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ANTAGONISTIC EFFECT OF SYMBIOTIC BACTERIA ISOLATED FROM MIDGUT OF HONEY BEE WORKERS AGAINST *ASCOSPHAERA APIS*, THE CAUSAL PATHOGEN OF CHALKBROOD DISEASE

Adham M. Moustafa*; Mohamed O.M. Omar; Mohamed A.A. Morsi** and Bassam F.G. Fahmy***

* Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt
**Plant Protection Department, Faculty of Agriculture, Assiut, Egypt
Corresponded author E-mail: adhm_mostafa@yahoo.com

ABSTRACT:

Six biotypes of fungal isolates belonging to *Ascosphaera apis* were isolated by three media from infested honey bee larvae. Two isolates (A_7 and A_{15}) were able to form sporocysts. However, the other four (A_3, A_4, A_8 and A_9) did not form sporocyts on cultivated Murashieg and Skoog medium (mMS). Six isolates from symbiotic bacteria associated with midgut of honey bee workers have been isolated from healthy workers. Four isolates from endospore-forming bacteria belonged to *Bacillus subtilis* (B_2, B_4, B_{10} and B_{100}) and two isolates from non endospore-forming bacteria to *Pseudomonas fluorescence* (P_1 and P_5) were isolated. Morphological features and physiological reactions of isolated bacteria were determined. Antagonistic effectiveness of both *Bacillus subtilis* and *Pseudomonas fluorescence* was tested against isolates of *Ascosphaera apis*, the causal pathogen of chalkbrood disease, *in vitro*. Data showed that *Bacillus subtilis* isolate (B_2) gave the highest antagonistic effect as inhibition zone and mycelial growth followed by *Pseudomonas fluorescence* (P_1). Highly significant differences among *Bacillus subtilis* (B_2), *Pseudomonas fluorescence* (P_1) and other bacterial strains were recorded. Scanning electron microscope was used to examined the fungal hyphae and mature sporocysts of *Ascosphaera apis* which isolated from infested larvae and grown on (mMs). Numerous distinguish differences were recorded. The examination showed that numerous bacterial cells of *Pseudomonas fluorescence* invaded fungal hyphae of *Ascosphaera apis* and caused disintegration the cell walls. Whereas *Bacillus subtilis* hyphae showed shrinking appearance. It could be conducted that such symbiotic bacteria can considered as a bioformula for controlling such disease in honey bee colonies.

INTRODUCTION:

Chalkbrood is a fungal disease of honey bee brood caused by *Ascosphaera apis*. This disease is now found throughout the world and there are indications that chalkbrood incidence may be on the rise.

Chalkbrood is an invasive mycosis in honey bees (*Apis mellifera* L.) produced by eterothallic fungus *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir (Spiltoir, 1955; Spiltoir and Olive, 1955) that exclusively affects bee brood. Although fatal to individual larvae, the disease

does not usually destroy an entire bee colony. However, it can cause significant losses in terms of both bee numbers (Harbo, 1995; Spivak and Downey, 1998 and Aronstein & Murray, 2010) and colony productivity (Bailey, 1963 and Wood, 1998) with reductions in honey production of 5-37% reported (Heath, 1982; Yacobson *et al.* 1991 and Zaghloul *et al.*, 2005). Chalkbrood is now found in honey bee colonies around the world, and there are some indications that the incidence of chalkbrood has increased in recent years (Heath, 1985 and Kluser & Peduzzi, 2007). The development and course of the disease in bee colonies vary as they are affected by many factors infectiousness, individual immunity of bee colony, genetic potential of queen, environmental conditions etc. (Harbo, 1995, Spivak and Dawney, 1998).

Fungal spores can be present on all surfaces within the beehive (Puerta *et al.*, 1994; 1995) and can remain viable for many years, providing a continual source of infection. Spores can be found in bee-stored pollen (Heath, 1982) in comb wax (Flores *et al.*, 2005) and in retail packs of honey (Anderson *et al.*, 1997 and Reynaldi *et al.*, 2003).

A broad range of chemotherapeutic compounds have been tested for their ability to control chalkbrood (Heath, 1982 and Liu, 1991). Considering that dependence on synthetic pesticides and antimicrobials could lead to general deterioration of the colony environment and bee health in general, it is advisable to minimize use of pesticides inside and outside of bee colonies (Bogdanov *et al.*, 1998 and Frazier *et al.*, 2008). There is a great interest in developing alternative control methods (Winston, 1995). Natural compounds for control of chalkbrood fungus would be a welcome alternative to synthetic fungicides. Numerous microbes associated with honey bees, such as certain *Penicillium, Aspergillus, Bacillus* species, showed inhibiting effects on growth of *Ascosphaera apis* in culture (Gilliam *et al.*, 1988 and Wood, 1998).

This research aimed to study symbiotic bacteria, isolated from gut of healthy honey bee workers as an inhibitory bioagent against *Ascosphaera apis*, the causal pathogen of honey bee chalkbrood disease *in vitro*.

MATERIALS AND METHODS:

Isolation and identification of the causal pathogen of chalkbrood disease was done at Mycological Center, Faculty of Science, Assiut University. Laboratory tests on the pathogen of chalkbrood disease and isolation of symbiotic bacteria were carried out at Plant Pathology Department, Faculty of Agriculture, Assiut University.

Isolation and identification of the causal pathogen from infested honey bee larvae:

Honey bee infested larvae samples were collected from naturally infested honey bee colonies. Diseased brood were washed with tap water, cut into small pieces (0.25-0.5 cm) and surface sterilized by dipping into 0.5% sodium hypochlorite solution for 3 minutes then rinsed for several times in sterile water. Disinfested brood samples were plated on sterile Petri dishes on potato dextrose agar (PDA) medium

containing 30 mg streptomycin sulphate/100 ml medium, and then incubated at 27°C for 48 hours. Identification of the causal pathogen was carried out. The resulted fungi were sub-cultured on the same medium until pure culture was established. Pure cultures were obtained by using morphological characteristics of mycelia as described by Chorbiński (2003).

Pure, single-spore cultures of each isolates were obtained by the methods described by Anderson and Gibson (1998).

Isolation and identification of certain symbiotic bacteria from healthy honey bee workers midgut:

Honey bee workers were collected from healthy and chalkbrood diseased colonies, kept over night in Petri dish in a refrigerator. The worker abdomen was cut off using a new single-edged razor blade for each bee and the internal tissues were removed for isolation of symbiotic bacteria in midgut as described by Jeyaprakash *et al.* (2003).

The workers were washed thoroughly with sterile water three times and dissected using sterile scalpel to free its gut. Guts were put in a sterile mortar with 1 ml of sterile distilled water, grounding for 1 minute and streaked on the surface of agar MS medium supplemented with 1 ml of glycerol and 0.1 g yeast extract. Plates were incubated at 25-26°C for 48 hours. Bacterial isolates were obtained in pure culture using the dilution method. Bacteria grown in separate colonies on the diluted plates were transferred to the aforesaid medium slants. Inoculated slants were incubated at 25-26°C for 48 hours. Cultures of isolated bacteria on slants were kept at 5°C until used. The plate diluting method has been applied for determination of quantitative bacterial counts of respective groups of bacteria in midgut of honey bee. Modified Murashige and Skoog medium (mMS) in Petri dishes have been inoculated with 1 ml of suspension obtained after cut off from abdomen workers samples streaking by using bacterial loop on the agar medium surface in four replications. Basical dilution (10^{-1}) was prepared as follows: 1 ml of content was added to the test tube containing 9 ml of sterilized distilled water.

Identification of bacterial isolates was carried out. According to Sneath (1986) the isolated endospore forming bacteria which isolated from honey bee midgut was identified as *Bacillus subtilus*. According to Bergey's manual (1984) and Grimont *et al.* (1996). The isolated non endospore forming bacteria was identified as *Pseudomonas fluorescence*.

The following tests were used for identification: shape of cells, motility, Gram's reaction, aerobiosis, starch hydrolysis, Gelatin liquefaction, nitrate reduction, Acetyl methyl carbinol production (V.P. test), fermentation reaction with mantol, glucose, sucrose, arabinose, xylose and lactos, pigment production.

Antagonistic effect of isolated bacteria against *Ascosphaera apis* in *vitro*:

The antagonism between the isolated bacteria and *Ascosphaera apis* was tested *in vitro*, using P.D.A. medium and isolate black buff (A_{15}) of the pathogen. Petri dishes

containing 10 ml. of the aforesaid medium were inoculated with equal disks (7 mm. in diameter) of *Ascosphaera apis* obtained from 4-day old cultures. For each antagonistic bacterium, a loopful of a 48 hours old suspension of bacterium grown at 27°C was streaked at opposite sides of the pathogen disk at the periphery. The inoculated plates in addition to plates inoculated with *Ascosphaera apis* only were incubated at 30°C. Four replicates were used for each treatment.

When the growth of *Ascosphaera apis* covered the plates surface (9.0 cm. in diameter) of control treatment, observation on the antagonism was recorded. The bacteria, which showed antagonistic effect against *Ascosphaera apis* were selected for further studies.

Scanning electron microscope studies:

Fine structure of the inocula of *Ascosphaera apis* and interaction of antagonistic bacteria *Bacillus subtilis* or *Pseudomonas fluorescence* in *vitro* against the causal pathogen was studied using (Jeol JSM-5400LV at the Electron Microscopy Unit, University of Assiut, Assiut, Egypt).

Black mummies were cut into 2 mm squares. The light part of the internal dead tissue was removed with a razor and the remaining black tissue containing sporocysts was placed on a nutrient medium of 2% potato dextrose agar (Difco) containing 0.4% yeast extract. Chlormophenicol at a concentration of 10 #g/ml of medium was added to prevent bacterial growth without preventing the growth of *A. apis* as it was known to have no inhibitory effects on the growth of *A. apis*. The fungus grew well; producing abundant sporocysts at 30°C during the 4th day after inoculation, mycelium with sporocysts were cut into 3 mm squares and transferred to vials containing 4% EM grade glutaraldehyde in 0.2 M sodium phosphate buffer, pH 7.4. Specimens were fixed overnight at 4°C and rinsed 3 times with the same buffer, and then post-fixed with 1% of OsO4 at 4°C in the same buffer at room temperature (20°C for 2 hrs.). Fixed specimens were dehydrated using a graded series of ethanol and preserved in 95% ethanol. Dehydrated specimens were dried in a critical point drier, mounted on a specimen stub, and repeatedly Pierced with a fine needle to break the sporocysts and to expose their interior structure. Specimens were coated with gold and examined.

Statistical analysis:

Data subjected to Advanced Analysis Package (ASAP): Analysis of variance t-test & F-test, simple and multiple correlations were used.

RESULTS AND DISCUSSION:

Six fungal isolates were isolated from infested honey bee broad samples. Isolated fungi were identified as *Ascosphaera apis* according to Heath (1982), Bailey &Ball (1991) and Bissett *et al.* (1996). Two isolates (A_7 and A_{15}) were able to form sporocysts on cultivated medium; however, isolates A_3, A_4, A_8 and A_9 did not forming sporocysts on cultivated mMS medium. Isolates of fungi were presented in Table (1).

Non harmful bacteria (symbiotic bacteria) have been isolated from midgut of healthy adult workers. The four endospore-forming bacteria (B_2, B_4, B_{10} and B_{100}) isolated from honey bee midgut could be identified as *Bacillus subtilis* (Chrenberg Cohn) according to Sneath *et al.* (1984). These isolates bacteria gave the following morphological features and physiological reaction which represented in Table (2).

Two non spore-forming bacteria (P_1 and P_5) isolated from honey bee midgut were identified as *Pseudomonas fluorescence*, according to the results reported by Bergey's Manual (1984) and Grimont *et al.* (1996a & b). These isolated bacteria isolates gave the following morphological features and physiological reaction (Table 3).

Data in table (4) and figures (1, 2) show that *Bacillus subtilis* isolate (B_2) gave the highest antagonistic effect against most strains of *Ascosphaera apis* followed by *Pseudomonas fluorescence* (P_1). B_2 isolate was pronounced in *vitro* on cultivated medium by inducing wide inhibition zones. The means of inhibition zones of B_2 isolate against *Ascosphaera apis* strains ranged from 14.75 to 23.00 mm with *A. apis* strains. *Pseudomonas fluorescence* isolate (P_1) followed the B_2 isolate which gave inhibition zones ranged from 12.00 to 21.25 with different *A. apis* significant differences were noticed among *Bacillus subtilis* isolate (B_2) and *Pseudomonas fluorescence* (P_1) in their antagonistic effect against isolates of *Ascosphaera apis* (A_3, A_7 & A_9). However, *Pseudomonas fluorescence* (P_1) gave the highest antagonistic effect against *A. apis* (A_8, Pale buff strain) with highly significant differences with (B_2).

Data in table (5) show that *Bacillus subtilis* (B_2) gave the highest inhibition effect against *Ascosphaera apis* strains mycelial growth followed by (P_1). Means of mycelial growth of *Ascosphaera apis* inhibited by (B_2) ranged from 5.25 to 9.00 mm. *Pseudomonas fluorescence* (P_1) followed by (B_2) in its inhibition effect on mycelial growth of *Ascosphaera apis.* Mean of mycelial growth of *Ascosphaera apis.* Mean of mycelial growth under antagonistic effect of (P_1) ranged from 6.75 to 8.50 mm. Significant differences were noticed among *Bacillus subtilis* isolate (B_2) and *Pseudomonas fluorescence* (P_2) in their antagonistic effect on mycelial growth of *Ascosphera apis* strains (A_3, A_4 & A_8). However (B_2) and (P_1) gave a similar effect on fungal strains (A_7, A_9 & A_{15}).

Table 1 : Types of isolated *Ascosphaera apis* the causal pathogen of honey bee chalkbrood disease

Isolate number	Isolate type	Sporocysts
A_3	White	-
A_4	White	-
A_7	Pale buff	+
A_8	White	-
A_9	White	-
A_{15}	Black buff	+

Table 2 : Morphological features and physiological reactions of endospore forming bacteria isolates B_2, B_4, B_{10} and B_{100}

Tests	Results
Bacterial cell shape	Rod
Motility	Motile
Gram staining reaction:	
Anaerobic growth	+
Catalase activity	-
Acid production from	+
Glucose	+
Xylose	+
Mannitol	+
Gas production from glucose	+
Vogas-preskauer test	-
Hydrolysis of:	
Casein	+
Starch	+
Esculin	+
Urea	-
Gelatin liquefaction	+
Tyrosinase activity	-
Nitrate reduction	+
Indole formation	-
Growth at pH:	
5.7	+
6.8	+
Growth in NaCl:	
2%	+
5%	+
7%	+
10%	Weak growth
Pigment production in:	
Nutrient agar medium	Cream to light brown
PDA	Cream to brown to black

Table 3: Morphological features and physiological reactions of non-spore-forming bacterial isolates P_1 and P_5

Tests	Results
Bacterial cell shape	Rod
Motility	Motile
Gram staining reaction	-
Aerobiosis	Aerobic
Voges-proskaur	-
Catalase activity	+
H_2S production	-
Acid production from:	+
Glucose	+
Sucrose	+
D-fructose	(+)
Glycerol	+
Aesculin	+
Mannitol	-
D-Ribose	+
Maltose	+
Gelatin liquefaction	-
Starch hydrolysis	-
Nitrate reduction	+
Indole formation	-
Growth at 40°C	-
Aesculin hydrolysis	-
Oxidation of gluconate	-
Citrate utilization	+
Pigment production	Yellow pigment with weak fluorescence light under ultraviolet light

+ = Positive reaction - = Negative reaction.

Table 4 : Antagonistic effect of *Bacillus subtilis* and *Pseudomonas fluorescence* isolates against some *Ascosphaera apis* strains, the causal pathogen of honey bee chalkbrood disease in *vitro*

Bacterial isolates		Means of inhibition zone (in mm) against *Ascosphaera apis* strains					
		White A_3	White A_4	Pale buff A_7	White A_8	White A_9	Black buff A_{15}
B_2		20.25	21.50	21.25	14.75	23.00	18.75
B_4		10.75	12.25	14.50	9.25	19.50	12.00
B_{10}		11.75	12.50	13.25	12.00	16.00	13.25
B_{100}		10.75	10.75	4.50	9.00	15.50	4.50
P_1		12.25	19.25	12.00	21.25	12.50	19.25
P_5		8.25	15.00	8.25	14.75	12.00	11.00
LSD	5%	2.76	2.47	6.25	3.91	4.08	4.18
	1%	3.81	3.42	8.64	5.41	5.64	5.78

Fig. 1: Antagonistic effect of *Bacillus subtilis* (Isolate No. B2) Against *Ascosphaera apis* (Black buff strain A15) the causal pathogen of honey bee chalkbrood. Note that the growth of the causal pathogen on culture medium was degraded by the effect of symbiotic antagonists' bacteria in vitro and an increase in inhibition zone is pronounced with such bacterial isolate

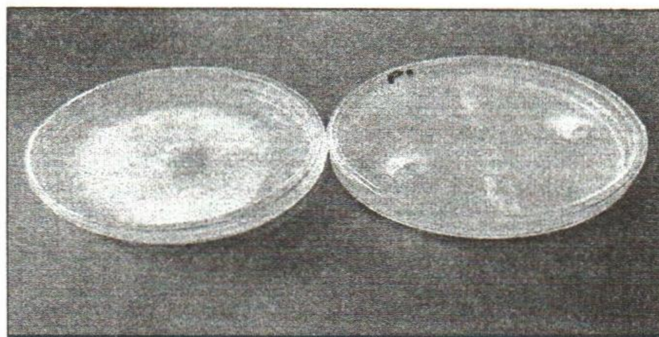

Fig. 2: Antagonistic effect of *Pseudomonas fluorescence* (isolate No. P1) Against *Ascosphaera apis* pale buff strain (A_8) the causal pathogen of honey bee chalkbrood. Note that the weak pronounced growth of the causal pathogen on culture medium due to the effect of symbiotic antagonists' bacteria in *vitro*

Table 5: Antagonistic effect of *Bacillus subtilis* and *Pseudomonas fluorescence* isolates against *Ascosphaera apis* strains, the causal pathogen of honey bee chalkbrood disease in *vitro*

Bacterial isolates		Means of mycelial growth inhibition (in mm) of *Ascosphaera apis*					
		White A_3	White A_4	Pale buff A_7	White A_8	White A_9	Black buff A_{15}
B_2		6.00	7.25	9.00	5.25	7.25	8.00
B_4		18.50	19.25	11.50	16.75	17.00	19.50
B_{10}		21.50	20.50	12.50	23.00	14.00	20.25
B_{100}		30.25	28.25	19.25	30.50	15.75	24.50
P_1		11.75	16.75	9.75	15.50	8.50	8.50
P_5		21.75	23.75	14.75	26.00	21.25	20.50
LSD	5%	4.15	3.73	5.07	3.66	2.90	2.10
	1%	5.73	5.15	7.01	5.06	4.01	2.91

These results were in agreement with the results obtained by Gilliam *et al.* (1988) and Wood (1998) who reported that numerous microbes associated with honey bees such as certain *Penicillium, Aspergillus, Bacillus* species, showed inhibiting effects on growth of *Ascospherae apis* in culture.

Chin-Woeng *et al.* (2000) told that certain *Pseudomonas* species have biocontrol properties, which able to produces a phenazine type antibiotic active agent against certain fungal plant pathogens.

Reynaldi *et al.* (2004) found ten bacteria strains that showed the best antagonistic effect to *A. apis.* They mentioned that the best antagonistic effect occurred with *Bacillus subtilis* strains.

Scanning electron microscope studies:

Data obtained by using the scanning electron microscope revealed that the mature sporocysts contained a large number of globules of varying sizes. However, sporocysts which formed in *vitro,* their walls of the immature stage were wrinkled and then became smooth as the sporocyst matured (Fig. 3). These results were in agreement with those obtained by Liu (1987), Chorbinski (2003), Chorbinski and Rypula (2003). The interior and exterior surface of the sporocyst wall had numerous distinguishable papillae. However, some globules in the developing sporocysts of *Ascosphaera apis* began to form immature spores, which aggregated to form spore balls. These results were in accordance with those described by Chorbinski and Rypula (2003) who reported that a typical feature of the genus *Ascosphaera* is production of spherical sporocysts abundant in ascospores forming spore balls. The species of *Ascosphaera* genus varied in the sporocysts size, as well as in the shape and size of ascospores.

As shown in Figs. (4, 5) the hyphae of *Ascosphaera apis* were invaded by numerous bacterial cells of antagonistic bacteria *Pseudomonas fluorescence* in *vitro*, and these bacteria have caused the disintegration and lysed the cell wall of the fungal hyphae. In this respect, mature sporocyst of *Ascosphaera apis* also was invaded by a numerous bacterial cells

of antagonistic bacteria ***Bacillus subtilis*** **in** ***vitro*** **these bacteria lysed the cell wall of the mature sporocyst, forming a cavity and aggregated to go inside it (Figs. 6, 7).**

Ascosphaera apis **hyphae showed shrinking appearance when grown in** ***vitro*** **with antagonistic bacteria** ***Bacillus subtilis*** **on cultivated medium Figures (8 a,b).**

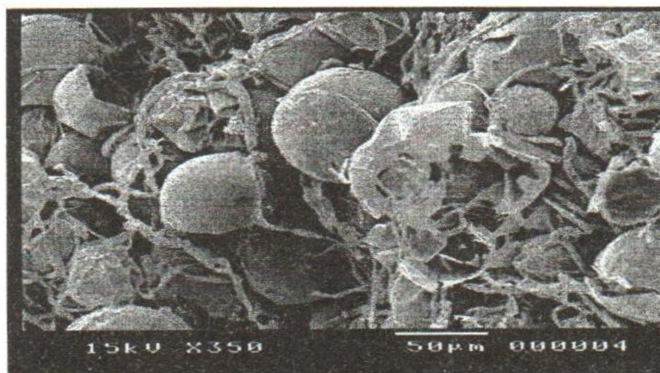

Fig. 3: The fine structure of ***Ascosphaera apis*** **using Scanning Electron microscope. Note that round globules sporocysts of various sizes were found. Non-mature sporocysts have wrinkled surface, however mature sporocysts have smooth surface. Bar= 50 μm**

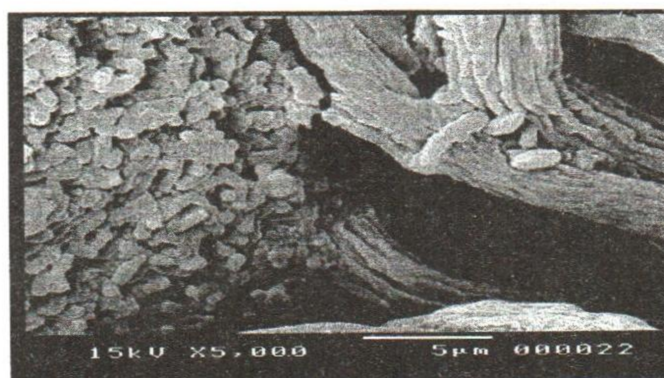

Fig. 4: ***Ascosphaera apis*** **hyphae invaded by a numerous bacterial cells of antagonistic bacteria** ***Pseudomonas fluorescence*** **in** ***vitro.*** **Note that the bacteria lysed the cell wall of the fungal hyphae. Bar= 5 μm.**

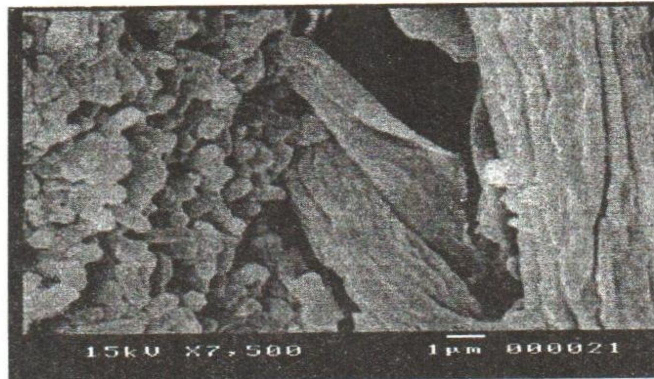

Fig. 5: *Ascosphaera apis* hyphae surrounded by a numerous bacterial cells of antagonistic bacteria *Pseudomonas fluorescence* in *vitro*. Note that these bacteria caused the distintegration of the cell wall of the fungal hyphae. Bar= 1 μm.

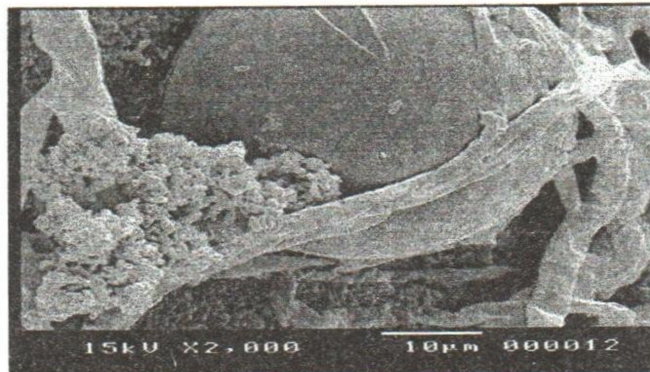

Fig. 6: Mature Sporocyst of *Ascosphaera apis* invaded by a numerous bacterial Cells of antagonistic bacteria *Bacillus subtilis* in *vitro*. Note that the bacteria lysed the cell wall of the mature sporocyst forming a cavity and aggregated to go inside it. Bar= 10 μm.

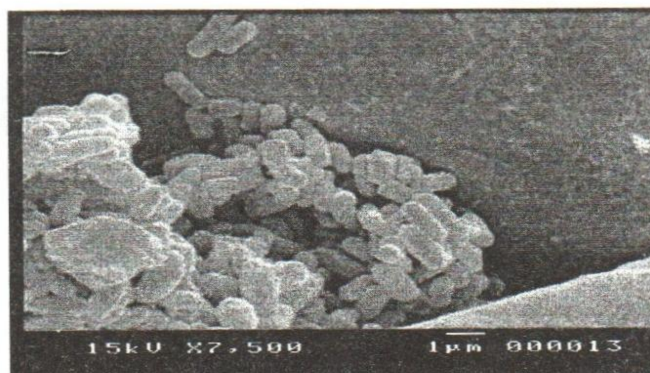

Fig. 7: A higher magnification of the outer surface of the mature sporocyst of *Ascosphaera apis* grown in *vitro*. Note that the antagonistic bacteria *Bacillus subtilis* lysed the wall of the sporocyst in *vitro* and this cavity was filled with the propagated and fused bacterial cells. Bar= 1 μm.

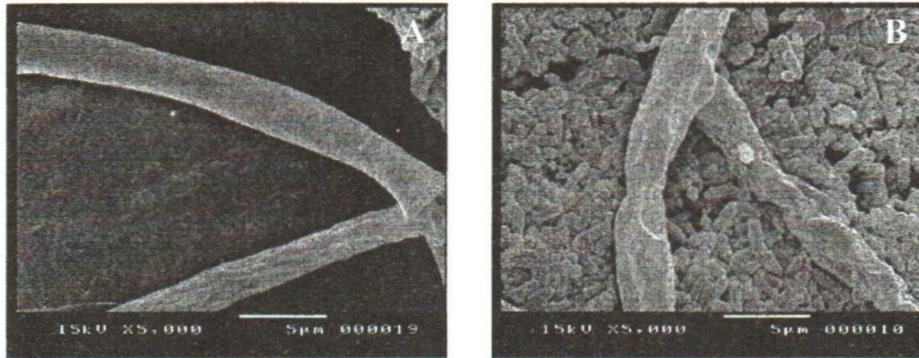

Fig. 8: (A) *Ascosphaera apis* normal hyphae grown in *vitro* on the cultivated medium. However, (B) *Ascosphaera apis* hyphae showing shrinking appearance due to the effect of antagonistic bacteria *Bacillus subtilis* grown in *vitro* on cultivated medium. Bar= 5μm.

The effect of symbiotic bacteria on the causal pathogen of honey bee brood mentioned by Traniello *et al.* (2002), Evans & Lopez (2004), Evans *et al.* (2006) and Fernandes *et al.* (2007) who reported that the genus *Bacillus* is a producer of lipopeptides, which represent a class of microbial surfactants. They pointed out that the genus *Bacillus* is a producer of these active compounds and among them *B. subtilis* produces surfaction, the most potent biosurfactant known. They found that these compounds can act as antibiotics, antivirals, antitumorals, immunomodulators and enzyme inhibitors. Their results demonstrated that lipopeptides have a broad spectrum of action, including antimicrobial activity against microorganisms with multidrug-resistant profiles and mentioned that the bacterial symbionts likely play roles in individual and colony fitness across the social insects. They mentioned also that recent evidence for a socially communicable defense against pathogens in termites might indeed reflect sharing of bacteria among termite colony members, rather than the proposed induction of host-specific physiological changes.

Tamehiro *et al.* (2002) demonstrated that a novel phospholipid antibiotic (Bacilysocin) have an antimicrobial activity, especially against certain fungi.

These results indicated that the inocula of the chalkbrood pathogen, which was antagonized by the symbiotic bacteria *Bacillus subtilis* and *Pseudomonas fluorescence* in *vitro*, must be worth to use it as biological control formulation to overcome the chalkbrood, which is considered a serious disease in the honey bee colonies.

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التأثير المضاد لبكتريا تكافلية عزلت من المعى المتوسط لشغالات نحل العسل تجاة أسكوسفيرا ابيس المسبب المرضى للحضنة الطباشيرية

أدهم مصطفي مصطفى [*] ، محمد عمر محمد عمر [**] ،
محمد أبو الفضل عبدالحميد مرسي [**] ، بسام فكري جلال [*]

*معهد بحوث وقاية النبات – مركز البحوث الزراعية – الدقي – الجيزة – مصر
** قسم وقاية النبات كلية الزراعة – جامعة اسيوط – مصر

استخدمت ستة عزلات تابعة للفطر المسبب لمرض الحضنة الطباشيرية في طوائف نحل العسل *Ascosphaera apis*، والتى تم عزلها من يرقات النحل المصابة بالمرض وتنميتها على ثلاث أنواع من البيئات الصناعية. ووجد أن هناك عزلتين (A_7،A_{15}) قادرتين على تكوين الحويصلات الجرثومية عند تنميتها على بيئة Murashige and Skoog (mMS) بينما الأربعة الأخرى (A_3,A_4,A_8,A_9) لم تكون الحويصلات الجرثومية. كما تم عزل ستة عزلات من البكتريا التكافلية الموجودة في المعى المتوسط لشغالات نحل العسل السليمة حيث ثبت أن أربعة منها (B_2,B_4,B_{10},B_{100}) تابعة لل ***Bacillis subtilis*** قادرة على تكوين جراثيم داخلية بالإضافة إلى عزلتين من نوع *Pseudomonas fluorescence* غير القادرة على تكوين جراثيم داخلية حيث تم تقدير الصفات المورفولوجية والفسيولوجية لها.

اختبر التأثير المضاد لعزلات نوعى البكتريا على الفطر المسبب لمرض الحضنة الطباشيرية خارجياً وأثبتت النتائج أن عزلة البكتريا (B_2) قد أعطت أعلى تأثير مضاد كمنطقة تثبيط ونمو ميسليومى تلاه فى التأثير العزلة (P_1) وقد سجل إختلاف معنوى عالى فى التأثير لكلا العزلتين (B_2,P_1) عن باقي العزلات البكتيرية على نوع الفطر المستخدم. تم فحص الحويصلات الجرثومية وهيفات الفطر المسبب لمرض الحضنة الطباشيرية *Ascosphaera Apis* والمعزولة من اليرقات المصابة بعد معاملتها بالبكتريا التكافلية باستخدام الميكروسكوب الألكترونى الماسح. أوضح الفحص أن العديد من خلايا بكتريا*Pseudomonas fluorescence*(P_1) . قد غزت هيفات الفطر وسببت تحلل جدر خلاياه كما أن بكتريا ***subtilis Bacillus*** قد أظهرت هيفات الفطر بمظهراً منكمشاً. ومن النتائج المتحصل عليها يمكن القول أن هذه البكتريا التكافلية يمكن أن تعتبر أساس لمكون حيوى يستخدم في برامج لمكافحة الفطرالمسبب *للحضنة* الطباشيرية في طوائف نحل العسل.