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Biological Control of *Botrytis cinerea* by *Bacillus* sp. Strain S7LiBe Under Abiotic Stress

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ABSTRACT

In this investigation, the isolate S7LiBe isolated from trefoil rhizosphere in Barbacha, Bejaia (northern Algeria), was selected on the basis of plant growth promoting attributes. This isolate identified as Bacillus sp. based on 16SrDNA sequence analysis, exhibits the highest similarity of 99.1% with *Bacillus pichinotyi* AF519463 andAF519460 and *Bacillus* sp. JX152775. *Bacillus* sp. S7LiBe has been subjected to the study of its antifungal activity against *Botrytis cinerea in vitro* and *in vivo* on the detached lettuce leaves. The effect of some abiotic factors on the performance of this bacterial strain as biocontrol agent and the production of some antifungal metabolites (siderophores, ammonia, hydrogen cyanide and chitinase) were also carried out. The results showed that *Bacillus* sp. S7LiBe strain inhibit the mycelial growth in high level (70%±1,33) and the fungal spores germination (82,54%±3,58). The strain produce siderophores, ammonia, hydrogen cyanide and chitinase. Its antagonistic activity was found to be optimal on CZA modified medium at a pH of 6.5 and a temperature of 30 ° C. The antagonist activity on the detached lettuce leaves showed that the bacterial strain inhibit 57.0% of the lesion extension.

Keywords: *Bacillus* sp., *Botrytis cinerea*, PGPR, Biocontrol.

I. INTRODUCTION

Pathogens affecting plant health are a major menace to food production and ecosystem stability worldwide [1]. It has been estimated that approximately one third of the food crop is destroyed every year due to attack by insects, pathogenic fungi, bacteria, and nematodes [2]. *Botrytis cinerea* is one of the most hazardous plant pathogen infecting a large number of vegetable plant. This phytopathogene infect leaves, stems, flowers and fruits of plants, either by direct penetration or through wounds caused by cultivation practices [3; 4; 5; 6].To control plant disease, producers had resort to the chemical products. However, many of the chemicals are hazardous and causes several negative effects on the environment and human health. For that reason, biocontrol has become an interesting alternative that are more "friendly" to the environment. Plant growth promoting rhizobacteria (PGPR) are the important group of microorganisms, which play a major role in the biocontrol of plant pathogens [7]. *Bacillus* species have been reported as plant promoting bacteria in a wide range of plants [8; 9; 10; 11], it´s one of the principals PGPR groups known for their application as biocontrol of several pathogenic fungi. *Bacillus* is the key bacterial genus that have shown greatest potential for *Botrytis* disease control [12], and that by several mechanisms which include; secretion of lytic enzymes, siderophores and antibiotics, competition for space and nutrient, defense plant stimulating and combination of mechanisms [7; 13]. In addition, *Bacillus* species produce spores that are resistant to heat and desiccation, which allows the preparation of more stable and durable formulations, this explains the greater availability of commercial products based on Bacillus [14; 15]. Commercial available biocontrol products include: Kodiak (*B. subtilis* strain GB03), Serenade (B. subtilis QST 713), YieldShield (*Bacillus pumilus* strain GB34), EcoGuard (*Bacillus licheniformis* strain SB3086) [16; 17]. and Subtilex (*B. subtilis* strain MB1600) which is active against *Botrytis* spp. infection of vines, strawberry, cucumber, powdery mildew of tomato, and brown rust of cereals [2; 17].

In the present investigation, Bacillus sp. S7LiBe strain isolated from trefoil rhizosphere in northern Algeria has been subjected to the study of its antifungal activity against *Botrytis cinerea in vitro and in vivo on the* detached lettuce leaves. Many abiotic factors, such as pH, temperature, moisture, inorganic and organic constituents, may influence the mechanisms of biocontrol, in this research we study the influence of some abiotic stress on the performance of *Bacillus* sp. S7LiBe against *B. cinerea. The* production of some antifungal metabolites (siderophores, ammonia, hydrogen cyanide and chitinase) was also carried out.

II. METHODS AND MATERIAL

A. Fungal strain and culture conditions

A strain *Botrytis cinerea* BC1 used in this study was obtained from the laboratory of Mycology, Bejaia University, Algeria. It was originally isolated, in the Laboratory of Plant Protection(INRA, STPV, Avignon, France), from tomato plants with typical symptoms of grey mold. The fungal strain was grown in Petri plates containing potato dextrose agar medium (PDA, Difco), The plates was incubated at 21ºC for 3-10 days, Stock cultures of *B. cinerea* was maintained on PDA medium and stored at 4°C.

B. Bacterial strain and inoculum

Bacillus sp.S7LiBe was isolated from trefoil rhizosphere in Barbacha, Bejaia (northern Algeria). This strain was selected for its ability to produce several promoting growth traits (production of siderophores, indole acetic acid, inorganic phosphate solubilization and heavy metal tolerance), and was identified by sequencing their entire 16S rRNA. Bacterial suspension was prepared by inoculating a loop full of cells in LB medium and incubating at 30°C for 24h.The bacterial concentration was calculated by serial dilutions and checked by optical density (OD_{600}), and then adjusted to 10^8c.f.u. /ml before use.

C. Molecular identification

The isolate S7LiBe was subjected to the molecular identifications, genomic DNA extraction from pure bacterial colonies was carried out using the FastDNA® SPIN kit in conjunction with the FastPrep FP120 instrument (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. The genomic DNA was further PCR amplified for 16S-rDNA gene sequencing using the flanking primer pair 616F (5´AGA GTT TGA TYM TGG CTCAG 3´) and 630R (5´ CAK AAA GGA GGT GAT CC 3´). For phylogenetic analyses the obtained 16S-sequences were aligned with the *Sina Aligner V1.2.11* on the Silva website *(www.arb-silva.de)* and phylogenetical allocated with the sofware package ARB [18]. Phylogenetic tree construction was performed by using the Maximum-Likelihood[19].

D. Effect of the antagonist on the growth of B. cinerea mycelium

The inhibitory effects of the strain on the growth of *B. cinerea* were tested by the agar diffusion method as described by [20]. $1cm^2$ fungal plug was inoculated in the center of PDA plate, each isolate was sown at a distance of 2.5 cm from the fungus. Plates without antagonist served as control. Three replications were performed for each confrontation experiment. The plates were then incubated at 25±2°C for 5 days and verified every day. The percentage of growth inhibition (PGI) of the fungus was recorded and calculated using the formula: PGI (%) = [(KR-R1)/KR] x100 where KR corresponds to the distance from the point of inoculation to the colony margin on the control dish (mm). R1 represents the distance (mm) of fungal growth from the point of inoculation to the colony margin on the treated dishes.

E. Effect of the antagonist on the germination of B. cinerea spores

20µl of spore suspension adjusted to 10^6 spores/ml and 20µl of 24h bacterial culture grown on LB medium adjusted to 10^8c.f.u./ml, were pipetted into an Eppendorf tube containing 1 ml of sterile distilled water with 5% of glucose and then incubated at 21°C for 24 h. Control tubes were inoculated only with fungal spores. The experiment was realized in triplicate. The germination studies were performed on hemocytometer using a light microscope (40X).

F. Effect of the antifungal volatiles on the growth of B. cinerea mycelium

The strains *Bacillus* sp. S7LiBe et *Pseudomonas* sp.S5LiBewere streaked on a Petri dish containing LB agar medium. a second Petri dish containing PDA medium, was inoculated with a 5 mm plug of the fungus *B. cinerea* in the center of the plate, and inverted and placed over the bacterial culture. The two plates were sealed together with parafilm and incubated at 25°C. The percentage of growth inhibition (n) of the fungus, was recorded and calculated using the formula: N= [(A-B)/A] x 100, where A corresponds to the diameter of the mycelium in the control (cm) and B represents the diameter of the mycelium in the plates inoculated with bacteria (cm).

G. In vivo antagonism experiments

The evaluation of the antagonistic activities of S7LiBe against *B. cinerea* is realized on the detached lettuce leaves. The test was done according to the protocol of INRA Avignon France with modification. Three lettuce leaves were deposed on box lined beforehand with absorbent paper soaked in distilled water, after this, the bacterial suspension (10^8 CFU/ml.) was sprayed on the surface of the detached lettuce leaves. The leaves were allowed to air dry in a sterile cabinet for up to 30 min, followed by inoculation with a mycelium plug (5mm) at the center of each leaf. A control was done with lettuce leaves sprayed with sterile distilled water and then with *B. cinerea* plug in the center. The leaves were then stored in a growth chamber at 24°C for 72 h. The percentage of disease reduction of the gray mold on the lettuce leaves was calculated using the following formula:

Reduction rate (%)=[(A-B)/A] X 100, Where; A is the lesion diameter recorded in untreated control, and B is the lesion diameter in the infected lettuce fruit treated with the antagonists [21]. For each treatment, nine leaves were assayed (three leaves as one replicate).

H. Production of antifungal compounds

1) HCN and NH3 production: Bacillus sp. S7LiBe was tested for the production of hydrogen cyanide by adapting the method of[22]. Bacteria were streaked on nutrient agar medium amended with 4.4 g glycine/l, a Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28°Cfor 4 days. A change in color of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong production of HCN by each strain, respectively.The production of ammonia was tested in peptone water. Freshly grown cultures were inoculated into 5 ml peptone water, after incubation period 48hrs at 30°C, 0.25 ml of Nessler's reagent was added to each tube. Development of brown to yellow color indicate production of ammonia [23].

2) Siderophores production: The experiments were carried out in Chrome Azurol S agar according to the method of Schwyn and Neilands [24]. CAS agar was prepared from four solutions, which were sterilized separately before mixing. At 50°C, after autoclaving, nutrition solution and casamino acid solution were added to the buffer solution. Indicator solution was added last with sufficient stirring to mix the ingredients. The bacterial strain was streaked on the surface of the bleu agar plates and incubated at 30°C for 72h and examined for growth and production of orange halos surrounding the colonies.

3) Chitinase Activity: Chitinase activity was determined as described by Kopečný et *al*. [25], Bacterial strain was inoculated in minimal salt medium containing 0.8% of colloidal chitin as carbon and energy source and incubated at 30°C. Clear zone around the colony indicate chitinas activity.

I. Effects of culture medium, pH and temperature on the antagonistic activity of S7LiBe

The influence of the culture medium; pH; salinity and incubation temperature on the antagonistic activity of the bacterial strain was studied according to the method cited in the section (D). Four culture medium (Potato Dextrose Agar PDA; Sabouraud Dextrose Agar SDA; Czapek Dox Agar CZA; Malt Extract Agar MEA) were tested. The culture medium which gave a high PGI%, was prepared at deferent pH value (4.5, 6.5, 8.5, 10.5) to determine the optimum pH. The optimum temperature was studied at, 4°C; 25°C; 28°C; 30°C; 37°C and 44°C,

using the optimum medium and optimum pH. After incubation, the PGI% was calculated as described previously.

III. RESULT AND DISCUSSION

A. Phylogenetic identification of S7LiBe

Comparative analysis of the 16S rDNA sequence of the strain S7LiBewith already available database showed that the Gram-positive isolate was member of the genus Bacillus and the 16S rRNA gene sequence was 99.1% similar toBacillus pichinotyi AF519463 andAF519460 and Bacillus sp. JX152775.The taxonomic positions of S7LiBe is shown in the phylogenetic tree (Figure 1).

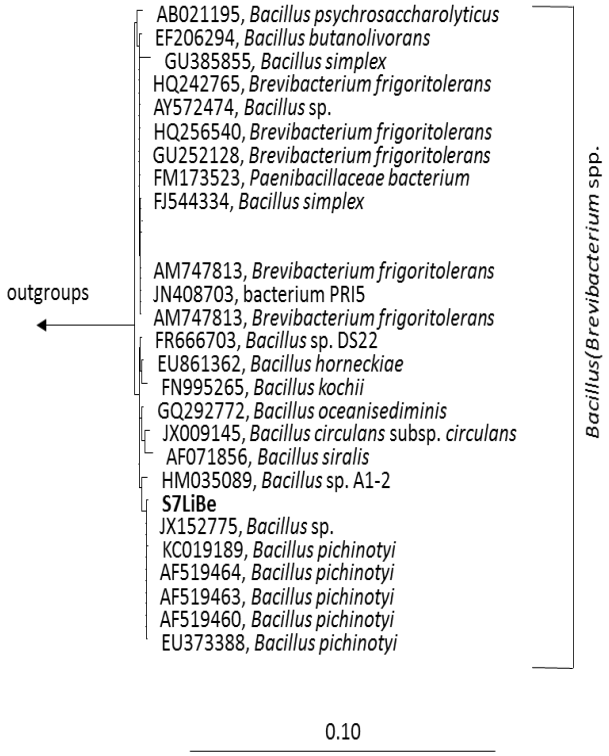

Figure 1 : Phylogenetic relationship of S7LiBe, Dendrogram based on maximum likelihood tree calculation.

B. Effect of the antagonist on the growth mycelium and the germination spores of B. cinera

Six days after incubation, the inhibitory zones were observed in the dual culture dishes (Fig. 2a and b2). *Bacillus* sp. S7LiBe inhibited 70%± 1.33 mycelia growth of *B. cinerea* and it was effective in suppressing germination of *B. cinerea* spores (Fig. 2c and 2d). The percentage spore germination was 25.65% ±6.69 after 24 h of incubation. These results indicate that *Bacillus* sp. S7LiBe grown on PDA plate released an extracellular diffusible metabolite(s) that inhibited the hyphal growth of *B. cinerea*. Several mechanisms have been proposed to explain the antagonist effect of *Bacillus spp.*, including antibiotic production, hydrolytic enzymes synthesis, competition for nutrients, or a combination of these mechanisms in synergy [26, 27, 28]. Alabouvette et al. (2006)[29], suppose that the inhibition of spores in soil germination is due to the competition for nutrients, especially the carbon. Elad and Stewart (2007) [12] showed the sensibility of *B. cinerea* to several antifungal produced by *Bacilus* sp. *Bacillus brevis* and *Bacillus polymyxa* produce the gramicidin S and the polymyxin B which inhibit *B. cinerea* germination *in vitro* and *in vivo.*

C. Effect of the volatile compoundson the growth of B. cinera mycelium

Although the absence of direct contact between bacterial strain and the fungi, a strain *Bacillus* sp. S7LiBe inhibited up to 65.81% ±4,06 of *B. cinerea* mycelium. These result approve that *Bacillus* sp. S7LiBe release a volatile compounds which affected fungi growth.Guetsky et al. (2002)[30] showed that the volatile compounds produced by *Bacillus pumilus,* inhibit the growth of *Botrytis cinerea*. Several studies have been demonstrated that the production of volatile compounds, e.g. HCN; NH3; 2,3-butanediol; acetoin, can influence growth of fungi[31, 32] and improve the plant growth [33, 34, 35, 36].

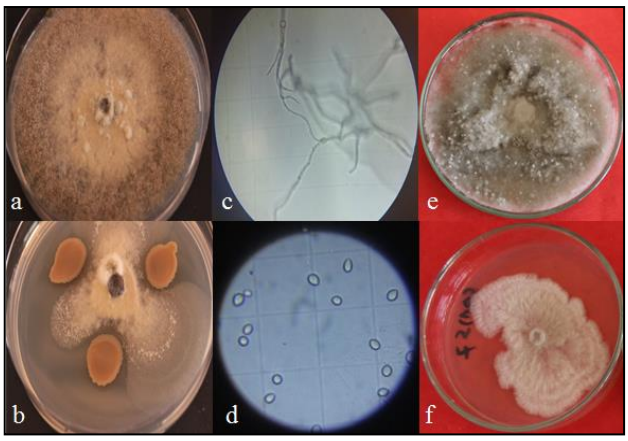

Figure 2 : Antifungal activity of *Bacillus* sp. S7LiBe against *B. cinerea.*(a, c and e) *B. cinerea*BC1 Control in

agar diffusion test, spores germination test and antifungal volatiles test respectively. (b, d and f) Strain *Bacillus* sp. S7LiBe in agar diffusion test, spores germination test and antifungal volatiles test respectively.

D. In vivo antagonism experiments

In vivo evaluation of *Bacillus* sp. S7LiBe as antagonists towards *B. cinerea* on the lettuce leaves showed a remarkable potential (Fig. 3).The antagonist inhibited up to 57% ± 1.65 of *B. cinerea*. These results confirm those obtained in the in vitro tests and those reported by Wang et al. (2009)[37], they showed the potential of *Bacillus subtilis* to inhibit up to 52.4% of *B. cinerea*. According to Elad and Stewart (2007)[12], *Bo. cinerea*, is sensible to the antibiotics produced by some *Bacillus* species in several host.

Figure 3 : Lesion extension in the lettuce leaves by *B. cinerea*. (S7) *Bacillus* sp. S7LiBe, (T) Control.

E. Production of antifungal compounds

1) HCN and NH3 production: In the present study, *Bacillus* sp. S7LiBe was a moderate producer (++) of cyanide and ammonia. Researchers have reported that many *Bacillus* species could secrete several antifungal metabolites such as CHN [33, 34, 38], and NH3 [34, 39]. According to Prashar et al. (2013) [38], the production of antifungal compounds diffusing in the culture medium or volatiles as cyanide and ammonia, affect negatively the fungi growth.

2) Siderophores production: Siderophores production is a major mechanism involved in bio-control by many PGPR groups, including *Bacillus* sp., which produces a large variety [40]. Siderophore production in the CAS agar is characterized by the production of orange halos surrounding the colonies (Fig. 4). In this investigation, *Bacillus* sp. S7LiBe produce an important zone around the colonies and the diameter was up to 4 cm ± 0.6.Several studies have demonstrated the production of siderophores by *Bacillus* sp.[41], the role of these molecules in the control of diseases has been well documented[42], they deprive pathogenic fungi of iron since the fungal siderophores have loweraffinity [43, 44]. These molecules may indirectly stimulate the biosynthesis of other antimicrobialcompounds by increasing the availability of these minerals to the bacteria [45].

3) Chitinase Activity: It has been reported that antifungal mechanism of antagonists has been attributed to the production of hydrolytic enzymes such as chitinase, [46, 47,]. In this investigation, *Bacillus*sp. S7LiBe produce a clear zone around the colonies, indicate the production of Chitinase enzyme. Chitinase-producing microorganisms have been reported as biocontrol agents for different kinds of fungal diseases of plants [48, 49, 50]. Some studies revealed a relationship between chitinases of *Bacillus Sp,* and its ability to inhibit *Fusarium oxysporum* and *Fusarium solani* mycelial growth [51].

F. Effects of culture medium, pH and temperature on the antagonistic activity of S7LiBe

Understanding which environmental factors are important and how these influence disease suppression is widely recognized as a key to improving the level and reliability of biocontrol [45]. However, little knowledge of how specific factors affect the interactions among soil borne plant pathogens and their antagonists[45, 52, 53].In this investigation, the PGI% obtained showed that *Bacillus* sp. S7LiBe inhibit the mycelial growth at different conditions tested (Fig. 4, 5 and 6), however, PDA, pH 4,5 and the temperature 4ºCwerethe least suitable condition for *Bacillus* sp. S7LiBe ($0{,}05 \geq p \geq 0{,}01$).Many factors have been discussed that may affect rhizosphere microbial communities and it is likely soil type, pH, temperature, salinity, organic carbon and inorganic nutrients, as well as the presence of other soil organisms will effect PGPR performance [54, 55, 56].

Carbon sources and minerals have been shown to have an important role in antifungal metabolite production by *Pseudomonas* BCAs, suggesting that nutrient amendments to formulations may also be a useful strategy for improving biocontrol efficacy [45].

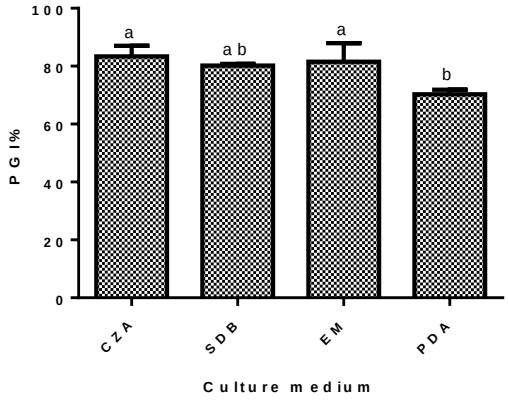

Figure 4: Effects of culture medium on the antagonistic activity of S7LiBe

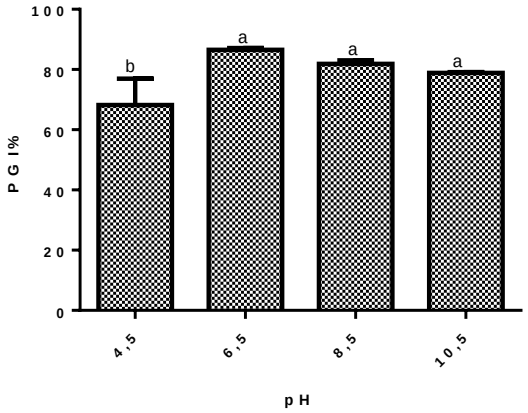

Figure 5 : Effects of pH on the antagonistic activity of S7LiBe

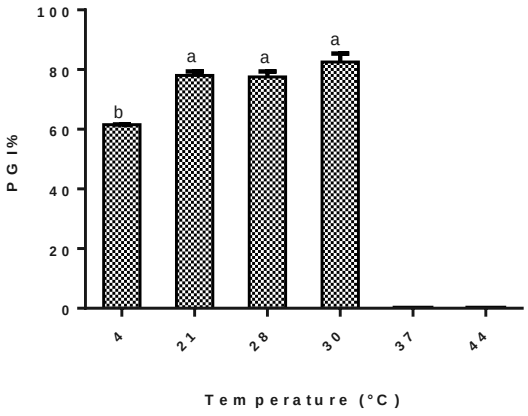

Figure 6 : *Effects of temperature on the antagonistic activity of S7LiBe*

IV. CONCLUSION

Thus the strain S7LiBe selected previously for its ability to produce several promoting growth traits, could be applied in the same time as a PGPR and a biocontrol agent, to improve crop productivity and to prevent fungal attack in crop plant.

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