عزل وتعريف أنواع من الجنس *Bacillus* من ترب سورية واختبار فعاليتها التضادية تجاه الفطر *Botrytis cinerea* في ظروف المخبر *in vitro*

Isolation and Identification of *Bacillus* spp. from Syrian Soils and Testing their Antifungal Activity Against *Botrytis cinerea in Vitro*

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الملخّص

تم عزل وغربلة 378 عزلة من الجنس *Bacillus* من ترب سورية، واختبرت قدرتها في تثبيط نمو الفطر الممرض *Botrytis cinerea* المسبب للعفن الرمادي، وذلك في ظروف المخبر (*in vitro*). أظهرت العزلة B307 أعلى فعالية تضادية في تثبيط نمو الفطر بنسبة بلغت 68 %، وأعلى فعالية في حلمهة الكيتين، وكذلك أظهر مستخلصها الخام الخالي من الخلايا والحاوي على بروتينات بأوزان جزيئية أعلى من 50 كيلو دالتون أعلى فعالية حلمهة للكيتين وتضادية تجاه الفطر *Botrytis cinerea*. تم تحديد هذه العزلة وفقاً لنتائج السلسلة للجينية 16S DNA على أنها *Bacillus lichenformis* بنسبة تشابه بلغت 99 %. أظهرت نتائج هذه الدراسة إمكانية استعمال السلالة *Bacillus licheniformis* B307 ومستخلصها الخام الخالي من الخلايا، والذي له فعالية في حلمهة الكيتين كعامل مكافحة حيوية للفطر *Botrytis cinerea*.

الكلمات المفتاحية: العفن الرمادي، *Bacillus licheniformis*، الفعالية التضادية، الأنزيمات المحلمهة للكيتين، المكافحة الحيوية.

Abstract

Three hundred seventy eight isolates of *Bacillus* spp. from Syrian soils were screened *in vitro* for their ability to inhibit the growth of *Botrytis cinerea*, agent of gray mold disease. The B307 isolate showed the highest antagonism activity with growth inhibition of 68%. In addition, B307 had chitinolytic activity, and the fraction from the isolate with more than 50KDa of its cell free crude extract showed the highest chitinolytic and antagonism activity against *B. cinerea*. This isolate was identified as *Bacillus licheniformis* according to the 16S DNA gene sequencing data with homology of 99%. The results of this study demonstrated that *Bacillus licheniformis* B307 and its free cell crude extract could be used as bio-control agent against *Botrytis cinerea*.

Keywords: gray mold, *Bacillus licheniformis*, antagonistic activity, chitinolytic enzymes, bio-control.

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Introduction

Botrytis cinerea, the causal agent of gray mold, has a wide range of hosts and may infect over 200 plant species in the field, greenhouse and warehouses (Holz *et al*., 2004). It has been considered as one of the common diseases of important crops such as bulb flowers, grape berries, legumes, strawberries and many other fruits and vegetables (Elad *et al*., 2007 ; Tripathi *et al*., 2008). This fungus infect the plant at every stage of its development (Beever and Weeds, 2007), and has been found in the entire parts of the plant, including leaves, fruits, flowers, petioles (Miclea *et al*., 2012). *Botrytis cinerea* is also one of the most important pathogens causing postharvest decay of fresh fruit and vegetables (Romanazzi and Feliziani, 2014). *B. cinerea* is necrotroph, inducing host-cell death which leads to progressive decay of the infected plant tissues. This pathogen produces abundantly sporulating gray mycelium on infected tissues (Staats *et al*., 2004). Economically, it causes annual loses of 10$ to 100$ billion worldwide (Boddy, 2016).

Prevention of pathogens is critically importance in agricultural production systems. In the last decades, the available methods of prevention for the conservation of sustainable agriculture have been evaluated, with emphasis on the importance of using environment friendly and safe methods. In this context, the biocontrol is good and safe alternative of synthetic fungicides, and fulfills consumer requirements for more natural and healthy food (Martinez-Romero *et al*., 2008 ; Gachango *et al*., 2012). The biocontrol has been shown to reduce Botrytis infections successfully on flowers and fruits in many crops using antagonistic microbes and has potential future (Donmez *et al*., 2011; Boddy, 2016).

In fact, many microorganisms have been recruited as biocontrol for various plant diseases, since they have the capability of synthesizing bioactive products that constitute a library of compounds with a large and privileged structural diversity, showing a variety of biological activities (Sihem *et al*., 2011). The search for new antifungal microorganisms with greater potency has been progressed slowly (Gupte *et al*., 2002). In purpose of screening for a new antifungal microorganisms, several researches were oriented towards isolation of new microorganism's species from different soils and ecosystems (Mellouli *et al*., 2003; Errakhi *et al*., 2007). Among the microorganisms, Bacillus species are one of the largest source of bioactive natural products (Emmert and Handelsman, 1999). Several studies have confirmed that *Bacillus* species have a wide range of antimicrobial activities (Nayak *et al*., 2017), since they are used as antifungal and antibacterial (Cukurovali *et al*., 2006 ; Yuan *et al*., 2012).

Bacillus species have the ability to form endospores and synthesize a wide spectrum of metabolites, and they are often considered beneficial and safe to both plants and environment (Shoda, 2000). *Bacillus* antagonistic effects against fungal pathogens may attribute to several compounds, such as antibiotics, volatiles and chitinolytic enzymes. Several *Bacillus* species produce enzymes that degrade chitin, an insoluble linear polymer of β-1,4-N-acetylglucosamine (GlcNAc), which is the second most abundant polysaccharide in nature and the major component of most fungal cell walls (Sadfi *et al*., 2001). Therefore, chitinases are used as potential biocontrol agents for many fungal pathogens through its chitin degradation activity (Gomaa, 2012). The aim of the present study is screening and testing the effectiveness of some local *Bacillus* isolates for being used as biocontrol agent for *B. cinerea*, the causal agent of gray mold disease, *in vitro*.

Materials and methods

B. cinerea isolation and spore suspension preparation

Botrytis cinerea was isolated from strawberry fruits (Akeed *et al*., 2018). The isolate was subcultured on potato dextrose agar (PDA) for 15 days at 25°C in a 12hrs light period to stimulate conidia formation. Spores suspension was prepared by flooding the culture with sterile distilled water containing 0.1% (v/v) Triton$_{X100}$, and dislodging spores from the hyphae by using a sterile glass spreader. The conidial suspension then filtered through sterile absorbent cotton wool plugs to remove any hyphal fragments, and the number of spores in suspension was counted using a hemocytometer and adjusted to 10^5 spores/ml by dilution with sterilized distilled water (Mónaco *et al*., 2009).

***Bacillus* strains**

Samples of soil were collected from different locations of Syria (Damascus, Damascus countryside, Sweida, Salamiyah, Deir ezzor) between 2015 and 2016. 10g of each soil sample were thoroughly mixed in 90ml of sterile distilled water in sterile flasks. Aerobic, Gram-positive spore-forming bacteria were isolated after heating the soil suspensions at 80°C for 10min in order to kill vegetative cells. Single bacterial colonies were obtained by plating of serial dilutions of soil samples on nutrient agar (NA). Colonies were streaked on successive NA plates to obtain pure cultures. Plates were incubated at 30°C until bacterial colonies developed, kept at +4°C and subcultured every two-months. Subsequently, selected isolates were maintained in 20% glycerol at -80°C. The inoculum from each of Bacillus isolates was prepared by inoculating 10ml of sterilized NB in 50ml test tubes with loop full of pure cultures and incubated overnight at 200 rpm and 30°C until the optical density at 600nm reached 0.15 which equal about 2×10^8 CFU/ml (Ammoneh *et al*., 2014).

Antagonistic activity of *Bacillus* isolates against *B. cinerea*

In vitro antagonism tests were performed on NA in 12×12cm Petri plates (divided to 9 parts) by applying a dual culture technique. 2ml of B. cinerea spore suspension were diffusion across the whole of the plate and incubated at 25°C for 24hr, the tested isolates of *Bacillus* were transferred from two-day old cultures to the center of every parts by using sterile toothpicks. After incubation for 5 days Bacillus isolates which have antagonistic activity formed inhibition zone (clear zone CZ) around the bacterial growth zone (GZ). Antagonistic efficiency of isolates was estimated by calculating the ratio of the diameter of clear zone to growth zone (CZ/GZ).

Secondary screening for selected isolates which have efficiency more than 1.5 was carried out, where 10ul of 24-48 hrs culture of *Bacillus* isolate (2×10^8 CFU/ml) were pipetted in the center of NA plate 9cm, and 10ul of *B. cinerea* spore suspension were cultivated in 2.5cm from the center of plate. Growth inhibition of *B. cinerea* was estimated by the formula: **GI= (R1-R2)/R1*100**, where GI is the percentage of growth inhibition, R1 is the radius of fungal growth from the point of inoculation to the colony margin towards the edge of Petri dish after 5 days of incubation as a control value, and R2 is the radius of fungal growth from the point of inoculation to the colony margin towards the center (*Bacillus* growth zone). Growth inhibition was categorized on a scale from 1 to 3, where 1= 1 to 25%; 2= 26 to 50% and 3= 51 to 75% growth inhibition (Sadfi *et al*., 2001).

Detection of chitinolytic activity

The selected isolates of *Bacillus* spp. exhibiting antifungal activity against *B. cinerea* were cultured on a synthetic medium containing (g/l): 1 g K_2HPO_4; 1g NH_4Cl; 0.5 g $CaCl_2.2H_2O$; 2 g Na_2HPO_4; 0.5g NaCl; 0.5g $MgSO_4.7H2O$; 0.5g yeast extract; 20g agar and 0.5% colloidal chitin. Isolates that have chitinolytic activity showed clearing zones on colloidal chitin agar, and the chitinolytic efficiency of isolates was estimated 5 days after incubation at 30°C by calculating the ratio of the diameter of clear zone CZ to growth zone GZ (CZ/GZ).

Colloidal chitin preparation

The colloidal chitin was prepared according to the method described by Rodriguez *et al*., (1983) with some modification: 20g of chitin were dissolved in 500ml of concentrated HCl. The chitin was added to the acid with stirring strongly at 25°C until dissolved (1.5-2 hrs). The mixture was incubated in water bath at 37°C with gentle stirring until the mixture became clear (0.5hrs), then it was filtered using glass wool to remove impurities and particles that did not dissolved. The filtrate was added to 5 liters of cooled distilled water with stirring for 0.5hrs, then placed at 4°C without stirring for 24hrs. The precipitate was collected and washed with distilled water using centrifuge until pH value 5-6, then stored in the dark at 4°C until used. 10ml of colloid chitin were taken and dried at 80°C for 24hrs to calculate dry weight and determine the concentration of chitin.

The inhibitory effect of cell free supernatant of Bacillus isolates (*in vitro*)

A liquid culture of selected isolates was performed on NB medium with 1% colloid chitin and incubated at 30°C with constant shaking at 200 rpm for 48hrs. The culture was removed and centrifuged at 8000g for 10min.

Cell free supernatant was obtained by filter (0.22µm) sterilized and tested for antifungal activity. Two wells (5 mm in diameter) were made in NA plate on the opposite side 2.5cm from the center of plate using a sterilized cork borer, subsequently wells filled with 200µl of filtered supernatant. 10µl of *B. cinerea* spore suspension were inoculated in the center of NA plate, and 10µl of bacteria suspension were inoculated on a distance 2.5 cm from the plate center. Plates were incubated for 5 days at 25°C.

For primary molecular detection of antagonistic and chinolytic enzymes in crud extract of selected Bacillus isolate, it was divided into three parts using concentration tubes (sartorius, vivaspin 2) and centrifuged at 8000g for 30min. The filtered crude extract concentrated first using a 30kDa MWCO tube, then the supernatant concentrated using 50kDa MWCO tube. These three fractions (less than 30kDa; from 30kDa to 50kDa; more than 50kDa) were tested on chitinase screening and antifungal testing plates. Protein determination in samples was performed according to Bradford (1976) and bovine serum albumin was used as a standard.

Bacillus strain identification

The primers BacF (5'-GTGCCTAATACATGCAAGTC-3') and BacR (5'-CTTTACGCCCAATAATTCC-3') flanking a highly variable sequence region of 545 bp towards the 5'end of the 16S rDNA region were used in polymerase chain reaction PCR (Nair *et al*., 2002). Genomic DNA was extracted and purified using DNA extraction kit according to the manufacturer's recommendations (Qiagen, Cat. NO. 69104). PCR mixtures were prepared using 20ng of template DNA, 0.2µM of each primer, and hotstar taq master mix kit (Qiagen, Cat. NO. 203446). Amplification was done under the following conditions: 5 min denaturation step at 94°C, followed by 40 amplification cycles (1min at 94°C, 1min at 55°C and 2min at 72°C) and an extra extension step of 10min at 72°C. PCR products were separated on a 1.2% agarose gel to which ethidium bromide was added and photographed under UV light. Amplification products were purified using QIAqick Gel Extraction kit (QIAGEN, Cat. No.28704) and sequenced on both strands using an ABI 310 sequencer machine (Department of Molecular Biology and Biotechnology, AECS). The sequences were subjected to a BLAST search against the full GenBank database available at NCBI public database using Basic Local Alignment Search Tool for Nucleotides (BLASTN).

Results and discussion

Screening of _Bacillus_ isolates for antagonistic activity against _B. cinerea_

Three hundred seventy eight isolates of *Bacillus* were tested for their efficacy in inhibiting growth of *B. Cinerea* and only 123 of them showed clear inhibition zone. This clearly suggested that these isolates possess antagonism against *B. cinerea* in vitro, that may attribute to the production of antifungal compounds which reduced the mycelial growth of *B. cinerea* by forming an inhibition zone. The results of the *in vitro* dual culture screening revealed that 54 isolates reduced the mycelial growth of *B. cinerea* with high efficiency, where 31 isolates of them were obtained from Damascus countryside, 18 from Deir ezzor, 3 from Salamya, and 2 from Sweida. These isolates were subject to additional screening step (Figure 1).

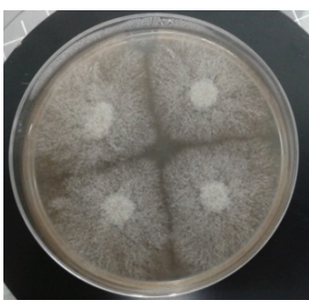
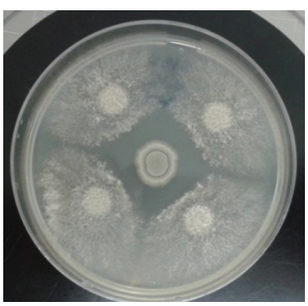
a   b

Figure 1. Secondary screening of selected isolates, example of _in vitro_ inhibition assay.

a: control; b: _Bacillus_ isolate showing their ability to inhibit growth of _B. cinerea_. Bacteria in the center and fungi in the four corners.

The results also showed that 7 isolates named B1, B19, B119, B190, B251, B262 and B307 were within category 3 (Table 1), and B307, isolated from Salamiyah, exhibited the highest growth inhibition of *B. cinerea* (68%) followed by the isolates B262, B119, B251, B190, B19, and B1 with an inhibition of 57, 56, 55, 53, 53, and 52% respectively. Several studies have pointed to the use of *Bacillus* species to inhibition of *Botrytis cinerea* growth (Table 2).

Table 1. Effect of *Bacillus* isolates from Syrian soils on *in vitro* growth of *Botrytis cinerea* (secondary screening).

***Bacillus* spp. Isolates**	**GI category[1]**	***Bacillus* spp. Isolates**	**GI category[1]**	***Bacillus* spp. Isolates**	**GI category[1]**
B 1	3	B 139	2	B 220	2
B 19	3	B 144	1	B 223	2
B 23	1	B 156	1	B 227	1
B 29	2	B 166	2	B 228	2
B 38	1	B 167	1	B 230	2
B 42	2	B 169	2	B 241	2
B 51	2	B 171	2	B 247	2
B 57	1	B 172	1	B 250	1
B 61	2	B 187	1	B 251	3
B 81	2	B 190	3	B 256	2
B 106	2	B 191	1	B 262	3
B 114	2	B 195	1	B 280	1
B 116	2	B 196	2	B 284	1
B 118	1	B 198	1	B 293	2
B 119	3	B 203	2	B 304	1
B 124	1	B 204	2	B 307	3
B 126	1	B 207	2	B 375	2
B 136	2	B 209	2	B 377	2

[1] **Percent growth inhibition was determined 5 days after incubation using Whipps' (1987) formula. Values were categorized on a scale from 1 to 3, where 1: 1 to 25%; 2: 26 to 50%; 3: 51 to 75%.**

Table 2. Antagonism activity of *Bacillus* spp. against *Botrytis cinerea*.

***Bacillus* spp.**	**Growth inhibition activity**	**Reference**
Bacillus sp.	53%	Kefi *et al*., 2015
B. mojavensis	65%	Nihorimbere and Ongena, 2017
B 307	68%	Current study
B. amylolyquefaciens	69%	Nihorimbere and Ongena, 2017
B. thuringiensis UM96	70%	Martínez *et al*., 2014
Bacillus subtilis strain GA1	70%	Toure *et al*., 2004
B.amylolyquefaciens	83%	Nihorimbere and Ongena, 2017
Bacillus subtilis	92%	Gao *et al*., 2017

Detection of chitinolytic activity

The results showed that among the seven isolates that exhibited the highest rate of inhibition of B. cinerea growth, only B307 was able to hydrolyze colloidal chitin and to form large clearing zones around the growth zone with efficiency 2 (figure 2), therefore this isolate was selected for further studies.

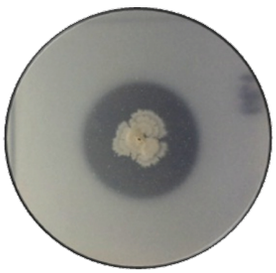


Figure 2. Chinolytic activity on agar medium showing clear zone around *Bacillus* growth zone.

***Bacillus* strain B307 identification**

Identification of isolate B307 was performed using 16S DNA gene sequences. The nucleotide BLAST similarity search analysis, based 16S DNA gene sequence revealed that this isolate belongs to the *Bacillus* genus. The closest phylogenetic neighbor according to the 16S DNA gene sequence data for B307 was *Bacillus licheniformis*, with 99% of homology (Accession number KY196419.1). Lee *et al,* (2006) have reported the use of strain *Bacillus licheniformis* N1 for the biological control for tomato gray mold caused by *Botrytis cinerea*.

***In vitro* inhibitory effect of cell free supernatant of *Bacillus licheniformis* B307**

Current study results showed that the cell free supernatant of *Bacillus licheniformis* B307 strain was sufficient to inhibit mycelium growth (figure 3a). However, for the other tested isolates, the inhibition of *B. cinerea* growth was restricted by the presence of bacteria cells (figure 3b).

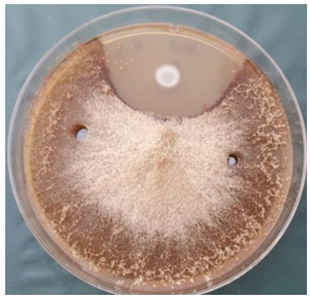
a   b

Figure 3. *in vitro* growth inhibition of *B. cinerea* growth by *Bacillus* on nutrient agar medium and by cell free culture supernatant, a: B307, b: B119.

Three major parts of B307 crude extract (2: less than 30kDa; 3: from 30kDa to 50kDa; 4: more than 50kDa) were fragmented and examined for their antifungal and chinolytic activity (Figure 4).

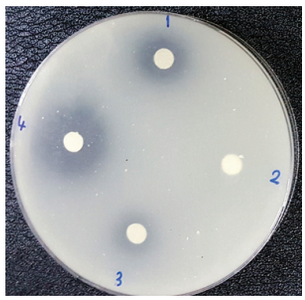
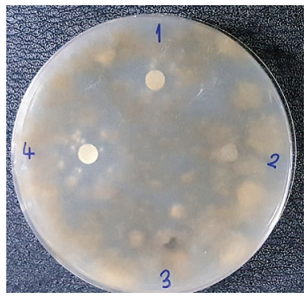
a   b

Figure 4. Test of chitinolytic (a) and antifungal activity (b) for: 1: crude extract; and fractions (2: less than 30kDa; 3: from 30kDa to 50kDa; 4: more than 50kDa). 50µl (protein concentration: 10µg/µl) from each part was added to each sterile filter paper 5mm.

The results also showed that fraction no. 4 had the highest chitinolytic and antifungal activity against *B. cinerea* whereas fraction no. 2 did not show any activity. Fractions no. 3 and 4 will be purified to determine their chitinase activities in a future studies. Many researches have been recorded isolation and purification of chitinases produced by *Bacillus licheniformis* of different molecular weights: 89, 76, 72, 66, 62, 59, 53, 49, and 42kDa (Kudan and Pichyangkura, 2009; Takayanagi *et al*., 1991; Trachuk *et al*., 1996). A number of studies have indicated the effectiveness of the produced chitinases from *Bacillus licheniformis* in inhibiting the growth of a number of plant pathology fungi (kim *et al*., 2007; Gomaa 2012).

Conclusion

The results of this study demonstrated the ability of the local Bacillus isolate *Bacillus licheniformis* B307 to inhibit growth of *B. cinerea in vitro*. This ability associated with a chitinolytic activity, and the free cell crude extract of its liquid culture showed ability to inhibit the growth of the fungus and production of chitinase. We will investigate the optimization of chitinase production from the selected strain B307 and test its antagonism against *B. cinerea in vivo*.

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Reference

- Akeed, Y., F. Atrash and W. Naffaa. 2019. Morphological and Molecular Identification of *Botrytis cinerea* the Causal Agent of Gray Mold Isolated from Strawberry Fruits. J. Arid Environ. (ACSAD).Vol.12 (1-2): 47-56.
- Ammoneh, H, M. Harba, Y. Akeed, M. Al-Halabi and Y. Bakri. 2014. Isolation and identification of local *Bacillus* isolates for xylanase biosynthesis. Iran J Microbiol, 6(2): 127.
- Beever, R. E and P.L. Weeds. 2007. Taxonomy and genetic variation of Botrytis and Botryotinia. In: Elad Y, Williamson B, Tudzynski P, Delen N, eds. *Botrytis*: Biology, Pathology and Control. Dordrecht, The Netherlands: Kluwer Academic Publishers, 29–48. http://dx.doi.org/10.1007/978-1-4020-2626-3_3.
- Boddy, L. 2016. Chapter 8 - Pathogens of Autotrophs, In The Fungi (Third Edition), edited by Sarah C. Watkinson, Lynne Boddy and Nicholas P. Money, Academic Press, Boston: 245-292.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72: 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3
- Cukurovali, A., I. Yilmaz, S. Gur and C. Kazaz. 2006. Synthesis, antibacterial and antifungal activity of some new thiazolylhydrazone derivatives containing 3-substituted cyclobutane ring. Eur J Med Chem, 41:201-207. http://dx.doi.org/10.1002/chin.200631115
- Donmez, M.F., A. Esitken, H. Yildiz and S. Ercisli. 2011. Biocontrol of *Botrytis cinerea* on strawberry fruit by plant growth promoting bacteria. J Anim Plant Sci, 21: 758-763.
- Elad, Y., B. Williamson, P. Tudzynski and N. Delen. 2007. *Botrytis*: Biology, Pathology and Control. Dordrecht, The Netherlands: Kluwer Academic Publishers. http://dx.doi.org/10.1007/978-1-4020-2626-3
- Emmert, E.A.B and J. Handelsman. 1999. Biocontrol of plant disease: A (Gram-) positive perspective. FEMS Microbiol Lett, 171: 1-9. http://dx.doi.org/10.1111/j.1574-6968.1999.tb13405.x
- Errakhi, R., F. Bouteau, A. Lebrihi and M. Barakate. 2007. Evidences of biological control capacities of *Streptomyces* spp. against *Sclerotium rolfsii* responsible for damping-off disease in sugar beet (*Beta vulgaris* L.). World J Microbiol Biotechno, 23: 1503-1509. http://dx.doi.org/10.1007/s11274-007-9394-7
- Gachango, E., W. Kirk, R. Schafer and P. Wharton. 2012. Evaluation and comparison of biocontrol and conventional fungicides for control of postharvest potato tuber diseases. Biol Control, 63(2): 115-120. http://dx.doi.org/10.1016/j.biocontrol.2012.07.005
- Gao, Z., B.Zhang, H. Liu, J. Han and Y. Zhang. 2017. Identification of endophytic *Bacillus velezensis* ZSY-1

strain and antifungal activity of its volatile compounds against Alternariasolani and *Botrytis cinerea*. Biol Control, 105: 27-39.
- Gomaa, E.Z. 2012. Chitinase production by *Bacillus thuringiensis* and *Bacillus licheniformis*: their potential in antifungal biocontrol. J Microbiol, 50: 103-111. http://dx.doi.org/10.1007/s12275-012-1343-y
- Gupte, M., P. Kulkarni and B.N. Ganguli. 2002. Antifungal Antibiotics. Appl Microbiol Biotechnol, 58 : 46–57.
- Holz, G., C. Sonja and B.Williamson. 2004. The ecology of *Botrytis* on plant surfaces, Springer, In: Elad *et al.* (eds.), Botrytis: Biology, Pathology and Control, 9 – 27. http://dx.doi.org/10.1007/978-1-4020-2626-3_2
- Kefi, A., I.B. Slimene, I. Karkouch, C. Rihouey, S.Azaeiz, M. Bejaoui and F. Limam. 2015. Characterization of endophytic *Bacillus* strains from tomato plants (*Lycopersicon esculentum*) displaying antifungal activity against *Botrytis* cinerea Pers. World J Microbiol Biotechno, 31: 1967-1976.
- Kim, J.H., S.H.Lee, C.S.Kim, E.K.Lim, K.H.Choi, H.G.Kong and B.J.Moon. 2007. Biological control of strawberry gray mold caused by *Botrytis cinerea* using *Bacillus licheniformis* N1 formulation. Int J Microbiol Biotechnol, 17: 438-444.
- Kudan, S., and Pichyangkura R. 2009. Purification and characterization of thermostable chitinase from *Bacillus licheniformis* SK-1. Biotechnol Appl Biochem, 157: 23. http://dx.doi.org/10.1007/s12010-008-8328-7
- Lee, J.P., S.W.Lee, C.S. Kim, J.H. Son, K.Y. Lee and B.J. Moon. 2006. Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. Biol control, 37: 329-337. http://dx.doi.org/10.1016/j.biocontrol.2006.01.001
- Martínez-Absalón, S., D.Rojas-Solís, R. Hernández-León, C. Prieto-Barajas, M.D.C. Orozco-Mosqueda, J.J. Peña-Cabriales and G. Santoyo. 2014. Potential use and mode of action of the new strain *Bacillus thuringiensis* UM96 for the biological control of the grey mould phytopathogen *Botrytis cinerea*. Biocontrol sci techn, 24:1349-1362.
- Martinez-Romero, D., M. Serrano, G.Bailen, F. Guillen, P.J.Zapata, J.M. Valverde, S. Castillo, M. Fuetes and D. Valero. 2008. The use of a natural fungicide as an alternative to preharvest synthetic fungicide treatments to control lettuce deterioration during postharvest storage. Postharvest Biol Technol, 47:54-60.
- Mellouli, L., R.B. Mehdi, S. Sioud, M. Salem and S. Bejar 2003. Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated Streptomyces sp. US24 strain. Res Microbiol, 154: 345–352. http://dx.doi.org/10.1016/S0923-2508(03)00077-9
- Miclea, R.V, L.A. Suciu and C.E. Puia. 2012. *In Vitro* Studies Regarding the Morphology of Botrytis Cinerea Pers. Isolated from Geranium Plants. ProEnvironment Promediu, 5: 60-66.
- Mónaco, C., G. Dal Bello, M.C. Rollán, L. Ronco, G. Lampugnani, N.Arteta and M. Stocco. 2009. Biological control of *Botrytis cinerea* on tomato using naturally occurring fungal antagonists. Archives of Phytopathology and Plant Protection, 42: 729-737.
- Nair, J.R, G. Singh and V. Sekar. 2002. Isolation and characterization of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. J Appl Microbiol, 93: 772-780. http://dx.doi.org/10.1046/j.1365-2672.2002.01756.x
- Nayak, S., C. Limsuwan, N. Chichurd, K.J. Kühlmann and S. Pungpang. 2017. Antimicrobial activity of partially characterized analytes from *Bacillus pumilus* (B2). Aquac Res, 48: 5606-5613. http://dx.doi.org/10.1111/are.13382
- Nihorimbere, V., and M.Ongena. 2017. Isolation of Plant growth-promoting *Bacillus* Strains with Biocontrol Activity *In vitro*. MRJMBS, 5: 13-21.
- Rodriguez-Kabana, R., G. Godoy, G. Morgan-Jones and R.A.Shelby. 1983. The determination of soil chitinase activity: conditions for assay and ecological studies. Plant Soil, 75:95-106. http://dx.doi.org/10.1007/BF02178617
- Romanazzi, G., and E. Feliziani. 2014. Chapter 4 - *Botrytis cinerea* (Gray Mold), In Postharvest Decay, edited by Silvia Bautista-Baños,, Academic Press, San Diego. 131-146.
- Sadfi, N., M. Cherif, I. Fliss, A. Boudabbous, and H. Antoun. 2001. Evaluation of bacterial isolates from salty

soils and *Bacillus thuringiensis* strains for the biocontrol of *Fusarium* dry rot of potato tubers. J Plant Pathol, 83: 101-117.
- Shoda, M. 2000. Bacterial control of plant disease. J. Biosci Bioeng, 89: 515-521. http://dx.doi.org/10.1016/S1389-1723(00)80049-3
- Sihem, B.M, E. Rafik, F. Mathieu, C. Mohamed and A. Lebrihi. 2011. Identification and partial characterization of antifungal and antibacterial activities of two *Bacillus* sp. strains isolated from salt soil in Tunisia. Afr J Microbiol Res, 5:1599-1608.
- Staats, M., P. Van Baarlen and J.A.Van Kan. 2004. Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Mol Biol Evol, 22: 333-346. http://dx.doi.org/10.1093/molbev/msi020
- Takayanagi, T., K. Ajisaka, Y. Takiguchi and K. Shimahara. 1991. Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. BBA Protein Struct Mol Enzym 1078: 404-410. http://dx.doi.org/10.1016/0167-4838(91)90163-T
- Toure, Y., M.A.R.C.Ongena, P.Jacques, A.Guiro and P. Thonart. 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J Appl Microbiol, 96:1151-1160. http://dx.doi.org/10.1111/j.1365-2672.2004.02252.x
- Trachuk, L.A, L.P.Revina, T.M. Shemyakina, G.G.Chestukhina and V.M.Stepanov. 1996. Chitinases of *Bacillus licheniformis* B-6839: isolation and properties. Can J Microbiol, 42: 307-315. http://dx.doi.org/10.1139/m96-046
- Tripathi, P., N.K.Dubey and A.K.Shukla. 2008. Use of some essential oils as post-harvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. World J Microbiol Biotechno, 24: 39-46. http://dx.doi.org/10.1007/s11274-007-9435-2
- Yuan, J., W.Raza, Q. Shen and Q.Huang. 2012. Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. Appl Environ Microbiol, 78: 5942-5944. http://dx.doi.org/10.1128/AEM.01357-12
- Whipps, J.M. 1987. Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. New Phytol, 107: 127-142. http://dx.doi.org/10.1111/j.1469-8137.1987.tb04887.x

N° ref: 915