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ANTAGONISTIC ACTIVITIES OF *Bacillus amyloliquefaciens* FROM PHYLLOSPHERE OF SUGAR BEET AGAINST *Cercospora beticola sacc.*

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ABSTRACT

Sixteen isolates of *Bacillus amyloliquefaciens* isolated from phyllosphere of sugar beet leaves were tested for its antagonistic activity against *Cercospora beticola* the causal agent of sugar beet leaf spot disease. The tested isolates showed a significant antagonistic potential against *C. beticola in vitro*. The inhibition of the radial growth of the pathogen ranged from 77.8% to 90%. The diffusible metabolites and volatile gases produced by *B. amyloliquefaciens* dramatically inhibited the pathogen growth. The antifungal proteins (AFP) extracted from culture filtrates showed an antagonistic effect on the pathogen and inhibition zones ranged from 8.17 mm to 16.45 mm. The antifungal metabolites (AFM) produced by the tested *B. amyloliquefaciens* isolates showed a high thermal stability. The activity of the AFM at pH 3.0 upto 5.0 reduced by 20% and started to increase at pH 6.0 then reached its maximum at pH 7.0. All the tested isolates produced Sidrophores and β1,3,glucanase while only three isolates were able to produce chitinase. In the greenhouse experiments, the AFM and bacterial cells treatments decreased the severity of cercospora leaf spot. It was found that the treatments with AFM or bacterial cells before inoculation with the pathogen was more efficient than the treatments after inoculation with the pathogen. This may be the first report on using *B. amyloliquefaciens* as a biocontrol agent against *C. beticola.*

Keywords: *Bacillus amyloliquefaciens,* Cercospora leaf spot, Antagonism, biological control.

INTRODUCTION

A wide range of microbes including bacteria (Wulff *et al.*, 2003), filamentous fungi (Punja and Utkhede, 2003), and Oomycetes (Picard *et al.*, 2000) have been employed as biological control agents. Biocontrol agents can be effective through the production of hydrolytic enzymes and antibiotics, niche colonization and competition for host nutrients, induction of plant host defense mechanisms, and interference with pathogenicity factors (Punja and Utkhede, 2003).

Integration of *Bacillus*-based biological control agents (BCAs) with breeding for disease resistance has proven to be useful in management of several disease problems, particularly where high yielding, highly resistant cultivars are not available. Published examples include Cercospora leaf spot (CLS) of sugar beet (Jacobsen *et al.*, (2002) and Larson, (2004) and other plant diseases showed that, disease control involving a *Bacillus*-based BCA was better on the more resistant cultivars. Larson, (2004) showed that a BCA, *Bacillus mycoides* isolate Bm J, provided control of Cercospora leaf spot equal to synthetic fungicides as measured by the area under the disease

progress curve (AUDPC) on a sugar beet hybrid that had a moderate level of resistance.

Antibiotic production by some bacteria plays a major role in disease suppression, including *Bacillus* spp. (Cook *et al.*, 1995). *Bacillus* spp. have been reported to inhibit the growth of a number of plant pathogens through antagonism, with multiple modes of action such as the production of antibiotics (iturin, surfactin, fengycin), enzymes that degrade fungal structural polymers (chitinase, β-1,3-glucanase), and antifungal volatiles (Leelasuphakul *et al.*, 2006). Strains of *Bacillus subtilis* also have been studied as biological control agents of plant pathogens (Cook *et al.*, 1995). Only a few antibiotics produced by some strains were isolated and identified, and their role in biological control has been studied (Asaka and Shoda, 1996). Many reports were founded on the use of *Bacillus subtilis, B. pumilus* and *B. mycoides* as a biocontrol agents for *Cercospora beticola* on sugar beet while no reports found for *Bacillus amyloliquefaciens* on *C. beticola* . However, reports found on its use on other plant pathogens *Rhizoctonia solani* (Yu *et al.*, 2002), *Botrytis cinerea* (Mari *et al.*, 1996) *Colletotrichum lagenarium* (Kim and Chung, 2004) *Sclerotinia sclerotiorum* (Mansour *et al.*, 2008).

B. amyloliquefaciens is closely related to *B. subtilis*. Several strains were reported effective for control of plant pathogens (Mari *et al.*, 1996; Yoshida *et al.*, 2001). Antibiotic production may play an important role in their biocontrol activity (BCA). Yu, (1998) ;Yu and Sinclair, (1997) and Yu *et al.*, (2002) showed that *B. amyloliquefaciens* B94 inhibited a variety of fungal plant pathogens suggesting that antibiotic production was involved in disease-suppression. Identification of the antibiotics produced may improve our understanding of the mechanism involved in this and other biocontrol systems.

Protection of sugar beet against CLS still lies mainly on fungicide application. The development of resistant or tolerant varieties can help to maintain crop yield under heavy disease pressure, reducing the quantities of fungicides applied. Sugar beet breeders find it still very difficult to obtain highly resistant hybrids with a root yield potential equal to the susceptible ones grown in the absence of *C. beticola*. In fact, CLS resistance is a quantitative trait, controlled by at least five loci, and it is characterised by a low heritability (Setiawan *et al.*, 2000).

The aim of this work is investigating some of the biological characteristics of the *B. amyloliquefaciens* antifungal compound, including its activity against *C. beticola*, and a suggested mode of action *in vitro* and *in vivo*.

MATERIALS AND METHODS

Source of *Cercospora beticola* isolate.

A virulent isolate of *C. beticola* (isolated from Kafr El-Sheikh governorate, Egypt) was obtained from the Cercospora collection in the Department of

Sugar Crops Pest and Diseases, Sugar Crops Research Institute, ARC, Giza to be use in this study.

Source of *B. amyloliquefaciens* isolates.

Sixteen *B. amyloliquefaciens* isolates formally isolated from sugar beet phyllosphere cultivated in different regions in Egypt (Behera governorate, BH; Dakahleia governorate, DK; Giza governorate, Gz; Kafr elshaikh governorate, Ksh and Sharkeia governorate, SHR) Egypt and identified in Sugar Crops Pests and Diseases Dept., SCRI, ARC, Egypt following the key for identifying bacteria, according to Fahy and Persely, (1983) and Garrity *et al.,* (2005). The results were confirmed by the analysis using the computer software Probabilistic Identification of Bacteria for Windows (PIB) developed by the University of Southampton, United Kingdom and available online at http://www.som.soton.ac.uk/staff/tnb/pib.htm. and the keys for Bacillus genus (Garrity *et al.,* 2005).

Antagonism of *B. amyloliquefaciens* isolates.

The development of bioantagonistic bacteria against *C. beticola* was tested *in vitro*. Interactions between antagonistic bacteria and *C. beticola* were determined by the novel ring method described by Kucuk and Kyvanc (2003). One 5-mm disk of a pure culture of *C. beticola* was placed at the center of a Petri dish containing PDA. A circular line, made with a 6-cm diameter Petri dish dipped in a suspension of bioantagonistic bacteria (5 x 10^9 cfu ml^{-1}), was placed surrounding the fungal inoculum. Plates were incubated for 14 days at 28ºC and growth diameter of the pathogen was measured and compared to control growth where the bacterial suspension was replaced by sterile distilled water. The Percentage inhibition was calculated using the following formula:

% inhibition = (1 - (Fungal growth(mm) / Control growth (mm)) x 100 .

Production of diffusible metabolites.

PDA plates, covered with a sterile nitrocellulose membrane, and inoculated in the center with 100 µL of a bioantagonistic bacterial suspension (5 x 10^9 cfu ml^{-1}). After incubation for 72 hrs at 28ºC, the membrane with the grown bacterial isolate was carefully removed (Fig. 1), and the plates were incubated for 24 hrs at 28ºC to assure sterility. A 10 ml of sterilized PDA layer (40ºC) added to the plates and left to solidify. The plates inoculated in the middle with a 5-mm in diameter disk of a pure culture of *C. beticola* and were incubated at 28ºC for 14 days. Each trial with a single bacterial isolate was run in triplicates. The percentage of inhibition of the growth of *C. beticola* was determined as mentioned before.

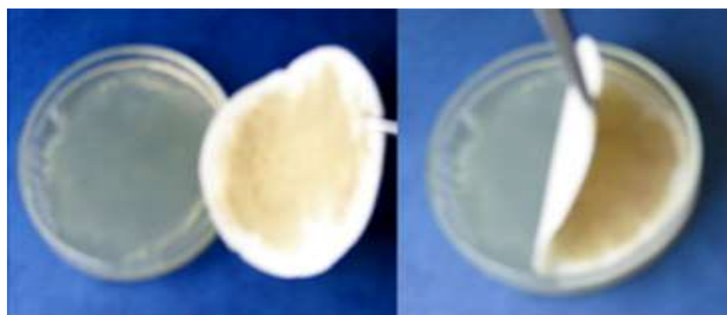

Fig.1. Production of diffusible metabolites by *B. amyloliquefaciens* in PDA.

Production of volatile antibiotics.

100 µL of a antagonistic bacteria ($5x10^9$ cfu ml^{-1}) were placed at the center of one half a Petri dish containing NA medium, and the lid of the dish containing PDA was inoculated at the center with a 5-mm disk of 30 days an old culture of *C. beticola.* the halves were placed face to face preventing any physical contact between the pathogen and the bacterial suspension, and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed (Arrebola *et al*., 2010). Plates were incubated at 28ºC for 15 days and the growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonist. Each experiment considering a single bacterial isolate was run in triplicate. The percentage of inhibition of *C. beticola* was determined.

Antifungal activity (AFA) of co-culture filtrates

C. beticola was grown in 100 ml nutrient broth for 10 days at 30 ºC, and then the bacterial isolates were inoculated and co-cultured, incubated on a rotary shaker (100 rpm for 3 days) at 30 ºC. After incubation, the bacterial and fungal cells were separated from the culture by centrifugation at 10.000 g for 20 min at 4 ºC. The resulted supernatant sterilized by filtration through 0.22 µm filter membrane.

Isolation of antifungal proteins (AFP) from culture filtrates

The total proteins in the resulted culture filtrate (300 ml) precipitated by adding solid ammonium sulfate to the culture filtrate to reach 30% saturation and incubated overnight at 4 ºC. The resulting precipitates were harvested by centrifugation (10,000g for 40 min) at 4 ºC. The pellets were dissolved in 50 ml of 25 mM of Tris–HCl buffer (pH 8.1) and dialyzed in the same buffer for 24 h with three changes at 4ºC. The resulting protein solution was precipitated by adding 2 volumes of cold acetone and kept in -20 ºC for 1 hours. The precipitated proteins were separated by centrifugation (10,000g for 20 min) at 4 ºC. The precipitated proteins were air dried and dissolved in 15 ml of sterilized phosphate buffer pH 6.5 then resterilized by filtration through 0.22 µm sterilized Millipore filter and kept in 4 ºC for further investigations (Son and Kim, 2002). The concentration of the extracted proteins were normalyzed using Bradford protein assay kit.

Activity of the isolated AFP.

In the center of a Petri dish containing PDA, a 0.5 cm diameter disc, taken from the colony edge of a 14-day culture (PDA). Two filter paper discs (1cm diameter), imbibed with 20 µL of AFP suspension immediately placed at a distance of 3 cm from the pathogen disc (Rahman *et al.*, 2007). Sterile distilled water was added to the control dishes. Plates were incubated at 30 ºC for 14 days. When the pathogen had completely grown in the control dish, the zone of inhibition of the pathogen was measured.

Heat and pH Stability of the antifungal metabolites (AFM).

Culture filtrates of the tested isolates were produced as mentioned before in nutrient broth. The filtrates were concentrated 5 times under vacuum. The concentrates were subjected to filter sterilization using 0.22 um Millipore filters.

The effect of temperatures (4, 30, 40, 50, 60, 70, 80, 90, 100 and 121 °C) on the activity of AFM was evaluated, an equal volume (1ml) of the concentrated AFM isolated from all the tested *B. amyloliquefaciens* isolates mixed together and divided to nine patches each patch treated with a different temperatures in a water bath and in autoclave in case of the 121°C treatment for 20 min. After allowing the heated samples to cool to 20 °C, all AFM subjected to a bioassay on *C. beticola* as mentioned before. The -4°C treatment was considered as the control treatment.

In case of pH stability test, an equal volums (5 ml each) of the AFM isolated from all the tested *B. amyloliquefaciens* isolates divided to nine patches each patch were adjusted to pH 3, 4, 5, 6, 7, 8, 9, 10, or 11 using 0.5N of NaOH or HCl, and incubated for 24 h at 20 °C. Each culture filtrate and the controls then were restored to pH 7 using bromothymole blue as a pH indicator. The samples of heat and pH stability test were subjected to a bioassay using the filter paper disc method on *C. beticola* as mentioned before.

Chitinase production.

The tested microorganisms were cultured in 250 ml conical flasks containing 50 ml of chitin–peptone medium for bacterial isolates (glucose 0.5%, peptone 0.2%, colloidal chitin 0.2%, (prepared from crab shell chitin (Sigma) according to Berger and Reynolds, 1958), K_2HPO_4 0.1%, $MgSO_4$-$7H_2O$ 0.05% and NaCl 0.05%, pH 6.8) (Lim *et al.,* 1991). All the bacterial cultures incubated at 28 °C for 3 days on a rotary shaker incubator 100 r.p.m.. After the incubation period, the cultures were centrifuged at 10.000 r.p.m. for 20 min. at 4°C and the supernatant was used as crude enzyme source. A mixture of crude enzyme source (1 ml) and suspension of colloidal chitin (1 ml from stock solution 0.1% in 50 mM sodium acetate buffer; pH 5) was incubated at 38°C in a water bath with constant shaking. After 2 hr, the release of *N*-acetylglucosamine in the reaction mixture was estimated by the method of Reissig *et al.,* (1955). The enzyme activity was determined using *N*-acetylglucosamine (Sigma) as the standard. Absorbance was measured at 660 nm using a Milton Roy Spectronic 1201. One unit of chitinase is defined as the amount of enzyme producing 1 µmol *N*-acetylglucosamine/min in 1 ml of reaction mixture under standard assay conditions.

β- 1,3 glucanase production.

Bacterial isolates were grown in 250 ml conical flasks containing 50 ml of peptone medium contained laminarin (0.2%) (From *Laminaria digitata*; Sigma) (Lim *et al.,* 1991), incubated at 28 °C for 3 days on a rotary shaker incubator 100 rpm.. The cultures then centrifuged as previously mentioned and the resulted supernatant used as crude enzyme source. The reaction mixture was the substrate laminarin (Sigma-Aldrich) (2.5% w/v) in 10 mM ammonium acetate, pH 6.0, and 1 mM DTT. The reaction incubated at room temperature for 24 hr. samples were assayed for the release of reducing sugars according to the Somogyi-Nelson method Nelson, (1944) modified by (Naguib 1964 and 1965). Absorbance was measured at 660 nm using a Milton Roy Spectronic 1201. Standard curve of glucose was used as reference (Lim *et al.,* 1991). β 1,3 glucanase activity was determined as 1 nmol of glucose released per minute per ml.

Siderophore production.

The tested bacterial isolates were grown in King's broth for 3 days at 28°C and centrifuged at 10000 r.p.m. for 10 min. The pH of the supernatant was adjusted to 2.0 with diluted HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. Five milliliters of ethyl acetate fraction was mixed with 5ml of Hathway's reagent (1.0 ml of 0.1M $FeCl_3$ in 0.1 N HCl to 100 ml distilled water then 1.0 ml of potassium ferricyanide). The absorbance for dihydroxy phenols measured at 700nm using Milton Roy Spectronic 1201 (Reeves *et al.,* 1983).

Effect of *B. amyloliquefaciens* isolates and its AFM on CLS severity under greenhouse conditions.

Greenhouse experiment was conducted in Sugar Crops Research Institute, at Giza, Egypt to evaluate the potential of *B. amyloliquefaciens* isolates on control of CLS disease.

Preparation of Bioagents

Isolates were grown on nutrient broth medium (pepton 10g, yeast extract 5g, Nacl 5g) for 3 days on rotary shaker (150 rpm) at 28°C . After incubation, the cultures were centrifuged at 10.000 rpm at 4C for 20 minutes. The supernatant was separated carefully and the growth was washed 2 times with sterilized distilled water and resuspended in distilled water. The cell density adjusted to $3x10^6$ cfu/ml by distilled water according to the method described by (Douglas *et al.* 2003).

Preparation of *C. beticola* Inoculum

C. beticola, inoclum was prepared according to the method described by Vereijssen *et al.,* (2003) and Esh, (2005) and diluted with sterilized distilled water to reach 3×10^4 cfu/ml.

Inoculation of Sugar Beet Plants

Sugar beet plants were grown in 30 cm diameter pots filled with sand: peat moss: clay soil (1:1:1) and cultivated with Sugar beet plants variety Kawmera. Plants were divided to four groups the first and the second group treated by the tested bacterial cells 2 times before inoculation with *C. beticola* in 7 days intervals and the second group treated with bacterial cells after 24 hours from inoculation with *C. beticola* 2 times in 24 hours intervals. The

third and fourth groups were treated with the same way but the bacterial cells replaced by the culture filtrates. *C. beticola* conidial suspension atomized on sugar beet leaves from all directions until run off. After inoculation with C. *beticola*, plants were irrigated and covered with transparent plastic bags to serve as a moist chamber and the greenhouse fog system was kept running for 5-days. Both procedures were to increase the greenhouse humidity to above 90%. After 5 days, the plastic sheets removed, and plants kept on the bench to allow disease development (Esh, 2005 and Taghian, Shadia 2008). The percentage of disease severity reduction was calculated using the severity readings in the formula ((untreated infected control-treatment)/untreated infected control)x100

RESULTS AND DISCUSSION

The antagonistic activity of *B. amyloliquefaciens* isolates.

Data presented in Table 1 and Fig 2 show that, all the tested isolates had significant potential antagonistic effect against *C. beticola*. The inhibition of the pathogen radial growth ranged from 77.8% (BmSHR2) to 90% (BmKsh2). No physical contact was observed between any of the bacteria tested and *C. beticola*; moreover, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria.

It has been described that *B. amyloliquefaciens* can secrete several antifungal metabolites which belong to the iturine family (Yu *et al*., 2002). Several strains were reported effective for control of plant pathogens (Asaka and Shoda, 1996; Mari *et al*., 1996; Yoshida *et al.,* 2001). Antibiotic production may play an important role in their BCA (Yu *et al*., 2002).

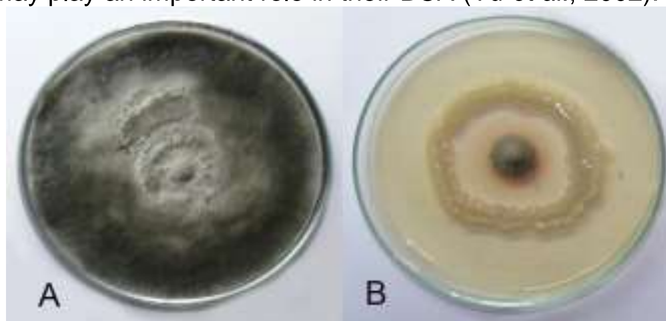

Fig.2: Antagonistic effect of *B. amyloliquefaciens* in dual culture (novel ring method) against *C. beticola*. A: *C. beticola* control B: *C. beticola* surrounded by *B. amyloliquefaciens* (BmKSH5) .

Production of diffusible antifungal metabolites (AFM).

The diffusible metabolites of the tested *B. amyloliquefaciens* dramatically affected the pathogen growth of the entire tested isolates Table 2 and Fig. 2&3. The diffusible metabolites completely (100%) inhibited the fungal growth except isolates BmDK1 (82%) and the isolate BmSHR2 (92.9 %). Culture filtrates of *B. amyloliquefaciens* KPS46 also inhibited mycelia growth of

various taxonomically diverse phytopathogenic fungi (Prathuangwong and Buensanteai 2007), indicating that compounds in the filtrates exhibit a wide spectrum of antimicrobial activity.

Bacillus spp., particularly *B. subtilis,* have been reported to produce many types of antimicrobial peptide substances, such as iturin A and surfactin (Leclere *et al.,* 2005). Yu *et al.*, (2002) purified three antifungal compounds from the culture broth of *B. amyloliquefaciens* strain B94, the three compounds identified as isomers of iturin A. These compounds were responsible for inhibition of *R. solani* growth *in vitro*. They sujested that iturin A may play an important role in disease suppression. *B. amyloliquefaciens* has been studied extensively as a producer of enzymes, such as (amylase, subtilisin (a protease), and barnase (a ribonuclease).

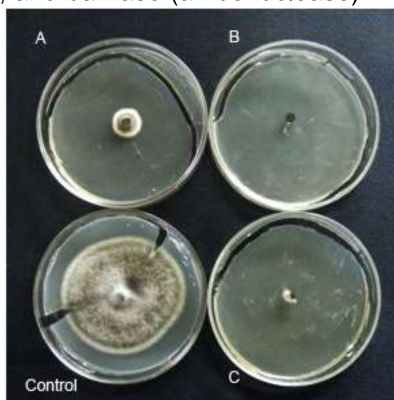

Fig. 2: Growth inhibition of *C. beticola* caused by the diffusible proteins of different *B. amyloliquefaciens* isolates A. BmDK1, B. BmBH, C. BmSHR2.

Table 1: Growth inhibition of *C. beticola* caused by direct antagonism in dual cultures (novle ring method), diffusible proteins and volatile gases of *B. amyloliquefaciens* isolates

isolates	Percentage of *C. beticola* Growth Inhibition		
	Novel ring method	Diffusible proteins	Volatile gases
BmBH	83.3	100.0	93.8
BmDK1	83.3	82.9	93.8
BmDK2	83.3	100.0	87.5
BmGz1	78.9	100.0	93.8
BmKsh1	83.3	100.0	93.8
BmKsh2	90.0	100.0	77.5
BmKsh3	83.3	100.0	93.8
BmKsh4	78.9	100.0	93.8
BmKsh5	86.7	100.0	55.2
BmKsh6	88.9	100.0	85.0
BmKsh7	81.1	100.0	81.3
BmKsh8	88.9	100.0	55.0
BmKsh9	84.4	100.0	68.8
BmSHR1	83.3	100.0	81.3
BmSHR2	77.8	92.9	55.0
BmSHR3	88.9	100.0	93.8
LSD (0.05)	1.5	-	3.2

The effect of volatile gases on radial growth of *C. beticola*.

Results presented in Table 1 and Fig.3 show that the effect of the volatile gases produced by the tested *B. amyloliquefaciens* isolates are similar to those obtained when testing the effect of diffusible antibiotics, all the tested isolates significantly inhibited the radial growth of *C. beticola*. The growth inhibition caused by the volatile gases produced by the tested isolates of *B. amyloliquefaciens* ranged from 50% to 93.8%. The isolates BmBH, BmDK1, BmGz, BmSHR3, BmKsh3, BmKsh4 and BmKsh1 recorded the highest inhibition percentage. While the lowest percentage of inhibition recorded by the isolates BmKsh5, BmKsh8, BmSHR2.

It worthy to mention that, no relation found between the location of the isolates and it's AFA. Many reporters reveled the effect of Bacillus species volatile gases and its relation to the plants as a factor increase the induced systemic resistance of the plants (Ryu *et al.*, 2004). Arrebola *et al.*, 2010 studied the AFA of volatile compounds produced by *Bacillus subtilis* and *Bacillus amyloliquefaciens* against *Penicillium digitatum* Sacc., *Penicillium italicum* Wehmer and *Penicillium crustosum* they found that the produced volatile gases from both tested bacteria inhibited the radial mycelia growth of *Penicillium* spp. *in vitro* and *in vivo.*

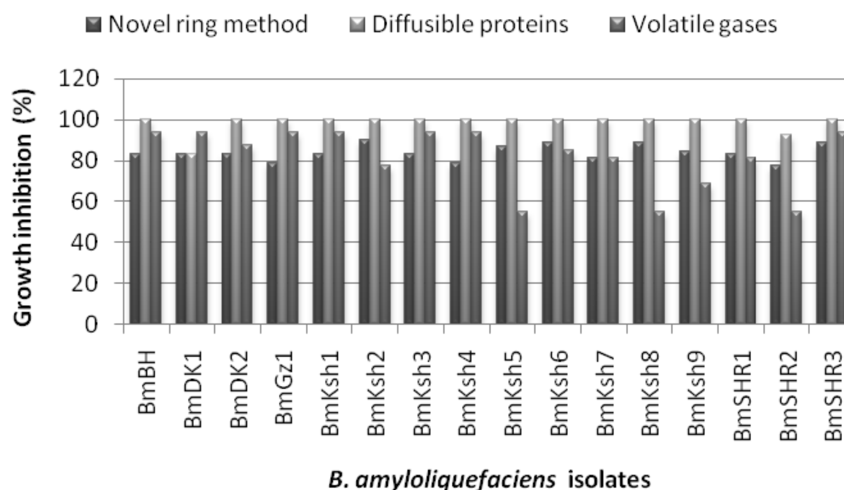

Fig.3: The antagonistic effect of Dual culture, diffusible proteins and volatile gases of *B. amyloliquefaciens on C. beticola in vitro*

Diverse authors have held responsible the action of one or various volatile components produced by bacteria for the inhibition and destruction of pathogenic fungi mycelia (De La Fuente *et al.*, 2001).

AFP activity against *C. beticola*.

The AFP extracted from culture filtrates of the tested *B. amyloliquefaciens* showed a remarkable activity against *C. beticola* growth *in vitro*. The inhibition zones resulted from the AFP ranged from 8.17 mm to 16.45 mm (Fig.4). All the isolates recorded an inhibition zones >10 mm except the isolates BmDK 1, BmKSH 9, BmSHR 1, BmSHR2 and BmSHR 3 which recorded a zone of inhibition < 10 mm. the presence of an inhibition zone indicated that the isolates were antagonistic to C. beticola. This observation is apparently in agreement with the reports of (Yoshida *et al.*, 2001).

The present study showed that antimicrobial substances can be produced by *B. amyloliquefaciens* isolates in nutrient broth which is peptone based. These media can be easily obtained, which is of advantage in mass production and commercialization of the biocontrol agent. Peptone appears to be a key nutrient for the production of antifungal compounds by *B. amyloliquefaciens* (Yoshida *et al.*, 2001). *B. amyloliquefaciens* is known to produce iturins, a family of cyclic lipopeptide antibiotics (Hiradate *et al.*, 2002). These molecules have seven a-amino acids and one b-amino fatty acid, and iturin A is produced as a mixture of up to eight isomers (Yu *et al.*, 2002). *B. amyloliquefaciens* strains producing iturin A have been used as biocontrol agents to suppress fungal plant pathogens (Yoshida *et al.*, 2001; Yu *et al.*, 2002, Mansour *et al.*, 2008 and Gacitua *et al.*, 2009).

Heat and pH Stability of the isolated antifungal proteins AFM.

The tested mix of the AFM produced by the tested *B. amyloliquefaciens* isolates showed a high thermal stability Fig. 5. No change in the activity of the AFM observed in the range of the tested temperatures from 4 to 60ºC. At temperature 70ºC and above, the activity of the AFM gradually decreased by temperature increase. It was noticed that, the boiled and 121ºC AFM still has antifungal activity ≤ 50% compared to the control (4 ºC). Many reporters detected a thermo-sTable antifungal metabolites and proteins produced by *B. amyloliquefaciens (*Mansour *et al.*, 2008 and Gacitúa *et al.*, 2009).

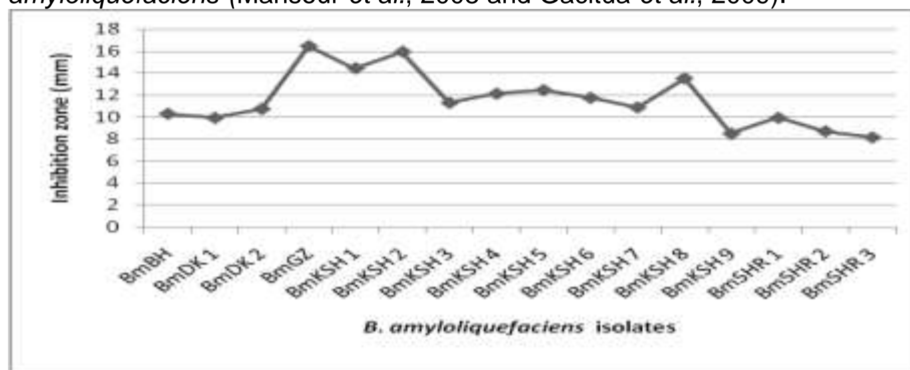

Fig. 4: Antifungal proteins activity of *B. amyloliquefaciens* on the growth of *C. beticola.*

From Fig. 6 it is clear that the activity of the AFM at pH 3 through 5 after 24 h at 20 °C reduced by 20% compared to the control treatment (pH7) and

started to increase from pH6 and reached its maximum at pH 7. At pH 11 the AFM lost about 50% of its activity compared to the control at pH 7.

Sidrophores production of *B. amyloliquefaciens* tested isolates *in vitro.*

Data in Table 2 show a significant difference between the tested *B. amyloliquefaciens* in its ability to produce sidrophores. Isolates BmGZ produced the highest amount of sidrophores that gave an adsorption at 700nm (1.19) followed by the isolates BmDK1, BmKSH2 and BmSHR3 (0.78, 0.64 and 0.52 respectively). siderophores are low weight compounds with high affinity for Fe+3 (Neilands, 1981), which are produced under limiting concentration of iron. These compounds are able to transport this element inside the cell for metabolic functions (Press *et al.* 2001), and microorganism which are able to produce sidrophores show competitive advantage as compared to those that do not produce them. From this point of view, the competence for iron increases in conditions where this element is limiting, but this condition is reverted when iron is added to the culture medium (Elad and Baker, 1985). Arias *et al.*, (2009) reported that *B. amyloliquefaciens* produce the cyclic lipopeptides surfactin, iturin A and fengycin as well as the iron-siderophore bacillibactin. There is evidence that siderophores fulfill an active role in the inhibition of a microorganism with another (Gacitúa *et al.*, 2009).

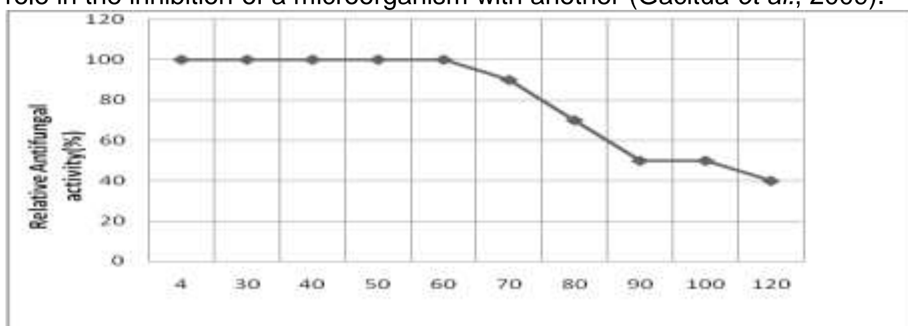

Fig. 5: Effect of temperature on the activity of total proteins isolated from B. *amyloliquefaciens* tested isolates on radial growth of *C. beticola in vitro*

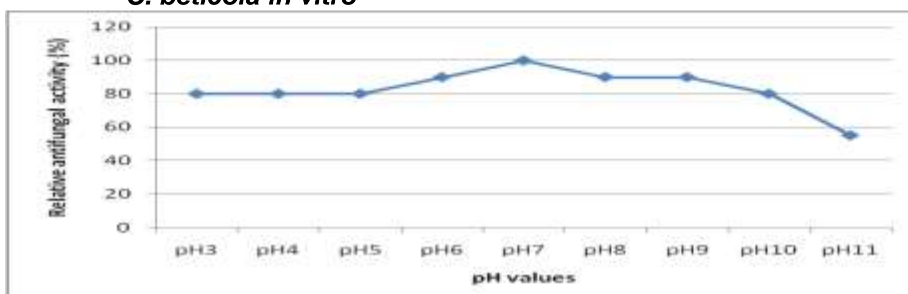

Fig. 6: Effect of pH on the activity of total proteins isolated from *B. amyloliquefaciens* tested isolates on radial growth of *C. beticola in vitro*

β 1,3 glucanase production of *B. amyloliquefaciens* tested isolates

Data in Table 2 show that all the tested *B. amyloliquefaciens* isolates produced β 1,3 glucanase. A significant differences was noticed between the tested isolates. Isolates BmKSH8 recorded the highest β 1,3 glucanase (236.2 uM/ml) while the lowest isolate produced β 1,3 glucanase was BmKSH4 (21.3 uM/ml). the amounts of β 1,3 glucanase produced by the other tested isolates ranged from (44.5 uM/ml) and 214.6 uM/ml).

In controlling fungal plant pathogens, a variety of mechanisms contribute to the biocontrol activity of microbes. Cell-wall-degrading enzymes, such as β-1,3-glucanases, cellulases, proteases, and chitinases, are involved in the antagonistic activity of some biological control agents against phytopathogenic fungi. In particular, numerous correlations between fungal antagonism and bacterial production of chitinases and/or β -1,3-glucanases have been noted (Kim and chung 2004)

The β-glucanases (endo-β-1,3_l,Cglucanases) of *B. subtilis* and *B. amyloliquefaciens* are both characterized by a substrate range similar to lichenase (EC 3.2.1.73) of germinating barley (Boriss *et al.*, 1981). However, the β-glucanase activities secreted by various strains of both taxa showed considerable variability (Borriss and Zemek, 1980). *B. amyloliquefaciens* MET0908 secreted an extracellular b-1,3-glucanase, which is a key enzyme in the decomposition of fungal hyphal walls (Lim *et al.*, 1991, Lahsen *et al.,* 2001 and Kim and chung 2004).

Chitinase production of *B. amyloliquefaciens* tested isolates

Data in Table 2 show that most of the tested *B. amyloliquefaciens* isolates wasn't able to produce chitinase in culture filtrates. Three isolates produced high amounts of chitinase BmDK1, BmSHR1 and BmSHR2. It was noticed that those isolates produced high amounts of chitinase produced low amounts of β 1,3 glucanase.

Chitin, a homopolymer of *N*-acetyl-D-glucosamine (Glc-NAc) residues linked by *â*-1-4 bonds, is a common constituent of insect exoskeletons, shells of crustaceans, and fungal cell walls (Gooday, 1977 and Deshpande, 1986). Some organisms that do not contain chitin also produce chitinases to degrade the polymer for nutritional or defensive purpose for example, a wide variety of bacteria actinomycetes, and plants produce chitinolytic enzymes in response to chitin-containing elicitors in their environments. Wang *et al.*, (2002) reported *B. amyloliquefaciens* V656 produced antifungal enzymes when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine waste. The antifungal enzymes displayed chitinase activities.

Table 2: Sidrophores, β 1,3 glucanase and Chitinase produced in *B. amyloliquefaciens* cultures

Bacillus amyloliquefaciens isolates	Sidrophores production	β 1,3 glucanase production(Micro mol of enzyme / ml)	Chitinase productionMicro mol of enzyme / ml)
	Adsorption at 700nm	(Micro mol of enzyme / ml)	
BmBH	0.12	103.1	0
BmDK 1	0.78	69.4	478.5
BmDK 2	0.19	107.5	0
BmGZ	1.09	44.5	0
BmKSH 1	0.14	144.5	0
BmKSH 2	0.64	199.4	0
BmKSH 3	0.14	113.1	0
BmKSH 4	0.49	21.3	0
BmKSH 5	0.18	214.6	0
BmKSH 6	0.27	117.6	0
BmKSH 7	0.06	158.9	0
BmKSH 8	0.19	236.2	0
BmKSH 9	0.16	85.1	0
BmSHR 1	0.36	69.4	444.9
BmSHR 2	0.16	67.2	436.9
BmSHR 3	0.52	71.7	0
LSD (0.05)	**0.13**	**3.45**	-

Effect of *B. amyloliquefaciens* isolates and its AFM on CLS severity before and after inoculation with the Pathogen under greenhouse conditions.

Data presented in Table 3 show that all the AFM produced by *B. amyloliquefaciens* reduced the severity of *C. beticola*. It was noticed that, the efficiency of the AFM increased when the treatment was before the inoculation with the pathogen. The percentage of disease severity reduction ranged from (23.4-63.8%). On the other hand, when the plants treated with the AFM after the infection the biocontrol efficiency was very low and the percentage of disease severity reduction ranged from (8.16 – 28.57%). In this work, we only used the normal concentration of culture filtrates and it showed a significant and remarkable activity in reducing disease severity. This suggest that this effect would increase if we used a concentrated culture filtrates.

Same results were obtained from plants treated with the bacterial cells Table 3. All the tested isolates reduced the CLS disease severity. Plants treated with the tested bacteria before the inoculation showed less disease severity than those treated after pathogen inoculation. The range of reduction in CLS disease severity in plants treated with the tested isolates before infection ranged from 48.94 - 72.34 % while the reduction in CLS disease severity reduced the range between 6.38 – 29.79% in plants treated with the tested bacteria after the inoculation with *C. beticola*. Generally, it was noticed that the range of disease severity reduction in plants treated with the bacterial cells was higher than those treated with the AFMs.

B. amyloliquefaciens is known to produce iturins, a family of cyclic lipopeptide antibiotics (Hiradate *et al.*, 2002). Yoshida el al., (2001) reported that application of culture filtrate of *B. amyloliquefaciens* RC-2 inhibited mulberry anthracnose caused *Colletotrichum dematium*, indicating that suppression was due to antifungal compounds in the filtrate. They also found that, the development of mulberry anthracnose on mulberry leaves was inhibited only when the culture filtrate was applied before fungal inoculation, and it was not inhibited by application after inoculation. These results suggest that the antifungal compounds in the filtrate exhibit a preventive effect on the disease.

Table 3: Percentage of CLS severity reduction caused by treating sugar beet plants with *B. amyloliquefaciens* isolates and its antifungal metabolites AFM before and after inoculation with *C. beticola* under greenhouse conditions.

Isolates	Percentage of CLS disease severity inhibition			
	Culture filtrates		Bacterial cells	
	Before Inoculation	After Inoculation	Before Inoculation	After Inoculation
BmBH	51.06	20.41	55.32	25.53
BmDK 1	34.04	20.41	61.70	19.15
BmDK 2	46.81	12.24	59.57	23.40
BmGZ	23.40	26.53	72.34	29.79
BmKSH 1	51.06	20.41	65.96	25.53
BmKSH 2	55.32	16.33	59.57	29.79
BmKSH 3	48.94	16.33	53.19	19.15
BmKSH 4	25.53	24.49	48.94	10.64
BmKSH 5	63.83	12.24	72.34	17.02
BmKSH 6	44.68	26.53	55.32	25.53
BmKSH 7	48.94	28.57	59.57	12.77
BmKSH 8	68.09	20.41	70.21	10.64
BmKSH 9	31.91	16.33	61.70	19.15
BmSHR 1	23.40	20.41	63.83	12.77
BmSHR 2	23.40	8.16	59.57	21.28
BmSHR 3	29.79	12.24	51.06	6.38
LSD (0.05)	**5.54**	**4.22**	**7.62**	**6.16**

Mari *et al.*, (1996) reported that *B. amyloliquefaciens* 2TOE reduced the severity of gray mold caused by *Botrytis cinerea* in pears. They suggested that the antifungal activity of the bacterium was due to competition for nutrients.

It is worthy to mention that no reports found in the literature concerning the use of *B. amyloliquefaciens* to control *C. beticola*. Therefore, *in vitro* activities of the *B. amyloliquefaciens* against C. *beticola* of sugar beet as well as the greenhouse results in this study suggested that the bacterium can be an effective biological control agent. The use of *B. amyloliquefaciens* as a biocontrol agent against C. *beticola* of sugar beet may be an economical as well as environmental safe way to suppress the disease. However, the form in which the bacterium can be applied on plants needed further investigation.

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النشاط التضادي لعزلات البكتيريا باسيلوس أميلوليكويفاسينس المعزولـه من أوراق بنجر السكر علي الفطر سيركوسبورا بيتيكولا

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تم إختبار نشاط المقاومه الحيويه لعدد 16 عزلة من البكتيريا *باسيلوس أميلوليكويفاسينس* المعزولـه من علي اسطح اوراق بنجر السكر علي المسبب المرضي سيركوسبورا بتيكولا المسبب المرضي لمرض التبقع السيركوسبوري في بتجر السكر. أظهرت كل العزلات المختبره قدره تضـاديه معنويـه علـي الفطـر *سيركوسبورا بتيكولا* في اختبارات الاطباق. تراوح تثبيط النمو الخطي للفطر الممرض في الاطباق المتسبب عن البكتيريات المختبره بين 77.8 – 90%. و قد ادت النـواتج الايضـيه و الغـازات الطيـاره الناتجـه من عزلات البكتيريا *باسيلوس أميلوليكويفاسينس* المختبره الي تثبيط نمو الفطر الممرض بشكل معنوي. و قد أظهرت البروتينات المضـاده للفطر و المستخلصه من البيئات السائله للبكتيريات المختبره قدره تثبيطيـه علـي الفطر الممرض و قد تراوحت مناطق التثبيط الناتجه عن استخدام البروتينات المضاده بين 8.17 -16.45 مم. و أيضا أظهرت النواتج الايضيه للعزلات المختبره من البكتيريا *باسيلوس أميلوليكويفاسينس* ثباتا عاليا في درجات الحراره المرتفعه. و قد ادي خفض الاس الهيدروجيني للنواتج الايضيه الي 3-5 الي اختزال قدرتها التضاديه بنسبة 20% و كانت اعلي قدره تضاديه لها عند الاس الهيدروجيني 6 و 7. أظهرت كل العزلات البكتيريه المختبره قدره علي انتاج السيدوفورس و انزيم بيتا 1،3 جلوكانيز بينما ثلاثة عزلات فقط كانت قادره علـي انتـاج انـزيم الكـايتينيز. فـي تجربـة الصـوبه ادي رش النباتـات بالخلايـا البكتيريـه او برواشـح المـزارع البكتيريه المختبره الي انخفاض شدة الاصابه بتبقع الاوراق السيركوسبوري تحت ظروف العدوي الصناعيه. و قد أظهرت النتائج ان معاملـه النباتات بخلايا او رواشح المزارع البكتيريـه المختبره قبل اجراء العدوي الصناعيه بافطر الممرض كان اكثر تاثيرا عنه اذا ما كانت المعامله بعد العدوي بالفطر الممرض. هذا العمل هو الاول في استخدام البكتيريا *باسيلوس أميلوليكويفاسينس* في المقاومه الحيويه للفطر *سيركوسبورا بتيكولا*.

قام بتحكيم البحث

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