Evaluation of the Antagonistic Potential of Bacterial Strains Isolated from Moroccan Soils for the Biological Control of *Sclerotium rolfsii* Sacc.

Michelle G. S. Bidima^{1,*}, Nouredine Chtaina¹, Brahim Ezzahiri¹, Mohammed El Guilli²

¹Plant Protection Unit, Hassan II Agronomic and Veterinary Institute, Rabat, Morocco.

²National Institute of Agronomic Research, Menzeh station, Kénitra, Morocco.

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*Corresponding author: Michelle G. S. Bidima, Plant Protection Unit, Hassan II Agronomic and Veterinary Institute, Rabat, Morocco.

Email: michelle.bidima@gmail.com

Abstract

Sclerotium rolfsii Sacc. is a polyphagous fungus responsible for root rots of many cultivated crops. In Morocco, the fungus is responsible of important damages on sugar beet crop mainly in the Doukkala region. The aim of our study was to evaluate the antagonistic potential in vitro and in vivo of 191 bacterial isolates isolated from Moroccan soils and a bacterial strain from the laboratory collection *Bacillus amyloliquefaciens* referenced as I3, in the control of *Sclerotium rolfsii*. 14 of bacterial isolates showed antifungal activity against *Sclerotium rolfsii*. Isolates referenced as O4, E10 and E7 were the most efficient in vitro tests with an inhibition rate of the mycelial growth of *Sclerotium rolfsii* of 98.16%, 96.22% and 87.81%, respectively. The inhibition of sclerotia germination in soil by the bacterial suspensions varied from 85% to 95% for the aforementioned 3 isolates. The antifungal activity of the bacterial culture filtrates at proportions [1/2]; [1/3]; [1/4]; [1/10] ([Filtrate Volume /PDA Volume]) showed the ability of these bacteria to produce secondary metabolites capable of inhibiting *Sclerotium rolfsii* mycelial growth and sclerotia germination with inhibition percentages ranging from 30.62% to 98.93% for mycelial inhibition and from 44.69% to 98.75% for sclerotia inhibition for isolate O4. The in vivo tests were performed by bacterization of seeds and the evaluation was done on 15 days old chickpea seedlings, statistical analysis revealed a very highly significant difference ($p < 0.05$) between the isolates. Disease incidence was 7.5% with the T-O4 bacterized seeds and disease severity 30 days after inoculation was 20% for the most effective T-O4 treatment while the T control treatment had a severity of 89%. The isolates, isolated from Moroccan soils have shown their ability to inhibit *Sclerotium rolfsii* especially isolates O4 and E10.

Keywords

Sclerotium Rolfsii, Sclerotia, Biological Control, Antagonist Bacteria

1. Introduction

Root rot due to the phytopathogenic fungus *Sclerotium rolfsii* is a major constraint to sugar beet production in the Doukkala region that can cause yield losses ranging from 50% to 80% and a decrease in sugar quality [1, 2]. The pathogen persists in the soil for several years in the form of sclerotia [3]. These sclerotia tolerate biological and chemical degradation due to their melanized cuticle, the disease is favored by moist soil with temperatures between 25 and 30 °C [4]. In addition, its prolific growth and ability to produce large numbers of sclerotia and its wide host range of about 500 plant species in over 100 plant families [5] make the control of this pathogen difficult. Control of this fungus, therefore,

remains difficult and must be based on strategies that can reduce the primary inoculum in the soil or prevent infection of host plants. Biological control with the use of antagonists represents a promising approach in the control of soil-borne pathogens while reducing chemical inputs and their effects on the environment. These antagonists can secrete bioactive molecules which have antifungal or fungistatic capacities, to improve plant growth and to induce plant resistance [6-8]. In the literature, several species of bacterial biological agents have been reported to be effective against soil-borne pathogens such as *Rhizoctonia solani* [9, 10], *Phytophthora* spp. [11, 12], *Pythium* spp. [13, 14]. Regarding *Sclerotium rolfsii*, few reports are found on the potential antagonistic activity of bacteria isolated from the soil for root rot management. In this context, the objective of our study is to isolate and to evaluate the bacterial antagonistic activity of strains isolated from Moroccan soils against *Sclerotium rolfsii*. This antagonistic activity was evaluated by confrontation and antibiosis tests on mycelial growth and sclerotia germination in vitro and in vivo on chickpea plants which was chosen because of its high susceptibility to *Sclerotium rolfsii*.

2. Materials and Methods

2.1 Origin of bacterial isolates

Bacterial isolates were obtained from soil collected at 10 cm depth in the rhizosphere of various agricultural soils in different agro-climatic regions of Morocco.

2.2 Isolation of antagonistic bacteria from soil

Isolation of potentially antagonistic bacteria was performed by the suspension-dilution method [15]. The soil was dried, crushed, and sieved through a 5 mm mesh size. Then 10 g of soil was mixed with 90 ml of sterile distilled water. After vortex agitation, dilution series up to $1/10^5$ were performed. Then 0.1 ml of each dilution was inoculated in sterile petri dishes on Casein-Soy-agar based culture medium amended with cycloheximide (2 ml/L) to inhibit fungus germination. The dishes were then incubated at 25°C in the dark for 24 hours. The strains purified from the developed bacterial colonies were stored at 4°C.

2.3 Origin of the *Sclerotium rolfsii* strain

The strain of *Sclerotium rolfsii* used in this study was isolated from an infected sugar beet root, collected in the Doukkala region at the Experimental Station of Agricultural Development (ESAD).

2.4 Production of *Sclerotium rolfsii* sclerotia

Sclerotia were disinfected with 1% sodium hypochlorite (NaClO) for 3 minutes, then rinsed thoroughly with sterile distilled water. A disinfected sclerotia is cultured in a petri dish containing 20ml of PDA (Potato-Dextrose-Agar) medium. The dish is incubated at 30°C for 72 hours, a whitish mycelium develops giving rise to sclerotia. These sclerotia are transplanted on other petri dishes amended with PDA medium and incubated for 1 month at 30°C to have mature sclerotia for the different experiments that will be performed.

2.5 Evaluation of the antagonistic potential of bacterial isolates in vitro

2.5.1 Dual culture assay with bacterial isolates and *Sclerotium rolfsii*

Bacteria isolated from the soil were tested for their ability to inhibit the mycelial growth of *Sclerotium rolfsii* in vitro by dual culture assay on PDA medium using a modified method of [16]. For each bacterial isolate, a plating was made on a petri dish with PDA medium in 4 equidistant striated lines along with the petri dish. Then a 5 mm diameter implant of *Sclerotium rolfsii* taken from a 7-day old culture was placed in the center of the petri dish. The dishes were incubated at 28°C for 96 hours. Plates without bacterial isolates were used as controls. The experiment was repeated 4 times for each bacterial isolate. The antagonistic capacity of the bacterial isolates is evaluated after 4 days of incubation at 28°C by calculating the percentage of inhibition of mycelial growth compared to the control using the following formula:

$$\text{Mycelial inhibition (\%)} = \frac{D1 - D2}{D1} * 100$$

D1: Diametric average growth of the pathogen in the control dishes

D2: Diametric average growth of the pathogen in the confrontation dishes

2.5.2 Effect of bacterial suspensions on germination and viability of *Sclerotium rolfsii* sclerotia in sterile soil

(1) Preparation of bacterial suspensions

The selected bacterial isolates were cultured on PDA medium for 5 days at 25°C and then the bacteria on the surface of each isolate were scraped and suspended in 6 ml of LB (Luria-Bertani) nutrient broth. Then the bacterial suspensions were incubated in the dark at 25°C under continuous agitation (75 rpm) for 72 h. After incubation, the biomass of bac-

terial isolates was collected by centrifugation at 3,000 rpm for 45 minutes and resuspended in 200 ml of sterile physiological water (NaCl, 9 g/l). The concentration of the suspension was determined using a Malassez cell and then adjusted to 10^8 cfu/ml by spectrophotometric measurement OD (Optical Density) 600nm.

(2) Evaluation of bacterial suspensions on the viability of sclerotia

The bacterial isolates most effective in the control of mycelial growth of *Sclerotium rolfii* were selected for evaluation of their effect on sclerotia viability in soil by the method developed by [17]. The soil was dried, crushed, and sieved, then sterilized in sterilization bags and autoclaved at 121°C for 45 min twice at 24 h intervals. Then 25 g of soil are placed in glass petri dishes and 20 mature sclerotia were introduced into each of the dishes. A quantity of 10 ml of each bacterial suspension (10^8 cfu/ml) was added to each petri dish, the control dishes were added to sterile physiological water. All plates were incubated at 30°C for 10 days, after which the sclerotia from each plate were recovered under a stream of water, disinfected with 1% sodium hypochlorite for 3 minutes, and rinsed thoroughly before being incubated again for 3 days at 30°C on PDA medium. The experiment was repeated 4 times for each bacterial suspension. Sclerotia that did not show mycelial development were considered non-viable. The percentage of sclerotia inhibition by bacterial isolates was calculated as follows:

$$\text{Sclerotia inhibition (\%)} = \frac{N - n}{N} * 100$$

N: Total number of sclerotia

n: Number of germinated sclerotia

2.5.3 Effect of bacterial culture filtrates on mycelial growth and germination of *Sclerotium rolfii* sclerotia

(1) Preparation of bacterial culture filtrates

The preparation of filtrates was done according to the method of [10]. To allow adaptation to the liquid medium of the bacteria and to activate their germination, precultures were prepared beforehand. The culture filtrate of each antagonistic bacterial isolate was obtained after the preparation of these precultures. The bacterial culture of each isolate was taken from a 7-day old culture and inoculated into sterile flasks containing 6ml of LB liquid medium, these flasks were incubated at 28°C in the dark under continuous agitation (75 rpm). After 48 hours, 250 ml of LB liquid medium were added to each flask containing the precultures and incubated under the same conditions for 72 hours. The biomass is then separated from the culture filtrate by filtration through a Whatman No. 1 paper filter. The supernatant is centrifuged at 4,000 rpm for 1 hour and sterilized with Millipore syringe filters with pore sizes of 0.45 µm to obtain the bacterial culture filtrate.

(2) Evaluation of bacterial culture filtrates on mycelial growth and germination of sclerotia of *Sclerotium rolfii*

Antibiosis results from the production of substances that act as antibiotics or antifungals and inhibit the growth of the pathogen. 20 ml of a bacterial mixture culture filtrate and PDA medium was poured into the sterile dishes at different dilutions [1/2]; [1/3]; [1/4]; [1/10] ([Volume filtrate / Volume PDA]). After medium solidification, a 5 mm diameter disc from a 7-day old culture of *Sclerotium rolfii* was placed in the center of each dish. The controls were inoculated with sterile distilled water only and the mycelial growth of the pathogen is measured by the average diameter of the colonies after 96 hours of incubation in each dish. The experiment was repeated 4 times for each dilution.

$$\text{Mycelial inhibition (\%)} = \left(\frac{1 - D2}{D1} \right) * 100$$

D1: Diametric average growth of the pathogen in the control dishes

D2: Diametric average growth of the pathogen in the confrontation dishes

Also, 20 sclerotia previously disinfected with 1% sodium hypochlorite for 3 minutes and rinsed thoroughly with sterile distilled water were germinated in petri dishes containing the mixture of culture filtrate and PDA medium at different dilutions [1/2]; [1/3]; [1/4]; [1/10] ([Volume filtrate / Volume PDA]) each treatment was also repeated 4 times.

$$\text{Sclerotia inhibition (\%)} = \left(\frac{N - n}{N} \right) * 100$$

N: Total number of sclerotia

n: Number of germinated sclerotia

2.6 Evaluation of the antagonistic activity of bacterial isolates against *Sclerotium rolfii* on chickpea under greenhouse

2.6.1 Soil and test plants

The sandy loam textured soil (Fine loam: 49.84% and Fine sand: 13.5%), used for all the experiments was collected at the Agricultural Development Station of the Doukkala region during the 2019-2020 agricultural season. It was col-

lected between 5 and 20 cm depth from the rhizosphere of a sugar beet crop and has the following physicochemical characteristics: pH: 7.98; EC: 1.218 Ms/Cm; Organic matter 3.42%; P₂O₅: 28.35 ppm; K₂O: 358.48 ppm. As *Sclerotium rolfsii* is a polyphagous fungus which attacks several cultivated plants, the chickpea was chosen for in vivo tests on potted plants under greenhouses for its high susceptibility to this fungus which cause visible collar rot.

2.6.2 Seed pre-germination

The seeds of chickpea were disinfected on the surface with 1% sodium hypochlorite (NaClO) and then rinsed thoroughly with sterile water before undergoing pre-germination. For this purpose, the seeds were soaked in glass petri dishes containing cotton wool moistened with water. The dishes are then placed in the dark in a germination chamber for 24 hours at 19°C.

2.6.3 Seeds bacterization

The pre-germinated seeds are gently soaked and shaken in 100 ml of the bacterial suspension (10⁸ cfu/ml) for 45 minutes. They are then removed from the suspension, placed on sterile filter paper, and left to dry in a laminar flow hood at room temperature 20-21°C for 4 hours. Bacterial cell counts on the seeds after treatment were checked by washing the seeds and counting them after dilution. The non-bacterized seeds served as controls.

2.6.4 Seedlings and inoculation of the pathogen

The soil is first dried at room temperature of 20-21°C, crushed, and sieved (5 mm diameter mesh) before being transferred to sterilization bags to be autoclaved at 121°C for 1 hour, twice at 24-hour intervals. After sterilization, the soil is moistened and then placed in oval plastic pots of 15 cm in diameter at a rate of 1 kg of soil per pot. Five seeds are sown in each pot according to the different treatments: **T-O4**: seeds bacterized with isolate O4; **T-E10**: seeds bacterized with isolate E10; **T-I3**: seeds bacterized with isolate I3; and **T**: seeds not bacterized (control). On 15-day-old seedlings (plants are more susceptible to infection in the early stages of development) raised from bacterial and non-bacterial seeds, 0.3 g of mature sclerotia (about 40 sclerotia) in germination are inoculated at the basal part of the crown and a depth of 5 cm at the root level of each plant in each pot. The pots are then incubated in a growth chamber at 28-30°C with 16 hours of light and 8 hours of darkness. Each treatment was repeated 4 times and the experiment was conducted twice.

The incidence of the disease was determined 30 days after inoculation of the chickpea plants. It was expressed by the proportion of attacked plants compared to the total number of plants and was determined by the following formula:

$$\text{Disease Incidence (\%)} = \frac{\text{Number of attacked plants}}{\text{Number of observed plants}} * 100$$

Disease severity was assessed at 15 and 30 days after inoculation for all plants using a scale from 1 to 5 [18]. With a disease classification as follows: 1: healthy; 2: Stem lesions only; 3: Up to 25% of the plant symptomatic (wilting, dead, or dying); 4: 26% to 50% of the plant symptomatic; 5: > 50% of the plant symptomatic. Severity was subsequently determined with the formula established by [19] as follows:

$$\text{Disease Severity (\%)} = \frac{\sum ab}{AK} * 100$$

a: Number of plants with the same degree of infection

b: Degree of infection

A: Total number of plants examined

K: The highest degree of infection

2.7 Statistical analysis

Data from the tests of the antagonistic potential of the bacterial suspensions or their filtrates on mycelial growth and germination of *Sclerotium rolfsii* sclerotia in vitro expressed as percentage inhibition (I) were analyzed using SPSS version 20 software. A one-way analysis of variance (ANOVA) was performed for the double-culture assay and the effect of bacterial suspensions on sclerotia viability in soil with treatments (Bacterial isolates) as a factor and percent inhibition (I) as a dependent variable. A two-factor analysis of variance was performed for the effect of bacterial filtrates on mycelial growth and germination of sclerotia with the treatments (bacterial isolates) and the different dilutions as factors studied with the percentage of inhibition (I) as a dependent variable. For the trial on potted chickpea plants, the experimental protocol was completely randomized with 4 replicates for each bacterial isolate. The comparison of the means of the different strains was performed by the Tukey test at p<0.05.

3. Results

3.1 Isolation of antagonistic bacteria from soil

A total of 191 bacterial strains were isolated from the soil rhizosphere (Table 1), a bacterial strain from the laboratory collection *Bacillus amyloliquefaciens* referenced as I3 was also included, which showed in previous research its ability to control wheat Septoria caused by *Zymoseptoria tritici* [20].

Table 1. Origin of bacterial isolates

Isolats	Crop or plantation	Region of origin
[A1-...-A19]	Argan forest	Souss
[T1-...- T74]	Sugar beet	Tadla
[O1-...- O32]	Date Palm	Tafilalet
[D1-...- D24]	Sugar beet	Doukkala
[E1-...- E42]	Grassland	Zaër
[I3] [Strain from the laboratory collection]	Wheat	Gharb

3.2 Inhibition of mycelial growth of *Sclerotium rolfii* by the isolated bacteria: double culture assay

Bacterial isolates were evaluated for their ability to inhibit mycelial growth of *Sclerotium rolfii* in vitro tests. This evaluation was done by determining the percentage of inhibition of mycelial growth of *Sclerotium rolfii* in presence of different bacterial isolates after 96 hours of incubation at 28°C (Figure 1). Among these bacterial isolates, 14 of them showed significant antifungal activity, the results are presented in Figure 2. The highest percentage of inhibition of 98.16% was observed by isolate O4 followed respectively by isolate E10 with 96.22% and E7 with 87.81%. While the isolate I3 showed a percentage of mycelial growth inhibition of 55.83%. The analysis of these results showed that there was a significant difference ($p < 0.05$) between the isolates on the basis of their effect on the mycelial growth of *Sclerotium rolfii*. Isolates O4, E10 and E7 constituted the best performing group of isolates and were retained for further experimentation along with the reference strain I3.

Figure 1. Inhibition of mycelial growth of *Sclerotium rolfii* in dual culture with isolate O4 after 96 hours of incubation at 28°C. A: Confrontation isolate O4 and *Sclerotium rolfii*; B: Control, *Sclerotium rolfii* in the absence of antagonistic bacteria.

Figure 2. Effect of bacterial isolates on the mycelial growth of *Sclerotium rolfii* after 96 hours of incubation in vitro. For each variable measured, the bars represent the standard errors of the means. The means with the same letters are not significantly different with the Tukey test at $p < 0.05$.

3.3 Effect of bacterial suspensions on sclerotia germination in sterile soil

Regarding the effect of bacterial suspensions of isolates O4, E10, E7 and I3 on the germination of sclerotia in sterile soil a loss rate of viability of variable proportions was observed. These isolates significantly ($p < 0.05$) inhibited sclerotia germination (Figure 3). From the results of ANOVA, the highest inhibition of sclerotial germination was performed by isolate O4 with a rate of 95.93%, while isolates E10 and E7 showed a percentage of sclerotial inhibition of 91.5% and 85.62%, respectively. The lowest sclerotial inhibition was observed with isolate I3 for a rate of 60.31%. Furthermore, multiple comparison of means with Tukey test ($p < 0.05$) gave 3 distinct groups of bacterial isolates according to the percentage of inhibition.

Figure 3. Effect of bacterial suspensions on the viability of *Sclerotium rolfii* sclerotia in sterile soil. For each variable measured the bars represent the standard errors of the means. Means with the same letters are not significantly different with Tukey's test at $p < 0.05$.

3.4 Effect of bacterial culture filtrates on mycelial growth and sclerotia germination

All bacterial cultures filtrates tested showed their capacity to produce compounds capable of inhibiting the development of *Sclerotium rolfii* in view of the results. Indeed, the mycelial growth of *Sclerotium rolfii* and the germination of sclerotia were significantly inhibited in the antibiosis tests and the analysis of the results by ANOVA revealed a very highly significant difference at $p < 0.05$. For all three isolates evaluated the minimum inhibitory concentration was reached with dilution [1/2]. The percentage of inhibition was progressively reduced with the decrease of the dilution factor of the filtrates and these showed heterogeneous levels of inhibition.

Figure 4. Effect of filtrates of bacterial cultures. A: On mycelial growth of *Sclerotium rolfii*. B: On the germination of sclerotia of *Sclerotium rolfii*. For each measured variable the bars represent the standard errors of the means. Means with the same letters are not significantly different with Tukey's test at $p < 0.05$.

After 96 hours of incubation on the 3 isolates tested, it appears that isolate O4 showed a very high inhibition, compared to the control. In both tests and for all dilutions ([1/2], [1/3], [1/5], [1/10]) with respective inhibition rates of 98.93%; 76.93%; 44.94% and 30.62% for inhibition of mycelial growth of the pathogen and 98.75%; 94.68%; 77.81% and 44.69% for inhibition of sclerotia germination. Isolate O4 was followed immediately by isolate E10 with 96.07%; 67.27%; 37.82%; 17.45% for inhibition of mycelial growth and 97.81%; 90.93%; 54.06%; 20% for inhibition of sclerotia germination always in the respective dilutions of ([1/2], [1/3], [1/5], [1/10]). Isolate E7 represents the isolate with the lowest inhibition rate in both tests with 92.81%; 87.5%; 34.37% and 6.87% for mycelial growth inhibition and 88.87%; 47.80; 20.07% and 7.96% for sclerotia germination inhibition.

3.5 In vivo experiment: Pot trial

The biocontrol trial of *Sclerotium rolfsii* by the four selected bacterial isolates O4, E10, E7 and I3 was conducted on 15-day-old potted chickpea seedlings derived from bacterized or non-bacterized seeds. These bacterial isolates were evaluated for their ability to control or reduce root rot induced by *Sclerotium rolfsii*. The results of the analysis are shown in Figure 5. These results show that seed treatments with these bacterial isolates provided control of *Sclerotium rolfsii*. Statistical analysis revealed a very highly significant difference ($p < 0.05$) between the different isolates. Comparison of the means allowed to distinguish 3 groups. The incidence of disease was 7.5% with treatment T-O4 which was the most effective, followed immediately by treatment T-E10 with an incidence of 12.5%. The E7 and I3 isolates were relatively less effective in controlling the pathogen with an incidence of 52.5% and 60% respectively. The severity of the disease was determined 30 days after inoculation and was 20% for the most effective treatment T-O4 while the control treatment T had a severity of 89%. For the T-E10, T-E7, and T-I3 treatments the severity was 26%, 40%, and 47%, respectively. Attacked plants showed yellowing of leaves and brown spots at the crown after 15 days of inoculation, wilting of plants due to weakening of the crown and roots completely damaged by rot after 30 days of inoculation with cottony growths of the fungus.

Figure 5. Biocontrol activity of bacterial isolates on chickpea seedlings: pot trial. For each variable measured the bars represent the standard errors of the means. Means with the same letters are not significantly different with Tukey's test at $p < 0.05$.

4. Discussion

The use of biological control agents in the management of soil-borne fungal diseases is a promising alternative to chemical pesticides both environmentally and economically. These biological agents can be applied to plants for the biological control of pathogens. In this study we were able to demonstrate through our results that bacterial isolates O4, E10 and E7 isolated respectively from Moroccan soil (region of Tafilalet and Zaër) showed an ability to produce anti-fungal secondary metabolites capable of inhibiting in a considerable way the development of *Sclerotium rolfsii* in vitro and in vivo. The tests of confrontation of bacterial isolates and *Sclerotium rolfsii*, and antibiosis tests showed that there was a significant difference between the percentages of inhibition generated by the different isolates ($p < 0.05$). A similar study conducted by [21] revealed an effective inhibition against *Sclerotium rolfsii* with the use of filamentous bacterial isolates. In all our experiments, it was found that bacterial isolate O4 was the most effective strain in controlling *Sclerotium rolfsii* up to 98% inhibition. These results suggest that it may play an important role in the biocontrol of *Sclerotium rolfsii* and agree with the results reported by [22-24] who have highlighted the efficacy of certain bacterial strains in the control of *Sclerotium rolfsii* and other soilborne pathogens. This effectiveness could be explained by the

mechanisms of action involved by antagonistic bacteria such as competition for nutrients [25], production of antibiotic secondary metabolites [26, 27] and induction of systemic resistance [28, 29]. With these different modes of action these antagonists can be used as biological control agent that can protect crops from soil-borne fungal diseases. The isolates isolated from Moroccan soils in our study have shown their ability to inhibit *Sclerotium rolfsii*. Isolates O4 and E10 were found to be the most effective in controlling of this pathogen. In order to use them as an effective biological control agent further experiments on the mechanisms of action related to this inhibition and the identification of these bacteria isolates are underway.

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