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Actinomycete as biocontrol agents against tomato gray mold disease caused by *Botrytis cinerea*

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Abstract

The present work aims to isolate actinomycete bacteria with antagonistic abilities towards *Botrytis cinerea*, the causal agent of gray mold, from a soil sample collected from the rhizosphere of a healthy tomato grove. *In vitro* confrontation led to the isolation of 104 actinomycete isolates; fifteen isolates have shown the most significant mortality rate of the mycelial growth of *B. cinerea* (>50%). Based on the results of this screening, representative strains were selected to verify their *in vivo* antagonistic activity on tomato fruits; the reduction of *B. cinerea* has a percentage ranging from 52.38% to 96.19%. Furthermore, the actinomycete isolates were evaluated for their plant growth-promoting (PGP) properties and their ability to produce biocontrol-related extracellular enzymes viz., amylase, protease, cellulase, chitinase, esterases, and lecithinase. Indeed, Ac70 showed high β-1,3-glucanase activity and siderophore production (17U/ml and 43% respectively), and the highest chitinase activity (39µmol/ml) was observed for Ac24. These results indicated that these actinomycetes might potentially control gray mold caused by *B. cinerea* on tomato fruits. Investigations on enhancing the efficacy and survival of the biocontrol agent in planta and finding out the best formulation are recommended for future research.

Keywords: Actinomycetes; *botrytis cinerea*; chitinase; siderophore; tomato.

1. Introduction

The fungus *Botrytis cinerea* causing gray mold disease on over 1000 plant species (Fillinger, 2016). It is a devastating disease on tomatoes, leading to severe economic losses in many countries worldwide. Annually *B. cinerea* causes $10 billion to $100 billion in losses worldwide (Boddy, 2016).

The control method's limitations are mainly due to the lack of plant sources of resistance, the difficulties of applying fungicides potentially harmful to the environment and health, and the evolution of pathogens evolution under different selective pressures. Even pesticides have good results in the short term, their side effects on the environment are worrying in the long term (Tomer *et al*., 2015). Biological control has proven to be one of the most eco-friendly mean due to its ability to use natural antagonists (Qessaoui *et al*., 2019).

These biological agents are of additional interest and have shown that the strains selected can have a growth-stimulating effect in the absence of pathogenic organisms (Chaurasia *et al*. 2018, Dashti *et al*., 2014). Actinomycetes can produce antimicrobial substances and compete spatially and nutritionally with phytopathogenic agents (Adelere., 2016). This

work aims to isolate the actinomycete strains from the tomato rhizosphere and screening for antagonistic effects on *B. cinerea*. Potential strains were tested *in vivo* for antagonistic activity on tomato fruits. After that, these bacterial strains were characterized to identify them in the future.

2. MATERIALS AND METHODS

2.1 Site of study

The study was conducted in the laboratory of plant protection (INRA, Agadir, Morocco) during the period ranged from March 2018 and May 2019.

2.2 Isolation of actinomycetes

Bacteria were isolated from the soil rhizosphere of healthy tomato plants, which were grown in an infested field. After drying and sieving (sieve 1mm in diameter), 1g of soil was used to prepare a suspension in 9ml of sterile physiological water. Once the homogenization was done, a series of dilutions were prepared in sterile physiological water. After agitation in an orbital shaker at 200rpm for 10 minutes, 0.1ml of each dilution was separately plated on Olson medium with 40µg/ml of cycloheximide and 10µg/ml of nalidixic acid. The Petri dishes were incubated at 28°C and observed for 5 to 25 days. After incubation, actinomycete isolates were distinguished from other microbial colonies by characteristics such as tough colonies partially submerged into the agar (Wang *et al*., 2016).

2.3 *In vitro* antagonistic effect of actinomycetes on *B. cinerea*

Each bacterial isolate was streaked as a band on the edge of a PDA 90-mm diameter plate and incubated 24 hours at 28±2°C. Then, a 10 mm diameter mycelial disc of *B. cinerea* was taken from the margin of a growing colony and placed onto the center of previously inoculated PDA plates. The Petri dishes were sealed by parafilm and incubated at room temperature in the dark at 28°C for 7days.

Plates containing only the fungal mycelial plug were maintained as control. The percentage of inhibition of the pathogen by the rhizobacterial strain over the control was estimated using the formula given by (Yun, 2018) as follows:

$$I\ (\%) = \left(1 - \frac{C_n}{C_o}\right) 100 \qquad (1)$$

Where I represents the percentage of inhibition of mycelium, Cn is the growth of mycelium in the treatment, and Co is the growth of mycelium in control.

2.4. *In vivo* antagonistic effect of actinomycetes on *B. cinerea*

The homogeneous mature tomato fruits were washed with tap water and artificially wounded of 3x3mm (diameter x depth) at 4 equidistant points around equators using a sterile nail. The wound sites were treated by applying 20µl of a spore suspension of *B. cinerea* (106spores/ml) after one hour of preincubation at room temperature. As a control, fruits also were inoculated with *B. cinerea* alone. The fruits were then transferred to an air-tight plastic bag to maintain high relative humidity and incubated at 20°C for 7days.

The percentage of disease reduction of gray mold on tomato fruits was calculated using the following formula:

$$I(\%) = (A - B)/A\ x\ 100 \qquad (2)$$

Where A is the lesion diameter in tomato fruit inoculated with *B. cinerea* alone, and B is the lesion diameter in infected tomato fruits treated with the antagonist. All *in vivo* antagonism assays were made in fifteen replicates (Berrada *et al*., 2012).

2.5 Production evaluation of extracellular enzymes, siderophore, and HCN

Selected actinomycetes were evaluated for their PGP and biocontrol traits including siderophore, chitinase, β-1,3-glucanase, HCN,cellulase, amylase, Esterase, Protease and Lecithinase.

Siderophore production was estimated on chrome azurol agar (CAS) plates using the method described by (Hu, 2011). Chitinase was estimated by amending agar plates with colloidal chitin suspension and mineral salts prepared by the method of (Lingappa & Lockwood, 1962), The ability of actinomycete isolates to produce β-1,3-glucanase was tested in a minimal medium supplemented with laminarin (Sigma) as described by (Valois *et al*., 1996).

HCN and cellulase were identified by using the approach of (Passari *et al*., 2015). Amylase has been done on Gause's agar using the method of Simair *et al*., 2017. Lecithinase activity was tested on the medium described by (Garrity, 2006). The ability of actinomycetes to produce proteases was assessed using milk agar and gelatin agar medium to detect caseinase and gelatinase (Fatema *et al*., 2016), respectively. The esterase activity of the isolates was evaluated according to the

3. RESULTS

3.1 Isolation of bacteria from the soil rhizosphere

The results obtained showed that actinomycetes represent 1.86x106 cfu/g. Their enumeration showed that they are present in the soil sample with a proportion of about 1.9% of the total flora.

One hundred and four isolates were selected. Among these 104 isolates, actinomycetes with white aerial mycelium are the most represented (79%). Actinomycetes with brown, red, or gray aerial mycelium are poorly represented (1%) and 40 isolates (38.46%) produce pigmentation of the medium.

3.2 *In vitro* antagonistic effect of actinomycetes on *B. cinerea*

The obtained results (Figure 1) showed that the fifteen isolates cause a very highly significant antagonistic activity on *B. cinerea*. The reduction rates were ranged from 50% (Ac83, Ac4, and Ac9) to 72 % (Ac70 and Ac33).

The confrontation of 15 isolates of actinomycetes with *B. cinerea* on the PDA culture medium allowed us to highlight the ability of these isolates to inhibit the mycelial growth of the pathogen.

3.3 *In vivo* antagonistic effect of actinomycetes on *B. cinerea*

The results obtained (Figure 2) showed a reduction rate of *B. cinerea* development in the presence of actinomycetes compared to control. These rates were ranged from 52.38% (Ac9) to 96.19% (Ac70). The in-vivo test showed significant efficacy of the actinomycete isolates against *B. cinerea* compared to positive control.

3.4 Extracellular enzymes, siderophore, and HCN production

Results of enzymatic activities of 15 actinomycete isolates (Table 1), all selected isolates produced siderophore (2-43%, Figure 3), chitinase (2-39µmol/ml, Figure 4), and the B-1,3-glucanase activity was detected in 12 isolates (1,4-17U/ml, Figure 5).

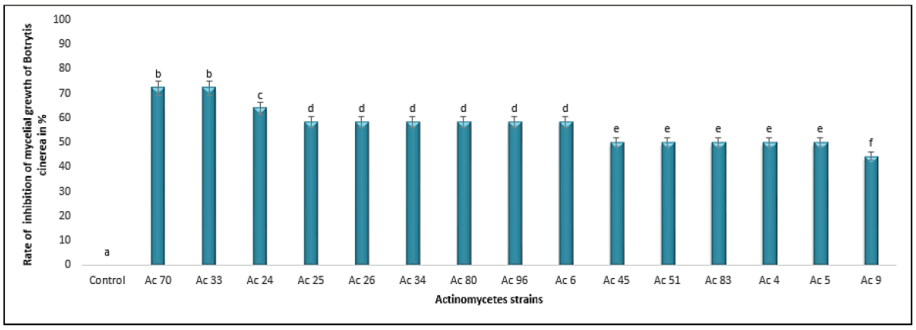

Fig. 1. Mycelial growth inhibition of *B. cinerea* shown by 15 actinomycete isolates revealed *in vitro* by dual culture technique. The inhibition rate (%) is the mean of three replicates (n = 3) ± standard error. Values followed by the same letters are not statistically different at $p \leq 0.05$.

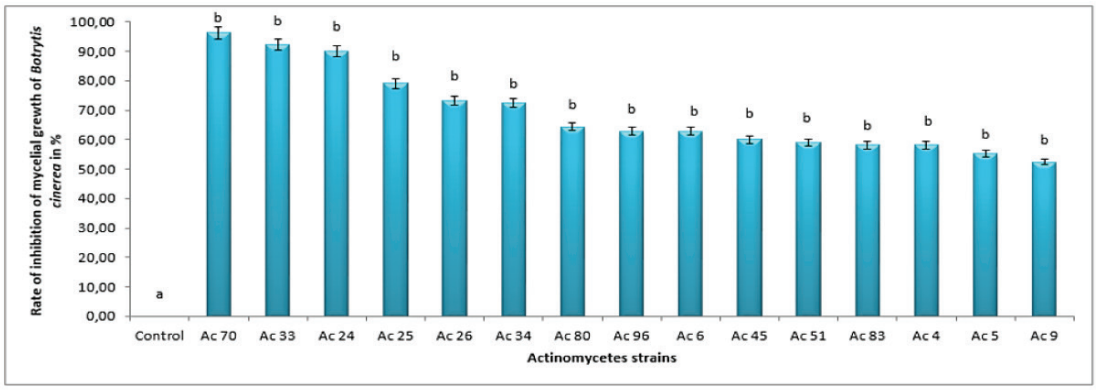

Fig. 2. Actinomycete isolates effect on the development of tomato gray rot *in vivo* test. Inhibition rate (%) are mean of 15 replicates (n = 15) ± standard error. Values followed by the same letters are not statistically different at $p \leq 0.05$.

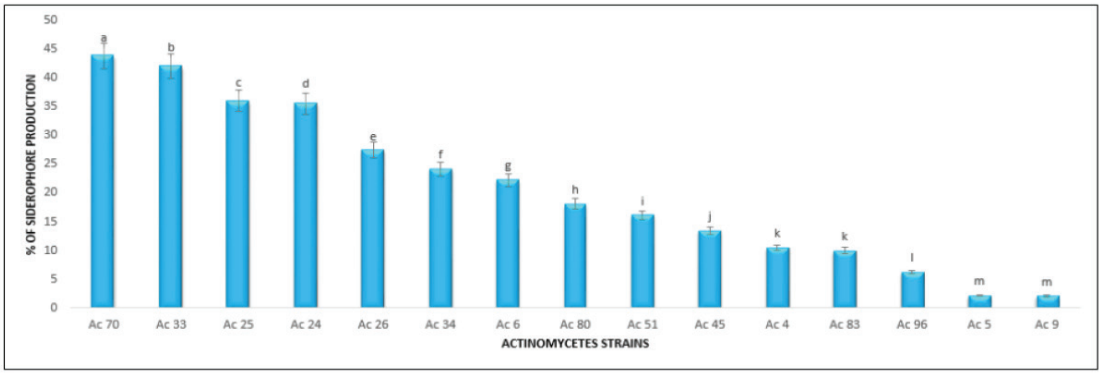

Fig. 3. Percent of siderophore produced by 15 actinomycete isolates. Values (%) are means of three replicates (n = 3) ± standard error. Values followed by the same letters are not statistically different at $p \leq 0.05$.

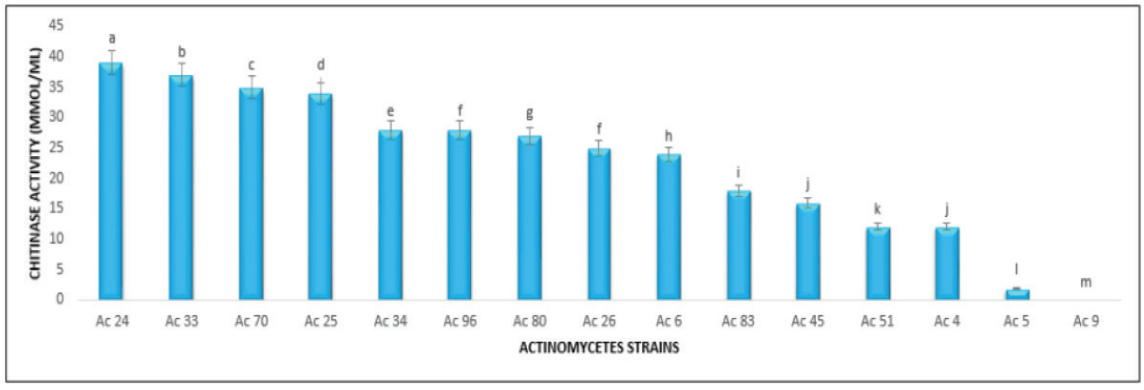

Fig. 4. Chitinase activity showed by 15 actinomycete isolates. Values (µmol/ml) are means of three replicates (n = 3) ± standard error. Values followed by the same letters are not statistically different at $p \leq 0.05$.

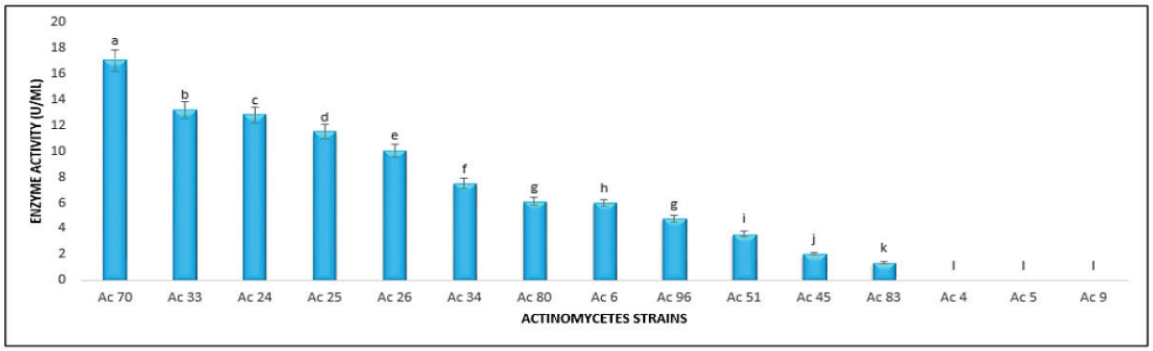

Fig. 5. The β-1,3-glucanase activity showed by 15 actinomycete isolates. Values (U/ml) are means of three replicates (n = 3) ± standard error. Values followed by the same letters are not statistically different at $p \leq 0.05$

Table 1. Properties of actinomycete isolates (+: positive; -: negative).

Actinomycetes isolate N°	Antagonistic properties					
	HCN	Amylase	Cellulase	Esterases	Lecithinase	Protease
Ac34	-	+	-	-	-	+
Ac96	-	+	-	-	-	+
Ac9	-	+	-	-	-	+
Ac33	-	+	+	-	+	+
Ac6	-	+	-	-	-	+
Ac24	+	+	-	-	-	+
Ac26	-	+	-	-	-	+
Ac45	-	+	-	-	+	+
Ac80	+	+	+	-	-	+
Ac51	-	+	-	-	-	+
Ac83	-	+	-	-	-	+
Ac25	-	+	-	-	+	+
Ac70	+	+	+	-	-	+
Ac4	-	+	-	-	-	+
Ac5	-	+	-	-	-	+

4. DISCUSSION

As reported by Al-Sane *et al*., 2002; Al-Zarban *et al*., 2002; and Al-Musallam *et al*., 2001, the ability of the selected actinomycetes to prevent or to reduce the action of phytopathogens fungi is related to the production of extracellular enzymes.

It has been shown that certain endophytic Streptomyces species have been reported to produce HCN, contributing to the reduction of the Fusarium disease and rice bacterial leaf blight pathogens.

It has also been reported that the *Streptomycetes* produce hydroxamate type's siderophores (Maglangit, 2019. Actinobacteria producing chitinase and β-1,3-glucanase are often employed in biocontrol processes and are widely utilized for the formulation of biopesticides (Gonzalez-franco *et al*., 2003).

Roopan *et al*., 2019, have shown protease activity in isolated actinomycetes. Amylase activity in actinomycetes has been reported by (Nithya *et al*., 2017). Cheriet *et al*., 2015, detected lecithinase activity in actinomycete isolates, and different cellulase activity in actinomycetes has been described by other authors (Viswanathan, 2019). Despite the good results obtained by these actinomycetes,

a certain difference exists between the *in vitro* and *in vivo* results. These results are convenient to those obtained by Whitaker & Bakker, 2019, who observed that the antagonistic effect expressed *in vitro* was not always transcribed in the soil or small-scale experimentation. Biological control trials that use these bacterial strains have shown that it is possible to limit the incidence of *B. cinerea* (Boukaew, 2017), even the level of protection is not important enough.

5. Conclusions and perspectives

Gray mold, caused by the necrotrophic fungus *B. cinerea*, damages a wide range of plants and affects many fruit and vegetable crops. In greenhouse horticulture, *B. cinerea* is also known for causing severe damage to tomatoes, affecting stems, flowers, and fruits. It is responsible for huge economic losses both in Morocco and around the world. The present work has isolated actinomycetes antagonists to the fungus from the rhizosphere soil of the tomato field. For 104 isolates of actinomycetes, 15 isolates have an inhibitory activity of mycelial growth of *B. cinerea*. These fifteen isolates also have the distinction of being as active *in vivo* tomato fruit rot disease. Indeed, these actinomycetes can reduce the inoculum of *B. cinerea* in the culture chamber in the presence of the fruit.

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