solani* (RsM) and Bacterized seeds, and (7) Mycelial plugs of *R. solani* Rs1 and seeds non- bacterized but treated with fungicide. Dishes were sealed with parafilm, The number of germinated seeds was recorded after 7 days of incubation at 25 °C. This trial was carried out twice over time with 4 repetitions for each treatment.

In vitro evaluation of the antibacterial activity of fungicides against B. subtilis Y1336

To evaluate the effect of the 4 fungicides on *B. subtilis* Y1336 growth, different doses of the tested pesticides (azoxystrobin, difenoconazol, tebuconazol, and trifloxystrobin) were added to LB media. 0.2 ml

of a bacterial suspension of the biocontrol agent (BCA) (1×10^4 CFU/ml) was spread onto the culture media. The number of bacterial colonies was counted after incubation at 27 °C for 48 hours in darkness. By counting the number of colonies, bacterial growth on LB amended with fungicides was compared to media without any pesticides. Each treatment contained 3 replications, and the trial has been conducted twice.

In planta experiments

Preparation of R. solani inoculum and pathogenicity test

Pathogenicity of the two *R. solani* isolates was evaluated according to the technique of Grisham and Anderson (Grisham and Anderson 1983). A quantity of 200 g of corn seeds was soaked in distilled water for 12 hours, autoclaved twice at 121 °C for 20 minutes in two successive days. The corn seeds were inoculated with ten plugs of 5 mm diameter of a 3-day old culture of *R. solani* grown on a PDA medium. The substrate was incubated at 25 °C for 6 weeks in darkness with daily shaking to ensure uniformity of fungus growth. Colonized corn seeds obtained were used as inoculum of the pathogen. Corn seeds not inoculated and prepared in the same way were served as control.

A soil mixture consisting of peat moss, sand, and soil (1: 1: 1) was autoclaved for one hour at 121 °C on two consecutive days. The soil was infected with the pathogen by adding 8 g of infested corn seeds for each kg of soil. Obtained soil was put into plastic pots of 20 cm in diameter at a rate of 3 kg of soil per pot. After 10 days of soil infection, twenty surfaces disinfected sugar beet seeds (cv**.** Barrosa) were planted in each pot. The pots were then placed in a greenhouse maintained at 25°C. Each treatment contained five replications in a randomized complete block design. The entire experiment has been conducted twice. The effectiveness of the BCA on pre-and post-emergence damping-off diseases of sugar beet seedlings was assessed by counting the number of seedlings that survived after 15 days (pre-emergence diseases) and 45 days (post-emergence disease).

Biological control activity of B subtilis Y1336 against R. solani

The Biological control potential of *B. subtilis* Y1336 against the highly aggressive isolates of *R. solani* (RsM) was examined under greenhouse conditions. The soil used for this test was inoculated with the pathogen as mentioned above. Seeds treated with fungicide difenoconazole (400 g/100 kg seed) were served as a positive control. After 7 days of soil inoculation, twenty sugar beet seeds (cv. Barrosa) were sown in each pot. In total there were 5 different treatments : (1) Non-bacterized seeds + soil not inoculated with *R. solani* ; (2) Non-bacterized seeds + soil inoculated (negative control) ; (3) Seeds treated with fungicide + inoculated soil (positive control) ; (4) Seeds bacterized with *B. subtilis* Y1336 + uninoculated soil ; (5) Seeds bacterized + inoculated soil. Pots were arranged in a complete randomized design with 5 replications, and the experiment was conducted twice over time. The number of healthy seedlings was recorded after 14 and 45 days after sowing. Then, the percentage of pre and post-emergence damping-off was calculated.

Statistical Analysis

Statistical analysis was executed with SPSS statistical software (version 20, IBM SPSS Statistics 20). Data were subjected to analysis of variance (ANOVA), and the least significant difference (LSD) at 5% level was used to compare mean values between treatments.

Results

Morphological characteristic of *R. solani*

Both isolates showed rapid mycelial growth so that the mycelium reached the limits of the Petri dish (90 mm) after only 3 days of incubation at 25°C in the dark. Fungal colonies had typical morphological characteristics of *R. solani*. On PDA medium, the young fungal colonies have a white and cream color, with many floccose aerial hyphae (Fig 1B). Furthermore, dark brown colonies with dark brown sclerotia were formed after 15 days of incubation. The majority of macrosclerotia (1,1 – 2 mm) are blackish-brown and appear as a very small sphere with a diameter ≤ 1.1 mm. While microsclerotia emerge as a thin crust on the culture medium surface. Microscopic examination showed that the mycelium of the pathogen exhibits right-angled hyphal branching with a constriction and a septum near the ramification, and is composed of multinucleate hyphae (4 to 9 nuclei per cell), 7.1-8.0 µm wide (mean 7.5 µm) (Fig 1).

Molecular identification of *R. solani*

Both *R. solani* isolates were confirmed by molecular sequencing of the ITS rDNA region, *R. solani* MZ854089 (Rs1) and *R. solani* OM955658 (RsM)**.** Based on the sequence analysis, a phylogenetic tree was obtained by the neighbor-joining method. Results clearly showed that both isolates were closely related to *R. solani* strains (Fig. 2). Collectively, soil-borne pathogens Rs1 and RsM should be identified as members of *R. solani*.

Effect of temperature on growth rates of *R. solani*

The effect of temperature on the mycelia growth of two strains of *R. solani* is **illustrated** in **figures 3 and 4.** In the darkness, **b**oth *R. solani* isolates showed maximum growth at 25°C on PDA medium. At this temperature, both strains of *R. solani* tested showed similar growth. The RsM strain also recorded its optimal growth at 30°C. A large difference in growth between the two strains of *R. solani* was recorded at 35°C, at this temperature the RsM isolate showed a radial growth equal to 5.79 mm/day. In general, both *R. solani* isolates showed better growth at 20°C compared to 35°C. The two tested strains of *R. solani* showed no growth at 5°C after 20 days of *in vitro* culture. Only the RsM strain showed mycelial growth at 40°C with a low rate (0.08 mm/day). This strain also showed a mycelial growth higher than that of Rs1 in the temperatures 10 °C, 15 °C, and 20 °C. To check if the temperatures of 5°C and 40°C were lethal or not for the two tested strains, their culture plates were transferred to 25°C. After 3 days of incubation in darkness, the two isolates showed mycelial regrowth.

Effect of fungicides on *R. solani* mycelia growth

Four fungicides: azoxystrobin, difenoconazole, tebuconazole, and trifloxystrobin were used to examine their antifungal activities against the two strains of *R. solani* isolated from two different regions of Morocco. The results are presented in figure 5. All fungicides revealed significant antifungal activities against both strains of *R. solani* tested in a dose-dependent manner, shown by reduced radial growth of the pathogen. The fungicides difenoconazol and tebuconazol showed total inhibition of mycelial growth of the strains tested at 500 µg/ml. whereas azoxystrobin completely inhibited the mycelial growth of the RsM strain from 50 µg/ml, but the Rs1 strain continues to grow even at 500 µg/ml of this fungicide. In this study, trifloxystrobin appeared to be the least effective fungicide in inhibiting the mycelial growth of both tested isolates. The Inhibition rate of mycelial growth does not exceed 78%, even at 500 µg/ml of trifloxystrobin. In parallel, this fungicide recorded the highest EC_{50} values, 1.96 µg/ml and 4.08 µg/ml, for Rs1 and RsM, respectively (Table 2), while difenoconazole showed the lowest value of EC_{50} (0.07 µg/ml for RsM).

In vitro evaluation of the antagonistic activity of *B. subtilis* strain Y1336

In vitro antagonistic activity and effect of the antagonist bacteria on the mycelial structure of R. solani

The commercially available *B. subtilis* Y1336 was tested for its antagonistic effect against *R. solani* employing dual culture trial. Inhibition rates were evaluated after 3 days of incubation. Obtained results emphasized that the evaluated strain exhibited an important antifungal effect against the two tested strains of *R. solani*. Mycelial growth of both pathogens strains Rs1 and RsM were inhibited, with inhibition rates of 74.43 and 73.27%, respectively. Microscopic observations of the zone of inhibition showed several forms of cytological alteration and damaged morphology occurred in *R. solani* hyphal compared to the untreated control (Fig. 6A). Generally**,** the alterations correspond to loss of structural integrity of the hypha and many varieties of deformations as swelling of parts of the hypha and thinning of others. Sometimes hyphal alteration was associated with the formation of conglobated clumps along the mycelia and vacuolation of the head's structure as a result of interaction with the antagonist. In addition, perforations and fragmentations of the hyphae were shown.

In vitro seed inoculation with B. subtilis

To evaluate the ability of *B. subtilis* Y1336 in controlling damping-off caused by *R. solaniin in vitro*. Co-cultivation of sugar beet (cv**.** Barrosa) seeds treated with the antagonist was performed in 50% PDA inoculated with the pathogen. After 7 days of incubation, the results showed that the two strains of *R. solani* resulted in total damping-off in the absence of the antagonist (Fig. 8). While, seedling emergence of 75.43 and 73.27% was observed in the combined treatment of seeds treated with *B. subtilis* Y1336 and the pathogen Rs1 and RsM, respectively (Fig. 9). No incidence of damping-off was shown in the controls not inoculated with the pathogen, and also, in the plates containing seeds treated only with the antagonist In the confrontation plates. *B. subtilis* limits the pathogen proliferation and causes a change in the color of the fungal mycelium in the zone of inhibition.

Impact of fungicides on the growth of B. subtilis Y1336.

To investigate the possibilities of biological damping-off control in sugar beet in combination with chemical fungicides, we examined the viability of the commercially available *B. subtilis* Y1336 in response to four active components having antifungal activities against the pathogen *R. solani*.. Among the 4 tested fungicides, difenoconazole and tebuconazole did not reduce the growth of the bacterial antagonist in the range of 0.1-500 µg / ml assayed (Fig. 4). However, 500 µg / ml of trifloxystrobin significantly decreased bacterial growth *in vitro*. Whereas, azoxystrobin significantly reduced the colonies of the antagonist bacteria from the concentration 100 µg / ml.

Pathogenicity test of *R. solani* and *in planta* antagonism of *B. subtilis* Y1336 against *R. solani*

Both strains of *R. solani* isolated in this study from infected sugar beet seedlings were found to be pathogenic and could induce pre-and post-emergence damping-off diseases of sugar beet seedlings (Fig. 11). Pots inoculated with *R. solani* show that the pathogen inhibits germination and causes lesions in the roots and hypocotyls whereas controls remained healthy. 'Koch's postulate was then verified by pathogen re-isolation and morphological identification. Based on the *in vitro* trials, commercially available *B. subtilis* Y1336 was subjected to greenhouse bioassay to verify its antifungal activity and its ability in minimizing the sugar beet damping-off disease caused by *R. solani*. In agreement with the *in vitro* results and as illustrated in table 3, the BCA *B. subtilis* Y1336 reduced the incidence of pre and post-inoculation damping-off incidence and significantly increased the number of emerged seedlings (compared to the inoculated control) under greenhouse conditions. The treatment of the seeds with *B. subtilis* Y1336 significantly controlled damping-off disease, caused by *R. solani*Rs1, by 58.75%. In soil not inoculated by *R. solani*, seed treatment with *B. subtilis* Y1336 did not show pre-or post-emergence damping-off diseases of sugar beet seedlings.

Discussion

Damping-off is primarily recognized as one of the serious threats in sugar beet cultivation in Morocco. To the best of our knowledge, this is the first detailed report of *R. solani* that causes damping-off of sugar beet in Morocco. Trials were performed in the laboratory and under greenhouse conditions to assess the ability of a commercially available *B. subtilis* Y1336 and 4 fungicides to manage seedling mortality in sugar beet. As a warmth-dependent fungus, *R. solani* is widely spread in the soil of Morocco. *R. solanI* isolated in this study from infected sugar beet seedlings were found to be pathogenic and could cause pre and post-emergence damping-off in sugar beet. These results are in accord with previous ones reported that *R. solani* is a highly pathogenic fungus causing damping-off in many crops including sugar beet (Buttner et al. 2004; Yangui et al. 2008; Mahmoud 2016b; Inokuti et al. 2019; Avan et al. 2021). This pathogen can cause seedling pre-and post-emergence mortality as well as root and crown rot in sugar beet (Liu et al. 2019). The two strains tested in this study cause damping-off and root rot in sugar beet (data not shown). So, the AG attributed to these two isolates could be *R. solani* AG 2–2. The mycelial growth of the two studied *R. solani* isolates was strongly influenced by temperature. From 10 to 25°C, the growth rate increased with increasing temperature up to 25°C for both isolates. Mycelial growth on PDA of RsM was faster than Rs1 over the range 10 to 20°C. Maximal growth of Rs1 on PDA was at 25°C and

30°C, while optimal growth of RsM was recorded at 25°C. The temperature effect observed in this study is in agreement with results reported by (Harikrishnan and Yang 2004). Whereas Kumar et al. (1999) showed that the most favorable temperature for the studied *R. solani* isolates was 25°C, and growth decreased significantly at 30°C.

The application of fungicides is the most used strategy to control *R. solani* on sugar beet. In this study, four fungicides were assessed for their effectiveness against two strains of *R. solani* obtained from two different sugar beet-growing regions in Morocco. According to the classification adopted by Martin et al. (1984), *In vitro* sensitivity of the pathogen to fungicides exhibited that the two isolates were extremely sensitive to azoxystrobin, difenoconazole, and tebuconazole, but they were moderately sensitive to trifloxystrobin. The maximum azoxystrobin concentration used (500 µg/ml) did not completely inhibit the mycelial growth of Rs1. Arabiat & Khan, (2016) noted, after 13 years of field treatment with fungicides, a dramatic increase in mean EC50 values for azoxystrobin and trifloxystrobin in tested *R. solani* strains that become tolerant to these two fungicides. In addition, forms of resistance to fungicides have been observed in crop fields due to the frequent application of azoxystrobin, which has led to a decrease in the sensitivity of other pathogens that are *Alternaria solani*, *Podosphaera fusca*, and *Pseudoperonospora cubensis* (Ishii et al. 2001; Rosenzweig et al. 2008). However, azoxystrobin has been the most widely used active ingredient to control *R. solani* on sugar beet since 1997, which is the year of its registration in the USA (Khan et al. 2017). Difenoconazole and tebuconazole were effective against both strains of *R. solani* tested *in vitro* trials. This is in accord with other studies that showed the efficacy of these two fungicides against *R. solani* (Shan-lin 2013; Datta et al. 2016). In addition, our results show that the cultivation of sugar beet seeds in PDA medium containing difenoconazole caused no damage to seedlings. *In planta*, damping-off protection by difenoconazole against *R. solani* was around 80%. Moreover, soil treatment with this fungicide did not lead to any damage to sugar beet seedlings in our experiments.

In Morocco, difenoconazole fungicide has been allowed to control Sigatoka diseases in sugar beet. Although, tebuconazole and difenoconazole have not been allowed to control damping-off diseases in sugar beet. Fungicide mixtures with two different modes of action can be further explored for the apparition of damping-off in sugar beet caused by *R. solani*. Indeed, for a long time, the application of fungicides with different modes of action has been generally accepted to improve diseases protections synergistically, as well as to minimize the emergence of fungicide resistant strains in the fields (Gisi 1996). Azoxystrobin and difenoconazole synergism was well confirmed during the management of *R. solani* damping-off and root rot in sugar beet (Bartholomäus et al. 2017).

Sugar beet producers generally use seeds coated with chemical substances to avoid the damping-off phase. Nevertheless, biological control recently has become a famous alternative to conventional synthetic chemical pesticides that are harmful to the environment and human health. Several species of the genus *Bacillus* have shown their ability to biocontrol, have been exploited as biopesticides (Fravel 2005), and have been widely reported as agents with the ability to protect many cultures against fungal infections (Choudhary and Johri 2009; Rong et al. 2020). The commercially available *B. subtilis* strain Y1336 showed great potential to control rose powdery mildew (Wang et al. 2018a), and to significantly

reduce the incidence of fire blight on apple and pear blossoms caused by *Erwinia amylovora* (Bahadou et al. 2017). *B. subtilis* Y1336, also, reduces significantly the incidence of Balloon flower stem rots caused by *R. solani* and *Sclerotium rolfsii* (Lee et al. 2012).

For the first time, the current study examined the effectiveness of *B. subtilis* Y1336 in biocontrol of the fungal pathogen known to cause damping-off. Their effect against *R. solani* was studied in dual culture, and tested by *in vitro* seed inoculation with *R. solani*, and by applying the BCA as seed coat under greenhouse conditions. *In vitro* test showed that the tested antagonistic BCA was able to reduce the growth of *R. solani*. Microscopic observation revealed altered mycelia morphology due to biological control activities. Additionally, *in vitro* inoculation of sugar beet seeds using *R. solani* and *B. subtilis* Y1336 showed that this combined treatment significantly increases the number of germinated seeds by 68.75%, compared to levels with co-cultivation of sugar beet seed and inocula of the pathogen alone. Under greenhouse conditions, coating the seeds with the BCA, significantly increased seed emergence and survival rates in addition to a significant reduction in damping-off disease severity caused by *R. solani*. *In vitro* and *in vivo* seed treatment with *B. subtilis* Y1336 did not show pre-or post-emergence damping-off diseases. Thus, this bacterium has no pathogenicity for sugar beet seedlings. As far as it is known, this is the first time that the commercially available *B. subtilis* Y1336 has been identified as a damping-off BCA. Pathogen management both on seeds and in the immediate environment of emerging seedlings with *B. subtilis* Y1336 as a seed coating or in-furrow application during seed sowing remains to be confirmed by field studies. There are several investigations on the application of *B. subtilis* to manage *R. solani* in several crops (Szczech and Shoda 2006; Ma et al. 2015; Mahmoud 2016b; Selim et al. 2017; El-Desouky et al. 2018). Various mechanisms have been suggested to elucidate the suppression of plant pathogenic fungi by *B. subtilis*, especially competition for space (Yu et al. 2011; Cao et al. 2012; Li et al. 2013), competition for nutrients (Moita et al. 2005; Li et al. 2013; Wang et al. 2018b), antibiosis (Landy et al. 1948; Stein 2005; Wang et al. 2018b), siderophore production (Yu et al. 2011; Singh et al. 2017; Khan et al. 2020) and induction of systemic resistance (Ongena et al. 2005, 2007; Wang et al. 2018b), or applied other biocontrol mechanisms.

Integrating fungicides with microbial antagonists can achieve protection against plant pathogenic by diminishing fungicide application. The combined application of *Trichoderma asperellum* SC012 and hymexazol was used to control *Fusarium oxysporum* causing cowpea wilt disease. Moreover, this combination could reduce the fungicide doses (Zhang et al. 2021). Fungicides with effective antifungal activity against phytopathogenic fungi must have no negative effect on the BCA growth. The BCA *B. subtilis* RB14-C used against *R. solani* was resistant to flutolanil, and the use of this bacterial antagonist reduced the quantity of flutolanil for effective management of tomato seedling mortality caused by *R. solani* (Kondoh et al. 2001). The control of *Sclerotium sclerotium* infecting witloof chicory was conducted by *Coniothyrium minitans* and also by a mixture of cyprodinil and fludioxonil fungicides. However, the application of this chemical mixture with *C. minitans* could not be suggested because 1 mg/ml of the mixture completely inhibited the mycelial growth of the antagonist (Benigni and Bompeix 2010). Our study showed that the commercial antagonist *B. subtilis* Y1336 was resistant to the 4 tested fungicides.

These results suggest that the antagonist *B. subtilis* Y1336 can be integrated with a mixture of the 4 chemical fungicides to control *R. solani* diseases.

It may be that the conditions under which the tests were carried out in the greenhouse were close to the optimal growth of *B. subtilis* Y1336. The effectiveness of this BCA needs to be verified and validated under field-like conditions such as cool temperatures at the start of crop establishment. Furthermore, determining optimal concentration of *B. subtilis* Y1336, any synergistic effects with other antagonists, and potential biocontrol effects of this bacterium against other sugar beet damping-off agents need to be studied to optimize the potential exploitation of this commercially available antagonist as a BCA of damping-off in sugar beet. In the field, the dynamics of *R. solani* epidemics on sugar beet is characterized by a polycyclic epidemic (Otten et al. 2003). There are two sources of inoculum that ensure *R. solani* epidemics: the primary resident or incoming inoculum and the secondary inoculum generated by infected seedlings and roots (Gilligan and Kleczkowski 1997). Overwintering mycelia or sclerotia of the pathogen serves as the primary inoculum for seedling mortality. Thus, measures that minimize sources of infection or suppress the spread of this pathogen can reduce damage and loss caused by *R. solani*. Generally, this should be conducted by removing primary and secondary inoculum. Therefore, it will be interesting to know if an application of seeds treated with *B. subtilis* alone, fungicides alone, or their mixture affects both the primary and secondary inocula of *R. solani* (Gilligan and Kleczkowski 1997). Moreover, Future studies should focus on investigating the effect of *B. subtilis* Y1336 on the generation, survival, and germination of *Rhizoctonia* sclerotia. Finally, we hope that further studies will lead us to examine the potential role of *B. subtilis* strain Y1336 in promoting plant growth as well as its biocontrol effect against crown and root rot caused by *R. solani*.

Declarations

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Tables

Table 1: Some characteristics of used fungicides

Active ingredient (a.i.)	Commercial name* (manufacture)	Chemical family	FRAC group	Fungicide group
Azoxystrobin	Ortiva 25 SC (Syngenta)	Strobilurin	11	QoI-fungicides (Quinone aoutside Inhibitors)
Difenoconazol	Score 250 EC (Syngenta)	Triazol	3	DMI-fungicides (DeMethylation Inhibitors)
Trifloxystrobin	Flint 50 WG (Bayer)	Strobilurin	11	QoI-fungicides (Quinone outside Inhibitors)
Tebuconazol	Supreme Super 200 EC (Upl limited)	Triazol	3	DMI-fungicides (DeMethylation Inhibitors)
Bacillus subtilis Y1336	WG	Biofungicide (*Bacillus spp.*)	44	-

** WG : Water* dispersible granule, SC = suspension concentrate. EC = Emulsifiable concentrate

Table 2: Mean effective fungicide concentration that inhibited mycelial radial growth by 50% (EC50) values of azoxystrobin, difenoconazol, tebuconazol, and trifloxystrobin for two strains of *R. solani* isolated from sugar beet fields in the Béni Mellal-Khénifra regions.

Fungicide	EC50 (µg/ml)	
	Rs1	RsM
Azoxystrobin	0.32	0.08
Difenoconazol	0.91	0.07
Tebuconazol	0.32	0.20
Trifloxystrobin	1.96	4.08

Table 3. Effect of *B. subtilis* Y1336 on controlling sugar beet seedling damping-off disease caused by the pathogen *R. solani* (Rs1) under greenhouse conditions.

Treatments	Number of healthy seedlings 15 DAS	Pre-emergence disease (%)	Number of healthy seedlings 60 DAS	Post-emergence disease (%)
Untreated seed without *R. solani*	*20 ± 0 a	0	20 ± 0 a	0
Untreated seeds + *R. solani*	4 ± 1.15 d	79.74	0.75 ± 0.95 d	81.25
Seeds treated with Difenoconazol + *R. solani*	17.25 ± 0.95 b	12.65	16 ± 0.8 b	7.24
Seeds treated with *B. subtilis* Y1336	20 ± 0 a	0	20 ± 0 a	0
Seeds treated with *B. subtilis* Y1336 + *R. solani* (Rs1)	14.25 ± 0.95b c	27.84	11.75 ± 2.06 c	17.54

*each value represents the average of two trials over time with four repetitions. Means within the same column followed by different letters are significantly different ($p \leq 0.05$) based on the least significant difference (LSD) test.

Figures

Figure 1

Infected Sugar beet seedling displaying symptoms of post-emergence damping off and morphological characters of *Rhizoctonia solani*.: A: Sugar beet seedling showing brown to black lesions on the hypocotyl (symptoms of damping-off disease caused by *R. solani)*; B: Growth of *R. solani* on PDA medium for 3 days at 25 °C; B: Microscopic observation (×40) of mycelium of *R. solani* with septate hyphae and a slight constriction at the base of the ramification.(scale bars = 10µm).

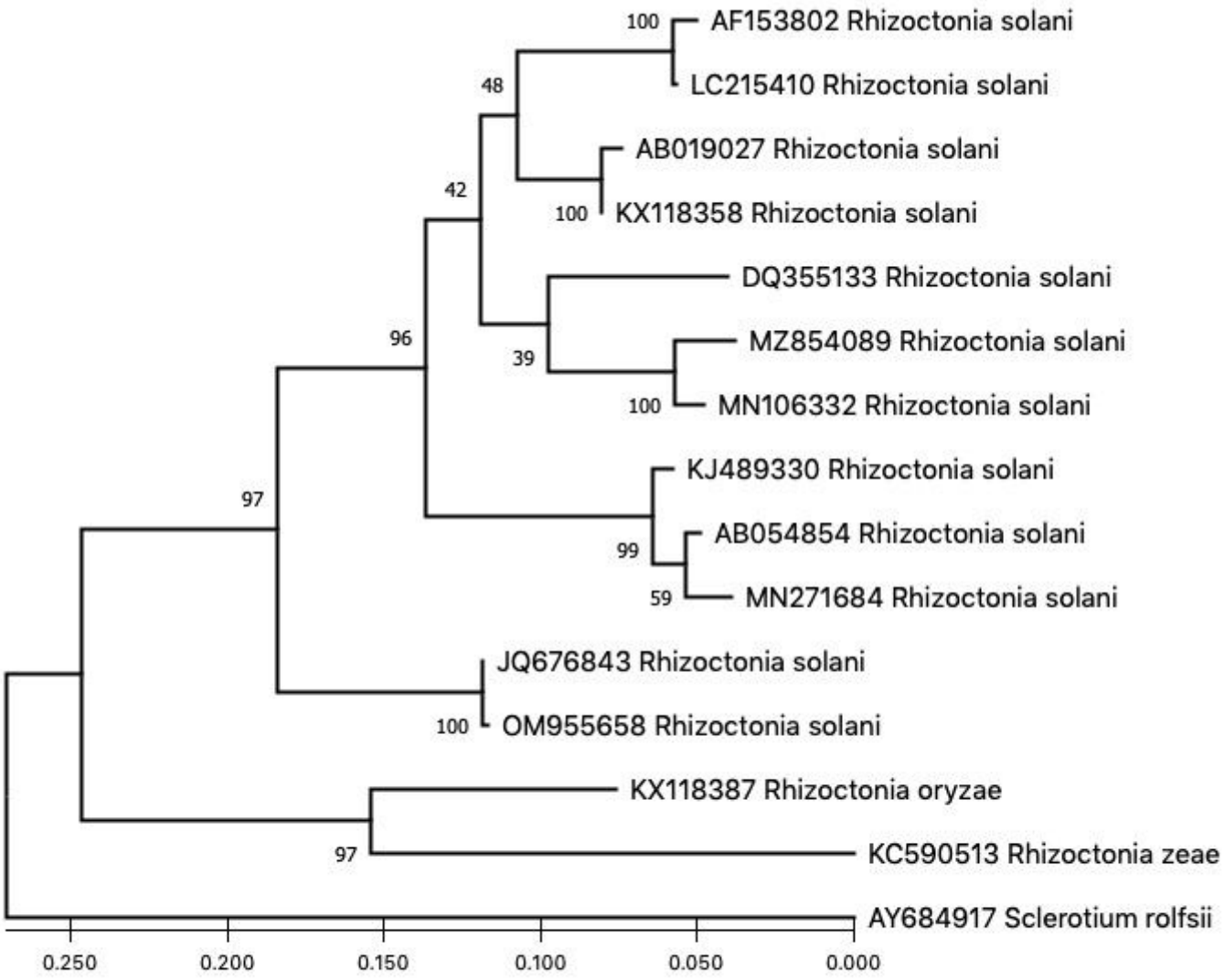

Figure 2

Phylogenetic tree based on ITS regions of ribosomal DNA sequence of sugar beet damping off. R. solani strains identified in this study (MZ854089 and OM955658) and *R. solani* reference strains using a Kimura 2 parameter model in MEGA X software. The phylogenetic tree was assessed via 1000 bootstrap replications and was rooted using Sclerotium rolfsii (AY684917).

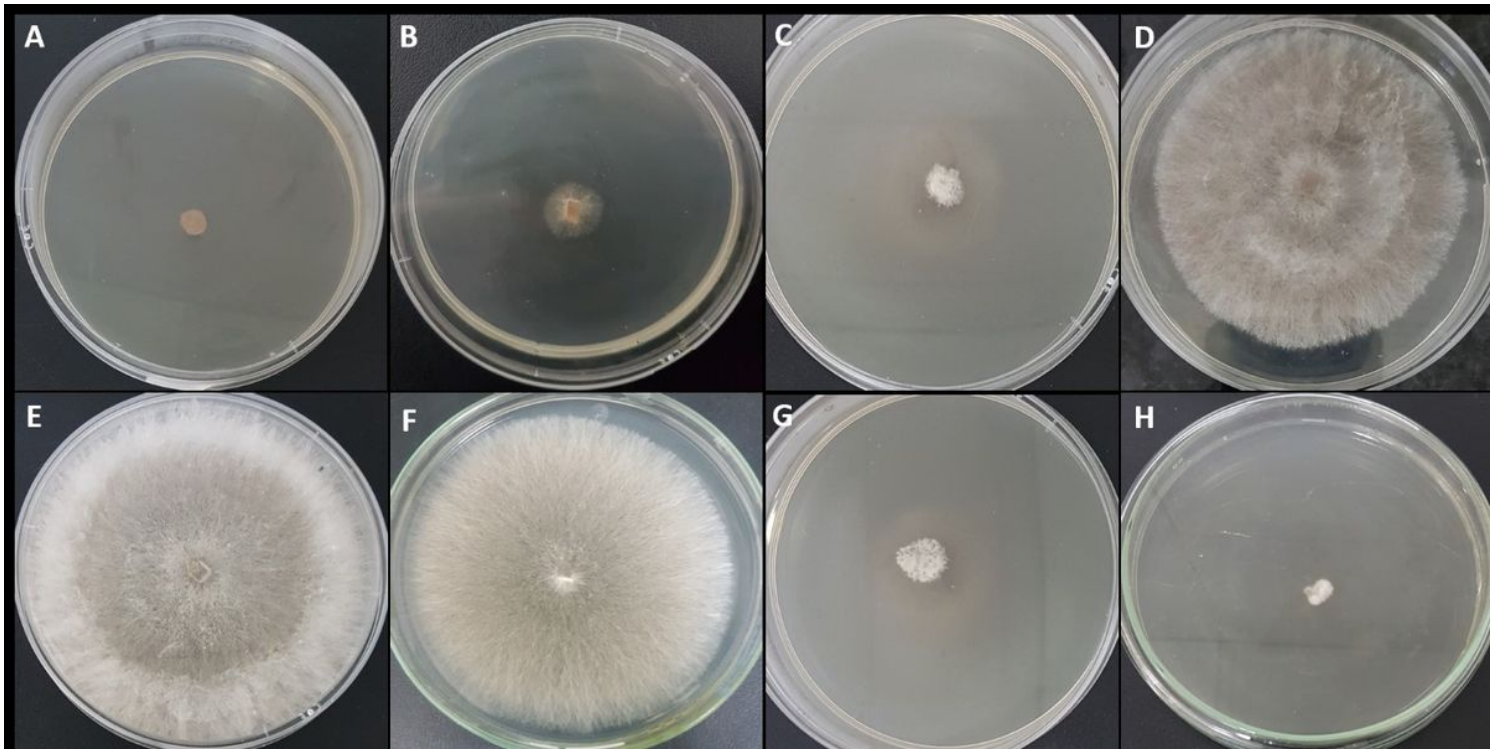

Figure 3

Influence of temperature on the growth of *R. solani* (RsM) after 72 hours of incubation at 5 (A), 10 (B), 15 (C), 20 (D),25 (E), 30 (F), 35 (G), and 40 ◦ C (H).

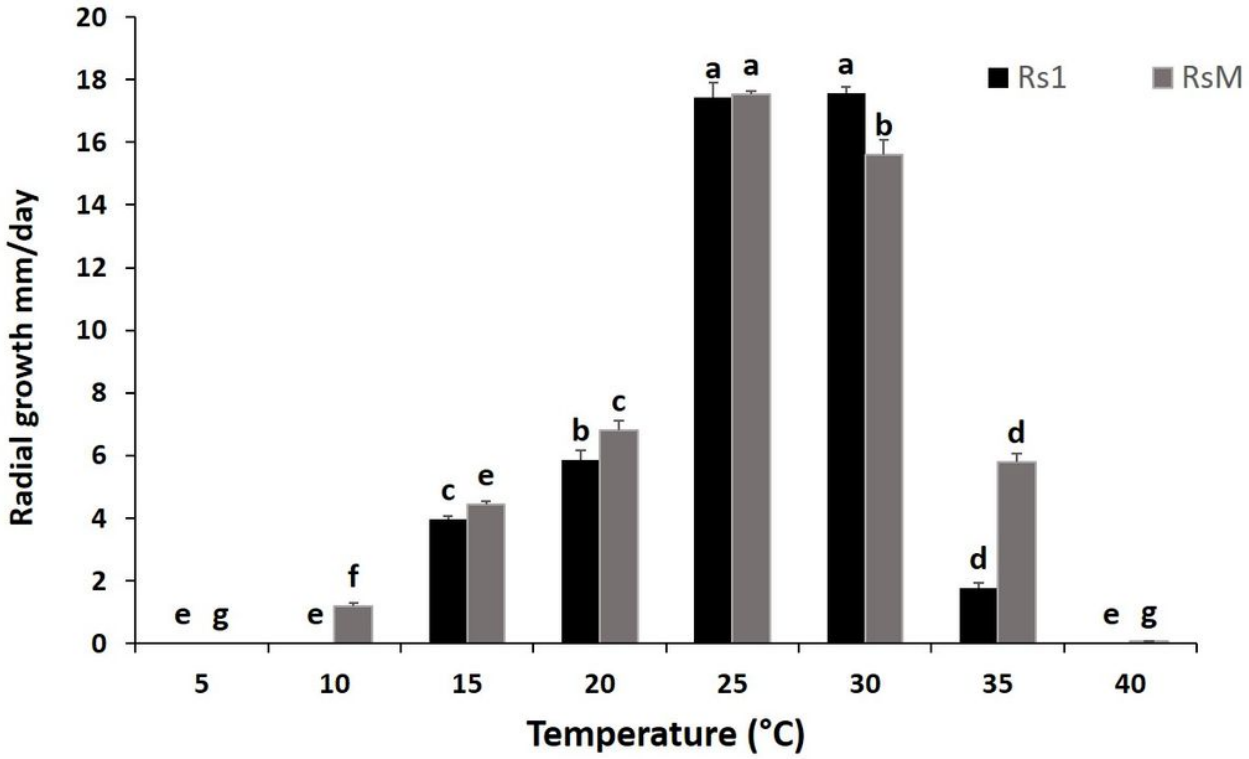

Figure 4

Radial growth of two isolates of *R. solani* (Rs1 and RsM) cultured on potato dextrose agar supplemented with streptomycin sulfate at 80 mg/liter at 5 to 40°C. Data represent mean of three replicates ± standard error of the mean. Means in each bar followed by the same letters are not significant differents as determined by Duncan's Multiple Range Test at $p \leq 0.05$. The test was conducted twice with similar results.

Figure 5

In vitro inhibition rate (%) of 4 fungicides (azoxystrobin,difenoconazol, tebuconazol and trifloxystrobin) against mycelial growth of two isolates of *Rhizoctonia solani* (Rs1 and RsM).
vertical bars indicate the standard errors. Common letters do not differ significantly from each other according to Duncan's multiple range test at $P < 0.05$.

Figure 6

In vitro dual cultures exhibiting antagonistic activity of the commercial Bacillus subtilis Y1336 against two isolates of *Rhizoctonia solani* on PDA medium 3 days of incubation at 25°C. Control without bacterial antagonist (A). The strain *B. subtilis* Y1336 against *Rhizoctonia solani* strain Rs1 (B) and *R. solani* strain RsM (C).

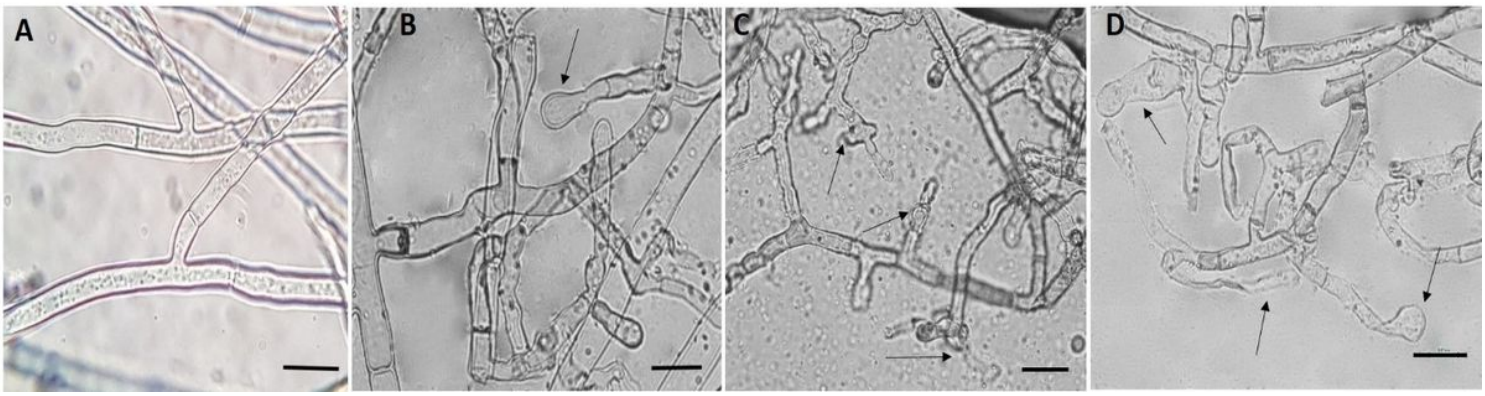

Figure 7

Microscopic examination (x40) of the mycelial structure of *Rhizoctonia solani* in co-culture with biocontrol agent (*Bacilus subtilis* Y1336). Control hyphae under a light microscope (A) shows equal widths and hyphal branching at almost right angles. Fungal hyphae from dual culture exhibited damaged

morphology and alterations in mycelial structure. So, (B) show hyphal deformation with head vacuolation due to the presence of the biocontrol agent. (C) indicate deformation of the mycelium with a large vacuole, numerous deformed and condensed structures, and a conglobated mass along the hyphae. (D) show cellular hyphae degradation as well as thinning hyphal endings. Scale bar = 10 µm.

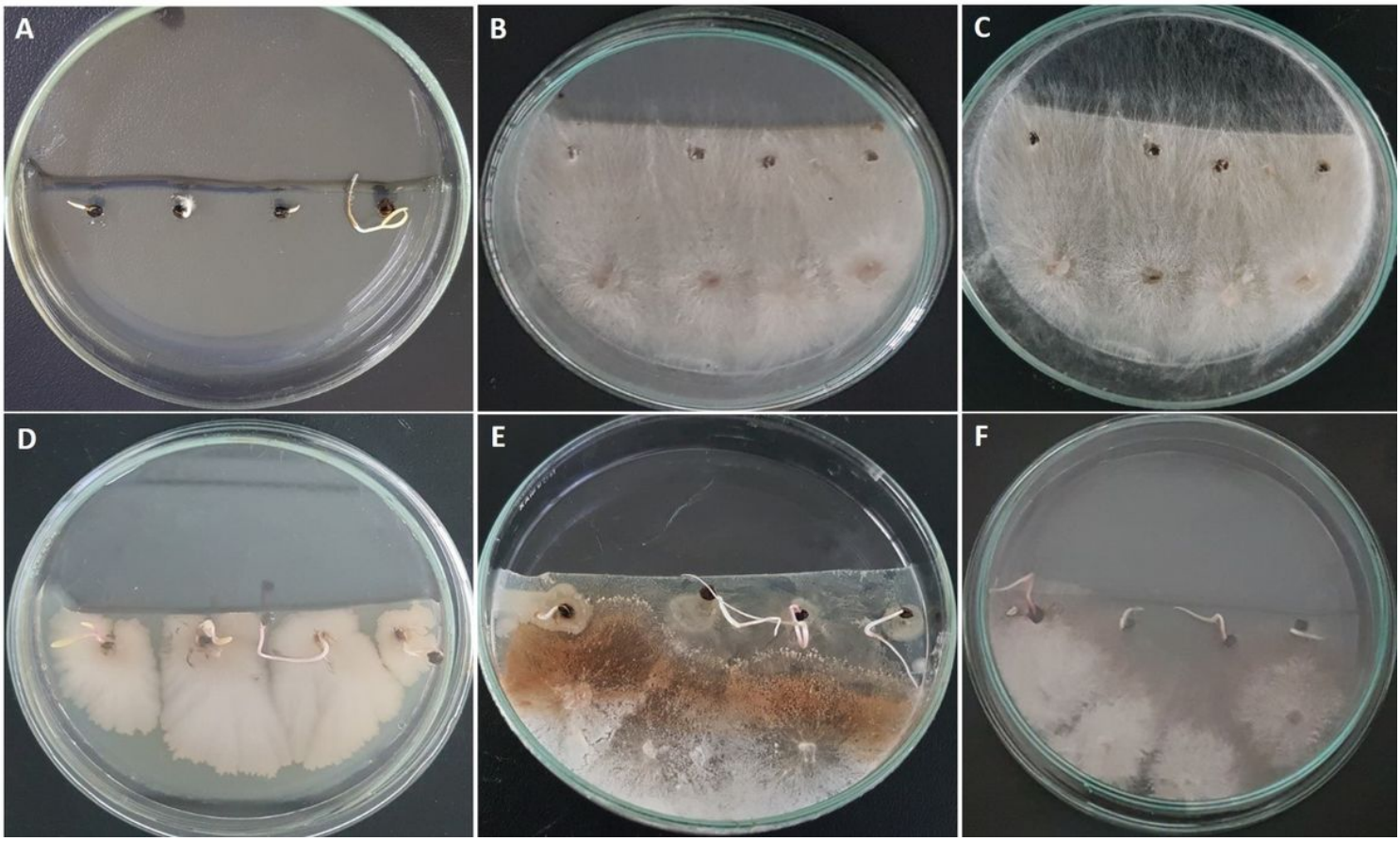

Figure 8

In vitro inoculation of sugar beet seeds on 50% PDA medium using four groups of inocula and a 7-day post-inoculation controls. A: plate contains only seeds (negative control) shows 0% damping-off. B and C: plates containing seeds and mycelia plugs of the pathogen (B: Rs1; C: RsM) show 100% damping-off. D plate contains seeds inoculated only with antagonistic bacterial strain Bacillus subtilis Y1336 exhibits 0% damping-off. E: plate contained combined treatment, seeds treated with *B. subtilis* Y1336 + mycelia plug of *R. solani* (Rs1) presents 0% damping-off. F: plate contained seeds treated with fungicide and mycelia plug buds of *R. solani* (Rs1) exhibits 0% damping-off.

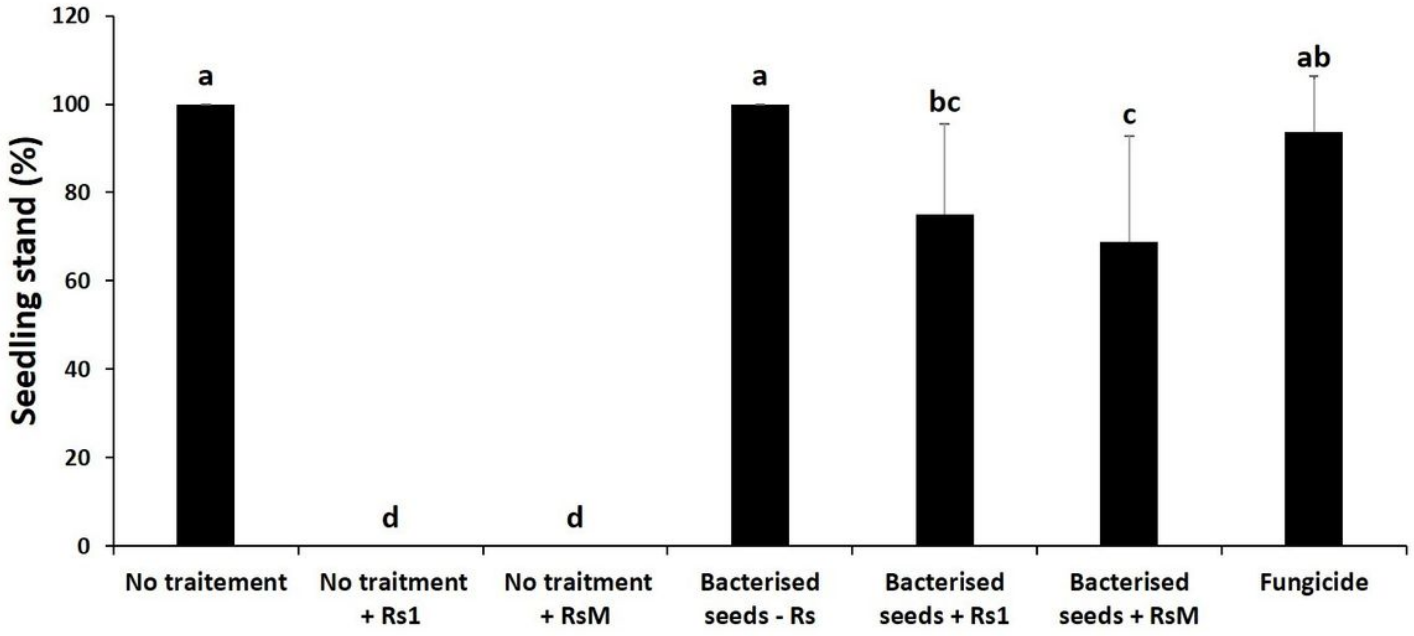

Figure 9

Efficiency of *B. subtilis* Y 1336 in control of damping-off caused by *R. solani* (Rs1 and RsM) under laboratory conditions. Means in each bar followed by different letters are significantly different ($p \leq 0.05$) as determined by Duncan's multiple range test.

Seeds of sugar beet (cv. Barrosa) were treated with bacteria formulated in CMC 1% and placed on 50% PDA plates. The inoculation with the pathogen was conducted by adding the mycelia plug of *R. solani*. The number of healthy seedlings was recorded at 7 days post-inoculation. Seeds not treated and those treated with difenoconazole (5 ug/ml) were used as controls. Data are the mean of four replicates. Means in each bar followed by different letters are significantly different ($p \leq 0.05$) as determined by **Duncan's multiple range test**. The test was conducted twice.

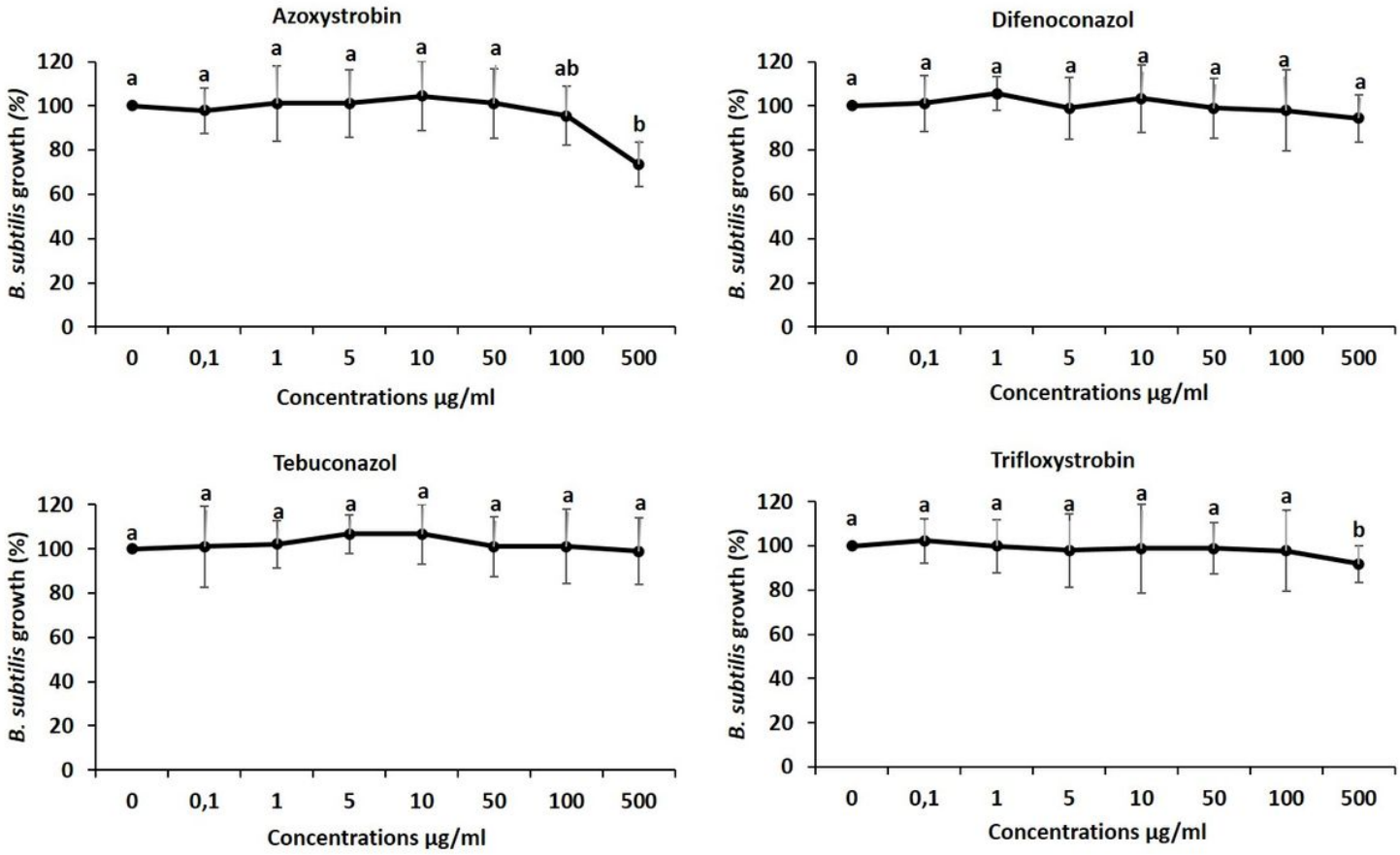

Figure 10

Effects of four active ingredients (azoxystrobin, difenoconazole, tebuconazole, and trifloxystrobin) on *in vitro* growth of biological control agent *B.subtilis* Y1336. Vertical bars indicate standard errors of the means are not significantly different at $p \leq 0.05$ (Duncan test).

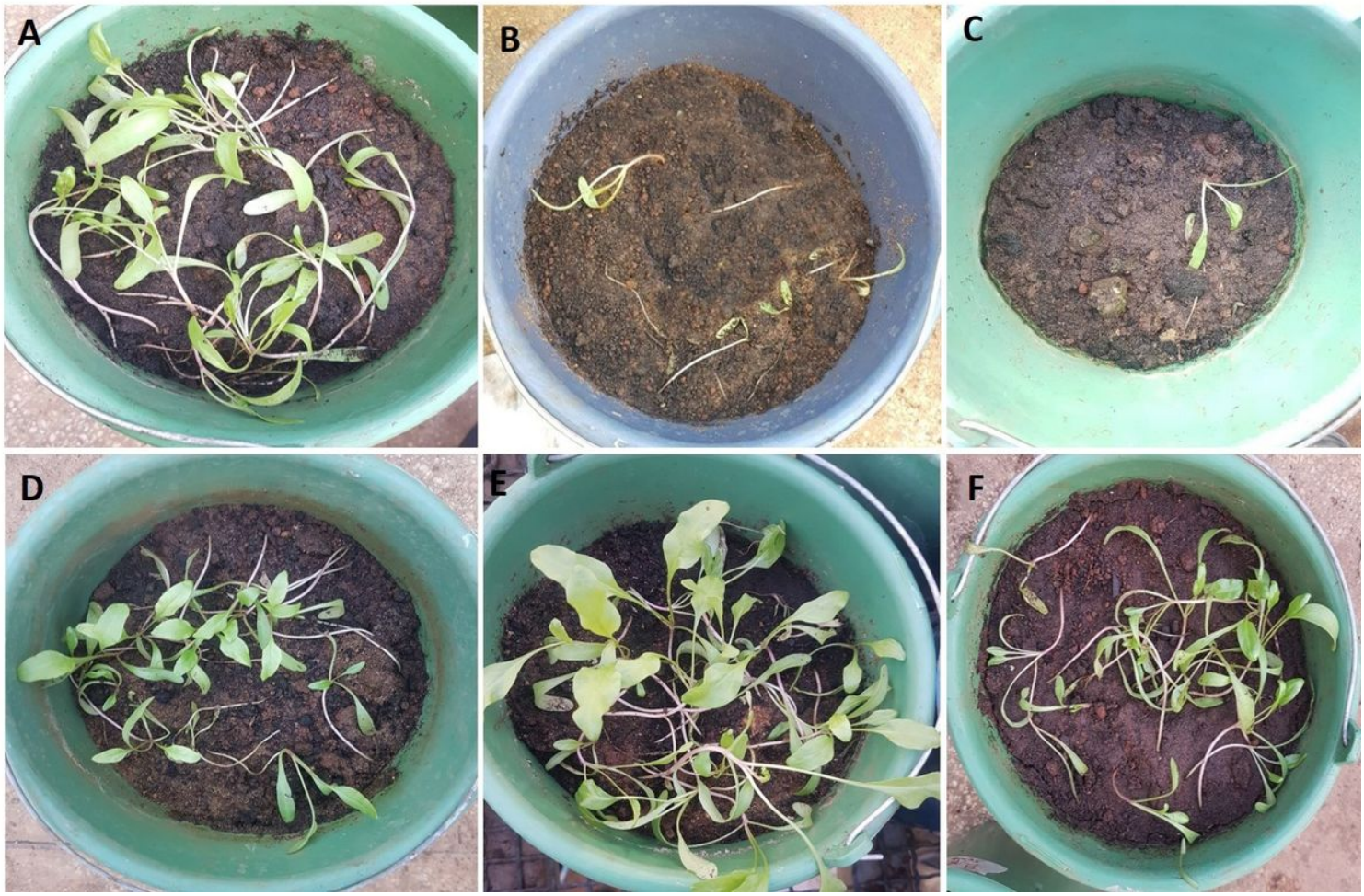

Figure 11

Pathogenicity of *R. solani* on sugar beet seedlings, and effect of *Bacillus subtilis* Y1336 in reducing damping-off disease caused by *R. solani* (Rs1), under **greenhouse conditions** at 45 days after sowing (DAS) A: Negative control (untreated seed + soil without *R. solani*); B and C: untreated seeds sowing in soil inoculated with Rs1 and RsM, respectively. D: Seeds treated with Difenoconazol in soil inoculated with Rs1; E: Seeds treated with *B. subtilis* Y1336 in soil non inoculated; Seeds treated with *B.subtilis* Y1336 in soil inoculated with highly aggressive isolates of *R. solani* (Rs1).