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MICROBIOLOGICAL STUDIES ON CHALK-BROOD DISEASE IN HONEYBEE COLONIES IN EGYPT

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ABSTRACT

Chalk-brood disease in honeybee larvae, caused by the heterothallic fungus *Ascosphaera apis*, was recently diagnosed in Egypt in 1995 and is regarded lately as a threat to the Egyptian honeybee keeping. Effective control of such disease is currently unavailable all over the world, and the most common practice is to re-queen from different genetic stocks. This work was undertaken to study some morphological, cultural and physiological characteristics of *Ascosphaera apis* isolated from honeybee colonies in Egypt. Microorganisms associated with chalk-brood mummified honeybee larvae were also examined using different culture media. The results showed that *A. apis* was the most prevalent fungus in chalk-brood mummified larvae, representing 33.3% of the isolated microorganisms from all the examined media followed by *Aspergillus. fumigatus* (27.4%) and *Aspergillus flavus* (14.1%). Bacteria and yeasts represented 12.6 and 10.6% of the total number of microorganisms isolated from mummified larvae, respectively. Potato Dextrose yeast Agar was the most proper medium for isolation, cultivation and characterization of this fungus followed by malt Agar and Saboruad`s Agar. The antifungal activities of essential oils from sweet marjoram (*Majorana hortensis*), basil (*Osinum basilicum*), peppermint (*Mentha peprita*) and spearmint (*Mentha viridis*) against *A. apis* were examined, both *in vitro* and *in vivo*. Marjoram and peppermint oils were the most effective against growth *in vitro* of the fungus. Feeding honeybees with sugar syrup containing 0.5 or 1% of marjoram oil resulted in reductions in the percentages of chalk-brood mummies comparable to those obtained with application of 40% formic acid solution. Screening of some microorganisms for antibiosis towards *A. apis* showed that *Bacillus spp.* (CHBr), isolated from mummified honeybee larvae, was the most potent antagonistic candidate compared to the other examined microorganisms. Preliminary investigation of *in vivo* antagonistic capabilities of this bacterium suggested a possible integration of such bioagent along with sweet marjoram essential oil into a management strategy for chalk-brood biological control.

Keywords: *Ascosphera apis, antifungal activities, essential oils, Bacillus antibiosis*

INTRODUCTION

Chalk-brood is a disease of honey bee larvae (*Apis mellifera*) caused by the heterothallic spore-cyst fungus *Ascosphaera apis*. It is common everywhere, particularly, during damp conditions (Bailey and Ball, 1991). The fungus is only found in association with bees from the subfamily Apinae, germinates in the larval gut (either pre- or post-capping), defeats the immunological barrier of the body cover and spreads a white mycelium over the whole larva body. Along with the appearance of spore cysts, the mycelium turns dark and the dead larva becomes mummified (Chorbinski and Rypula,

2003). The development of the disease in bee colonies are affected by many factors, e.g. strain infectiousness, individual immunity of bee colony, genetic potential of queen and environmental conditions (Harbo, 1995; Spivak and Downey, 1998). Spore germination occurs when colony temperature falls below the optimum range (32–35°C) for more than 2hr (Bailey and Ball, 1991). Ventilation and relative humidity are among the most important factors affecting infestation with chalk-brood (Curry, 1951). Some chalk- brood-resistant honey bee colonies have been shown to exhibit a hygienic behaviuor (Gilliam *et al.*, 1983) which is genetically controlled by simple two separate, unlinked loci (Lapidge *et al.*, 2002). The resistant colonies show reduced *A. apis* contamination in other areas of the colony such as stored pollen, honey and in the gut of nurse bees. In contrast, poor hygienic bees have *A. apis* in a wider variety of hive substrates such as stored food and wax combs (Gilliam *et al.*, 1983).

Chalk-brood has not been diagnosed in Egypt until it was registered, for the first time, by Matheson (1995). The disease was regarded, as a threat to honeybee keeping in Egypt (Hussein, 2000: Ali, 2001).The causative agent was isolated, identified and related to the fungus *A. apis* (Mohamed *et. al.*, 2001; Owayss, 2002). However, there is a scarcity in knowledge about the morphological, cultural and physiological characteristics of *A. Apis* isolated from Egypt, as the previous investigations focused on the entomopathology and chemical control of chalk-brood. Moreover, effective chemical control of chalk-brood is currently unavailable all over the world and the most common practice for dealing with infected colonies is to re-queen from different genetic stocks and dispose of heavily infected equipments (Koenig *et al.*, 1986).

Some essential oils from botanical origin have been reported to exhibit broad-spectra antifungal activities against a wide range of bacteria and fungi. *A. apis* growth inhibition due to essential oils extracted from basil (*Ocinum basilicum*), thyme (*Thymus volgaris*), and sweet marjoram (*Majorana hortensis*) have been reported (Heath, 1982; Colin *et al.*,1989:Higes *et al.*, 2000; Owayss, 2002). However, their role in combating chalk-brood infestation, as an alternative approach for biological control of the disease, needs more investigation (Chantawannakul *et al.*, 2005). On the other hand, very little is known about the antagonism of other microorganisms towards *A. apis* and further research is needed to identify potent antagonistic microorganisms in order to be integrated into a biological control strategy.

The present work was carried out to study some morphological, cultural and physiological traits of *A. Apis* isolated from Egypt. The microorganisms associated with mummified larva were isolated purified and characterized. In an approach for potential and environmentally-safe biological control of such disease, the antifungal activities of some essential oils against the entomopathogen were examined both *in vitro* and *in vivo*. In addition, the antagonism between *A. apis* and some microorganisms was *in vitro* examined. The most antagonistic bacterium was preliminarily examined *in vivo* as a biological control agent.

MATERIALS AND METHODS

Microorganisms

A number of microbial isolates and strains from the cultural collection of the Department of Microbiology, Faculty of Agriculture, Cairo University were examined for antibiosis towards *A. apis*. These microorganisms are: *Bacillus subtilis, Micrococcus spp., Mycobacterium phlie, Pseudomonas Spp.* (No.,1), *Pseudomonas Spp.* (No.,2) *Pseudomonas fluorescence, B. thuringensis, Actinomycetes spp., Saccharomyces cereviciae, Aspergillus niger. Trichoderma reesei* NRRL 11236 and *Phanerochaete chrysosporium* NRRL 6361 as well as *Trichoderma viride* EMCC 107 from the Egyptian Microbial Culture Collection (EMCC), Microbiological Resources Center (MIRCN) were also included. A bacterium isolated from chalk-brood mummies, and identified as *Bacillus* spp., was examined as well.

Cultural Media:

Fungal strains other than *A. apis* were cultivated, propagated and maintained on Potato Dextrose Agar (PDA) (Difco, 1984). The medium supplemented with 0.4% yeast extract (PDYA) (Bailey, 1981) was used for isolation, cultivation and maintenance of *A. apis*. Growth characteristics of *A. apis* were examined on Nutrient agar, Nutrient glucose agar, Yeast Glucose Phosphate broth, Malt Agar medium (0.5-2.0%malt), Martin Agar and Saboruad's Agar Medium. Spore suspension of *A. apis* was prepared by growing the fungus on PDYA slants containing 100μg streptomycin at 25°C for two weeks and scraping the culture in sterile tap water.

Isolation and characterization of *Ascosphaera apis*

Isolation of *Ascosphaera apis* from white and black mummified larvae collected from infected brood cells and hives in the apiaries of Bee Researches Department, Institute of Plant Protection, ARC, was performed according to Bailey and Ball (1991). *A. apis* isolates were identified macroscopically and microscopically (10X and 40X magnifications) following their morphological traits, growth and asco-spore production as described by Bailey and Ball (1991) and using reference images from Anderson *et. al.* (1998). Further identification of *A. apis* isolates was carried out by comparing growth of the morphologically-identified isolates on different media with that occurred on PDYA at 30°C as recommended by Bailey (1981).

Microorganisms associated with chalk-brood mummies

A suspension of ground chalk-brood mummies in peptone water was used for inoculating 100 ml aliquots of the following media: nutrient agar, nutrient glucose agar, PDA, PDYA, martin agar, malt agar and Saboruad's agar. Ten plates from each inoculated medium were incubated at 30°C for 15 days where the developed colonies were counted and isolated. The isolates were examined microscopically and categorized into bacteria, yeast and fungi. Bacterial and fungal isolates were identified according to Buchanan and Gibbons (1974) and Barnett (1960), respectively. The average percentages of fungal yeasts and bacterial isolates were calculated for each medium as the

following example: Average% of bacteria associated with chalk-brood mummies = total No. of bacterial isolates obtained from 10 plates / total No. of all microorganisms developed on 10 plates.

Effect of some essential oils on *in vitro* growth of *Ascosphaera apis*

Essential oils of some aromatic plants *i.e.*, sweet marjoram (*Majorana hortensis*), Basil (*Ocimum basilicum var basilicum*), Peppermint (*Mentha peperita*) and spearmint (*Mentha viridis*) were extracted by steam distillation. The essential oils were diluted using 10% ethanol solution in water and filter-sterilized through sterile, non-pyrogenic and hydrophilic cellulose acetate syringe filter of 25 mm-diameter and 0.2 μm pore size (ALBET-JACS-020-25, Spain).The disc diffusion technique (Sleigh and Timburg, 1981) was applied using filter paper (Whatman No. 1) discs (5mm diameter) for determination of the antifungal activities of four concentrations from each examined oil *i.e.*, 0.5, 1.0, 5.0 and 10 % (v/v). Discs containing 10% ethanol solution were included for comparison.

Antibiosis *in vitro* towards *Ascosphaera apis*

A number of 14 microbial isolates and strains was screened for antibiosis towards *A. apis* grown on PDYA medium according to Brock (1973). The inhibition zone diameters were measured (mm) after 5 days of incubation at 30°C.

Control of chalk-brood in honeybee colonies using essential oils

Eighteen honeybee colonies were arranged into 6 sets each of three replicates in a completely randomized design. Four sets were fed with a sugar syrup (1/1 w/v) containing either 0.5 or 1% oil from either sweet marjoram (*Origanum majoranum*) or Peppermint (*Mintha peperita*). Another set was treated with 40% formic acid solution at a rate of 2.5 ml/comb covered with bees using an absorbent cardboard (10x20 cm) inserted onto the brood combs. A set of three colonies received plain sugar syrup was included for comparison. Treatments were repeated after 5 days and the number of mummified larvae was recorded at 2-week intervals throughout 3 months. The infestation percentages were calculated for each colony.

Control of chalk-brood in honeybee colonies using *Bacillus spp.*

Eight chalk-brood-infested honeybee hives were arranged into 4 sets in a completely randomized design and the numbers of mummies were counted 3 times at a week interval. Two sets were sprayed with spore suspensions of the *Bacillus spp.* (CHBr) containing either 10^7 or 10^9 spores ml^{-1} at a rate of 50 ml/comb. Sterilized tap water was used for spraying a set of two colonies and sterilized broth medium was sprayed on another set where these 2 sets were used for comparison. Treatments were thereafter repeated twice after 3 successive days. The number of mummified larvae was counted at 7-day intervals after treatments application throughout 60 days.

ANOVA was carried out according to the procedures reported by Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Isolation and characterization of *Ascosphaera apis*

Isolation of the fungus was successfully achieved on PDYA medium and none of *A. apis* isolates could be obtained on a plain PDA medium. The obtained isolates from both types of cadavers (mummified larva), either black or white, exhibited the same identical traits. A strong vegetative growth with aerial hyphae occurred on PDYA after 72-hr-incubation period at 30°C covering the plate surface within 15 days with abundant fruiting occurs as early as the 5th day of incubation. The mycelium was fluffy white to light pink, compact or slightly floccose, aerial, silky and covered with liquid droplets. Darkening was noticed after the production of spherical spore cysts about 60 μ in diameter. Branching of hyphae was scarce with septa and external spore cysts appeared on aerial mycelia. These observations are in harmony with those reported by Anderson *et. al.* (1998); Hornitzky (2001); Chorbinski and Rypula (2003) for *A. apis* isolated from chalk-brood larvae in different geographical regions.

Morphological identification of *A. apis* is quiet easy provided that one succeeded in growing strains producing spore cysts. However, strain identification is often a hard task, due to mixed fungal infections as well as the occurrence of non-spore-cyst-producing strains. In addition, determination of the biochemical and enzymatic activities of the fungus is a great help in strain identification which is related to the invasiveness and infectiousness of *A. apis.* strain (Gilliam and Lorenz, 1993). Scanning electron microscopy (SEM) is also a useful tool for *A. apis.* strain identification through studying spore morphology and structure (Chorbinski and Rypula, 2003)..

Growth of *Ascosphaera apis* on different culture media

Identification of the isolated fungus was further confirmed by comparing their growth characteristics on some cultural media with those obtained on PDYA medium as recommended by Bailey (1981). It appears that Nutrient agar, Nutrient glucose gar and PDA were not the suitable media for cultivation of *A. apis* since no colonies could be observed on these media after 10 days of incubation at 30°C. In contrast, mycelia growth could be observed 24 hrs after inoculation of the spore suspension into PDYA plates. Colonies developed on this medium reached 4cm in diameter by the 2nd day of incubation and sporulation was more abundant after 5 days. Also, successful cultivation of the fungus on malt agar was achieved. A colony diameter of 4cm and spore abundance were observed by the third day of incubation; however, growth was not as good as that occurred on PDYA. A semi- solid medium (yeast glucose phosphate) was also examined in 250-ml Erlenmeyer flasks. A subsurface growth of mycelium was initially observed by the 4th day followed by vigorous aerial mycelium after 7 days of incubation. Dark green spore cysts were not observed before the first week of incubation.

Martin agar medium with or without streptomycin was also examined for cultivation of *A. apis.* Very small colonies (0.5 cm diameter) were observed when the fungus was seeded in the medium with streptomycin. The spread of mycelia was restricted and colonies did not exceed 1.5cm in diameter.

Sporulation started in the center of the colonies and did not appear within the first week of incubation. Streptomycin-free medium was more suitable for growth of the fungus where colonies appeared 4 days earlier compared with that supplemented with streptomycin, though the spreading of colonies was limited. Also, slow development of *A. apis* colonies was observed on Saboraoud's agar within 7-9 days of incubation with small diameter of colonies developed after 7 days. *A. apis* growth inhibition was previously observed by Chorbinski and Rypula (2003), probably due to the antibiotic in the medium formulation.

The growth characteristics of the isolated fungus on the examined cultural media were similar, if not identical, to those reported by Bailey (1981), Bailey and Ball (1991) for the entomopathogenic fungus *A. apis*, the causative agent of chalk-brood in honeybee larvae. Three representative out of 15 isolates were selected and examined for their infectiveness on three honeybee brood combs. Larvae in the three examined combs showed the diagnostic features of chalk-brood diseased mummies within 3 days after spraying the combs with a spore suspension of the specific isolate indicating a severe infestation by the disease.

Many techniques have been used to cultivate *A. apis* for identification purposes (Bailey and Ball, 1991; Anderson *et. al.* 1998; Ruffinengo *et al.*, 2000). Despite their success in cultivating *Asc. apis*, studies on chalk-brood infection have typically used a 'whole ground mummy' inoculation protocol which involves grinding entire mummies in a solution, and collecting the released spores for inoculation purposes (Gilliam *et al.*, 1983; Gilliam *et al.*, 1988; Tarpy, 2003). Such inocula possibly contained a combination of other fungi, yeast and bacteria (Johnson *et al.*, 2005).

Microorganisms associated with chalk-brood

In addition to *A. apis*, the mummified larvae harbored a number of microorganisms, fungi, yeasts and bacteria, were isolated purified and morphologically identified. Fungi, including *A. apis*, were the most prevalent microorganisms averaged 76% of total isolates followed by bacteria (12.6%) and yeasts (10.6%) (Table,1). The most common fungal isolates with distinct morphological traits according to Barnet (1976) were identified as *Asp. fumigatus* (average 27.4%) and *Asp. flavus* (14.1%). The highest average percentage of isolated fungi was that belonging to the entomopathogen *A. apis* (33.3%). The growth of this fungus was completely absent on nutrient agar and nutrient glucose agar despite the higher frequency of isolates from the other two species. Almost similar observation was recorded with plain PDA medium; but with slow development of a very few number of colonies representing only 10% of the total isolates. On the contrary, yeasts and bacteria associated with chalk-brood mummies were more frequently isolated from the aforementioned media. Yeasts were the least encountered microorganisms compared with fungi and bacteria. The other examined media supported good growth of all fungi, particularly, *A. apis*.

Bacteria were isolated on all the tested media except with those containing antibiotics where bacterial growth was completely suppressed. Their growth and colony development were more pronounced on nutrient agar

and nutrient glucose agar, compared to other media. The fact that such none specific media did not support growth of fungi might explain the decreasing number of their colonies that developed on such culture media indicating the competitive abilities of bacteria.

The cultural, morphological and physiological tests were conducted for three representative bacterial isolates as described by Buchanan and Gibbons (1974) being considered as *Bacillus spp.* These results agree with those reported by Gilliam *et al.* (1988); Gilliam (1997); Mohamed *et. al.* (2001) and Johnson *et al.* (2005) as many microorganisms have been shown to live in symbiosis with honey bees. These microorganisms are usually found in the intestines of worker bees and include Gram-variable pleomorphic bacteria, *Bacillus spp.*, Enterobacteriaceae, as well as several moulds and yeasts (Gilliam *et al.*, 1988). Certain microorganisms play an important role in the biochemical and microbiological conversion of pollen to bee bread and in assisting the preservation of honey. These additional bacterial agents may have been found in the larva while it was still alive colonizing it after death from chalk-brood infection, or both. Some of these microbes are thought to be important for disease resistance in honey bees by acting as antagonists against certain honeybee pathogens (Gilliam *et al.*, 1988).

Table (1): The percentages of the different microorganisms isolated from chalk-brood mummified honeybee larvae on different cultural media.

Microorganisms / Cultural media	Fungi				Yeasts	Bacteria
	A. apis	Asp. fumigatus	Asp. flavus	Total		
Nutrient agar	0	34	18	52	22	26
Nutrient glucose agar	0	45	26	71	13	16
PDA	10	38	26	74	11	15
PDYA	42	21	14	77	9	14
Martin agar	65	23	12	100	0	0
Malt agar	54	12	7	73	10	17
Saboraoud`s Agar	62	19	10	91	9	0

Antibiosis (*in vitro*) towards *Ascosphaera apis*

Table (2) presents different antagonistic capabilities of various tested microorganisms towards *Asc. apis*. The most antagonistic candidate was a *Bacillus spp.* (CHBr) that isolated in the present work from chalk-brood-mummified larvae. An inhibition zone of 22 mm diam was recorded when this isolate and *A. apis* was grown together in a plate. *Bacillus subtillus* and *B. thuringensis* showed also potent antibiosis against the entomopathogen, inhibition zones measured 17 and 15 mm in diameter, respectively. Isolates of Pseudomonas showed lower antagonistic activities, being the lowest for *Ps. Fluorescence*.

Different antagonistic microorganisms against *A. apis* have been previously isolated from chalk-brood mummified larvae, *i.e. Aureobasidium sp.*

(Gilliam *et al.*, 1988), *Bacillus spp.* (Mohamed *et al.*, 2001), and *Bacillus atrophaeus* (Nabil, *et. al.*,1998). In this regard, Johnson *et. al* (2005) stated that ground mummies inocula used for isolation of pure *A. apis* cultures contain other microorganisms of some antagonistic capabilities which might affect the infectiveness of the entomopathogen when examined *in vivo* for inoculation purposes.

The growth of *A. apis* on PDYA was preceded by growth of all the examined bacterial isolates having generation times shorter than *A. apis*. On the other hand, cultivation of some microorganism in the middle of a PDYA plate inoculated with *A. apis* favored the mycelia growth of the fungus. The development of heavier mycelia growth began earlier in the regions around the wells inoculated with *Actinomyces spp.* or *Sacchoromyces cereviciae*. Some metabolic, enzymatic and fermentative activities of these microorganisms probably resulted in a release of some by- products or growth factors which stimulated growth of the fungus. To the contrary, plating *A. apis* with either *Aspergillus niger*, *Trichoderma resie NRRL 11236*, *T. viridi EMCC 107* or *Phanerochaete chrysosporium NRRL 6361* on a PDYA plate did not affect growth of either microorganisms.

Figures are averages of 4 replicate plates

Table (2): Antibiosis (*in vitro*) of some microorganisms (inhibition zone diameter mm) towards *A. apis* on PDYA.

Microorganism	Diameter of inhibition zone(mm)	Microorganism	Diameter of inhibition zone(mm)
Bacillus subtilis	17	Pseudomonas flouresence	3
B. thuringensis	15	Saccharomyces cerviciae	0
Bacillus spp.(CHBr)	22	Actinomyces spp.	0
Micrococcus spp.	8	Aspergillus niger	0
Mycobacterium phlei	8	Trichoderma resie NRRL 11236	0
Pseudomonas spp. (1)	4	T. viridi EMCC 107	0
Pseudomonas spp.(2)	4	Phanerochaete chrysosporium NRRL 6361	0

Inhibition zones measured after 5 days of incubation at 30°C.

The *in vitro* antifungal activities of some essential oils against *Asc. apis*

The *in vitro* antifungal activities of four essential oils with different concentrations against *A. apis* were examined on PDYA (Table, 3). Growth of the fungus was not affected by 0.1 and 0.5% oil solutions of either sweet marjoram or basil. Growth inhibition was more pronounced when the concentration of the two oils was increased to 5 and 10% where the diameters of growth inhibition zones were 17, 18 and 10, 15 mm respectively. On the other hand, the fungal growth inhibition occurred with all the examined concentrations of peppermint and spearmint, with larger growth inhibition zones appeared in plates containing higher concentrations of these oils.

The obtained results confirm those reported by Colins *et.al.* (1989) for essential oils from thyme, geranium and *Satureia montana*, Higes *et al.* (2000) for oils from sag, Melissa, winter savory, sweet marjoram and lemon tree, Chantawannakul *et al.* (2005) for crude extracts of some medicinal plants. Both *In vitro* and *in vivo* studies by Owayss (2002) proved the antimycotic activities of basil essential oils against *A. apis*.

Control of Chalk-brood in honeybee colonies using essential oils

The efficacies of the essential oils of sweet marjoram and peppermint were *in vivo* examined for control of chalk-brood compared with that of 40% formic acid solution. The percentages of chalk-brood-infested mummies recorded at all the experimental periods were higher in control hives than in those received either essential oil or formic acid (Figure, 1). In the former hives, the disease infestation percentage recorded at the beginning of the experiment (2.17%) was increased gradually with time progressing to reach 2.86 at the last period. In contrast, colonies treated with 40% formic acid solution showed the lowest rates of disease infestation which was significantly ($P >_{0.05}$) decreased to a minimum of 0.04% by the end of the experimental periods. Application of the essential oils also resulted in significant reductions in the disease infestation %, particularly, when 1.0% solution of either sweet marjoram or peppermint oil was applied. In this regard, feeding honeybee with a sugar syrup contained 1.0% of marjoram oil resulted in an ultimate reduction comparable to that achieved with 40% formic acid solution. Despite the significant reductions in chalk-brood-diseased mummies% in colonies treated with the essential oil from peppermint, the *in vivo* antifungal activity of this oil seemed to be lower when compared with that of sweet marjoram or formic acid.

A broad range of chemicals has been previously tested either *in vivo* or *in vitro* for control of chalk-brood (Heath, 1982), however, an effective chemical treatment is not currently available all over the world (Johnson *et. al.*, 2005). The data obtained in the in the present work emphasize the earlier findings by Abou Lila and El-Sisi (1999) on the superiority of formic acid as an antifungal agent for *A. apis* control. Nevertheless, the dependence on the use of chemotherapeutic agents has created several problems in honeybee keeping practices in USA (Jendrejak and Kopernicky, 1998) and in Australia (Chorbiński, 2003). The evolution of chemical-resistant-honeybee pathogen populations and the increased toxicological hazards to beekeepers and bees as well as chemical contamination of some honey crops are among the problems associated with chemical control strategies. These aspects combined with the lack of any effective agent for chalk-brood control, increased interests in the investigation of alternative chalk-brood control strategies (Hornitzky, 2001).

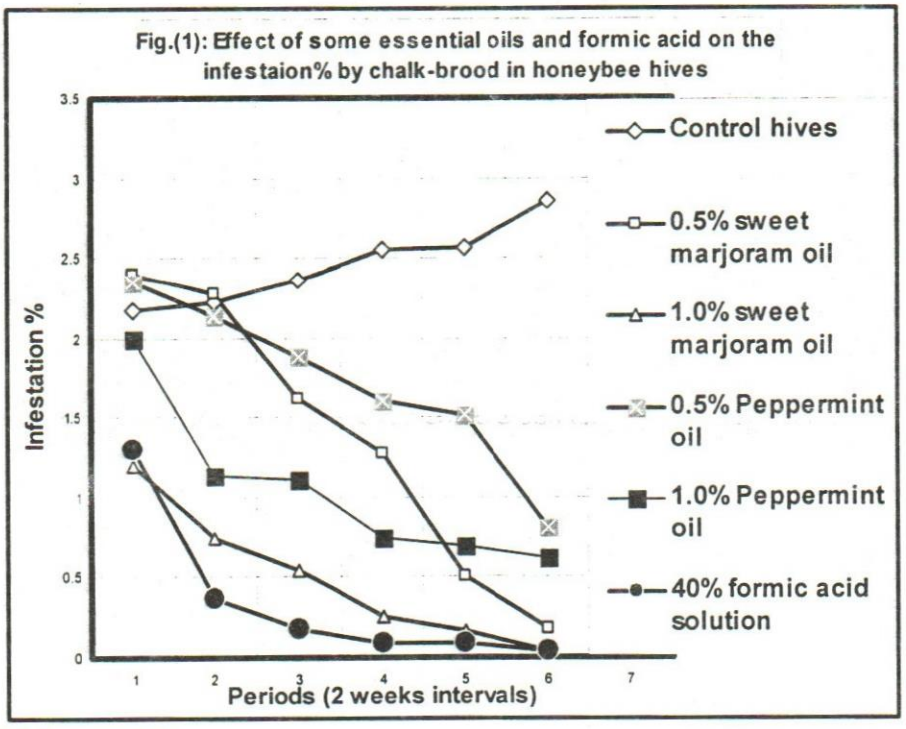
In this regard, data of *in vitro* studies with plant extracts by Colin *et. al.* (1989); Omar, *et. al.* (2001) and crude medicinal plant extracts by Chantawannakul *et. al.* (2005) recommended the possible application of such biological extracts in control of the disease *in vivo*. However, translating the *in vitro* studies into a successful management strategy require the resolution of several problems. These include: a) demonstration of *in vitro* activity at levels

that are not toxic to honeybee larvae, pupae and adults and which do not affect the flavor of honey; and (b) the development of adequate delivery system. In addition, reduced honeybee consumption of supplements containing extracts at concentrations above 0.5% was reported (Hornitzky, 2001) and the feeding formulations must be competitive with naturally occurring nectar and pollen. For these reasons, sweet marjoram and peppermint oils were fed in the present investigation in sugar syrups containing 0.5% or 1.0% oil, although their lower *in vitro* antifungal activities compared to those of more concentrated oil solutions (Table, 2).

Control of chalk-brood in honeybee colonies using *Baillus spp.*

The bacterium isolated in the present work from mummified honeybee larvae, identified as *Bacillus spp.* and examined *in vitro* for antibiosis against *A. apis* was preliminarily examined as a bioagent for chalk-brood control.

Data illustrated in Figure (1) show that the average number of mummified larvae in all hives before treatment application was ranged between 62 and 106 mummies. In colonies received plain broth medium or tap water, no significant differences (L.S.D.$_{P<0.05}$ = 29) could be recorded between the averages number of mummified larvae throughout the experimental period.



L.S.D. $_{P>0.05}$ = 1.23

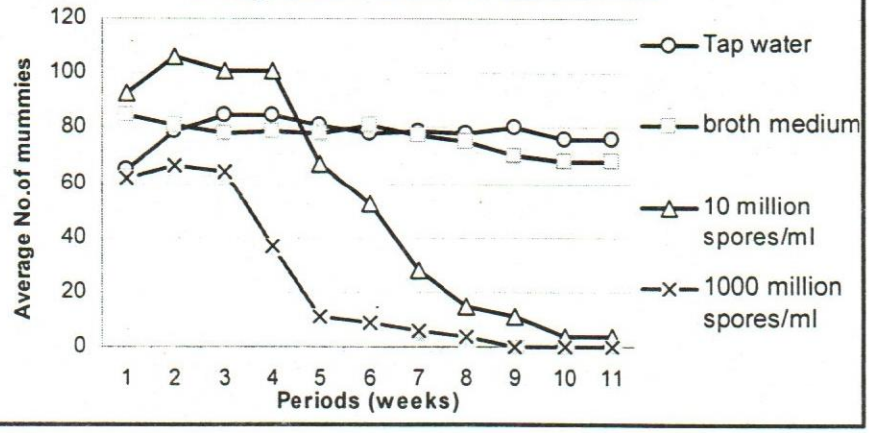
In contrast, application of *Bacillus spp.* spore suspensions resulted in significant reductions in the numbers of chalk-brood mummies recorded only

one week after application. Thereafter, the infection was drastically reduced reaching its minimum by the end of the last week. The disease was completely disappeared in colonies treated with the spore suspension containing 10^9 spores ml^{-1}.

Despite the huge number of experimental work that has been carried out to find out a potential chemotherapeutic agent for chalk-brood control, there is a scarcity in knowledge about *in vitro* antagonistic capabilities of microorganisms against *A. apis*. Moreover, data from field studies on the use of bacteria for biological control of chalk-brood were not available for us during the course of the present study. However, the antagonistic capabilities of a *Bacillus spp.* isolate against *Acremonium strictum* , the causative agent of Acremonium wilt in grain sorghum, were previously reported *in vitro* (Ali *et. al.*, 2004) and *in vivo* (Ali *et. al.*, 2005).

In conclusion, the data from the present work suggest a possible integration of sweet marjoram essential oil along with the antagonistic *Bacillus spp.* isolate (CHBr) in a management strategy for biological control of chalk-brood in the Egyptian honeybee keeping. Work is in progress concerning further investigation of the interactions among *A. apis*, *Bacillus spp.* (CHBr) and sweet marjoram essential oil in honey bee feeding formulations for eradication of chalk-brood disease. Further work needs to be carried out to study the physiology of the antagonistic bacterium in order to clarify its mode of action against growth of the fungus.

Fig. (2): Effect of *Bacillus spp.* spore suspensions on the average number of chalk-brood-mummies



L.S.D. $_{P<0.05}$ = 29.0

REFERENCES

Abou Lila, S.M. and El-Sisi, A.G. (1999): Controlling chalk-brood disease that infesting honeybee (*Apis mellifera*) colonies in Egypt using some local natural substances. Proceed. Apimondia 99, Congress XXXV1, Vancouver, 12-17 Sept., Canada.

Ali, M.A.M. (2001): Biological and ecological studies on certain pests of honeybees. PhD thesis (Entomology), Plant Protec. Dept., Fac. of Agric., Ain Shams Univ.

Ali, M.A; Osman Ettimad A , Ibrahim Thanaa, F; Khalafalla, G. and Amin, G. (2004). *In vtro* antagonism between soil and rhizosphere microorganisms and *Acremonium strictum*, the causal fungus of Acremonium wilt in grain sorghum. J. Agric. Sci. Mansoura Univ.29 (10):5821-5833.

Ali, M.A; Osman Ettimad A, Ibrahim Thanaa F; Khalafalla, G. and Amin, G. (2005). *In vivo* antagonistic capabilities of various *Bacillus spp.* formulations against *Acremonium Strictum*. J. Agric. Sci. Mansoura Univ., 30, 2: 851-860.

Anderson, D. L; Gibbs, A. J; Gibson, N. L. (1998): Identification and phylogeny of spore-cyst fungi (*Ascosphaera spp.*) using ribosomal DNA sequences. Mycological Research 102:541–547.

Bailey, L. (1981): Honeybee pathology. Chapter 5, Fungi, pp: 40-46.Academic Press. INC., London, LTD.

Bailey, L. and Ball, B. V. (1991): Honey bee pathology. Academic Press; London, UK.

Barnett, H.J (1960): Illustrated Genera of imperfect fungi. Burgess, Minneapolis, USA.

Brock, T. D. (1973): Basic Microbiology with Application. Prentic Hall Inc., Englewood Cliffs, New Jersey, USA.

Buchanan, R. E. and Gibbons, M. E. (1974): Bergy`s Manual of Determintive Bacteriology. 8th ed., The Williams and Wikins Corp., Baltimore, USA.

Chantawannakul, P; Puchanichanthranon, T. and Wongsiri, S. (2005): Inhibitory effect of some medicinal plant extracts on the growth *of Ascosphera apis* . In proceeding of the III WOCMAP Congress on Medicinal and Aromatic Plants - Volume 4: Targeted Screening of Medicinal and Aromatic Plants, Economics and Law , Feb. 2005.

Chorbinski, P and Rypula, K. (2003): Studies on the morphology of strains of *Ascosphera apis* from chalk-brood disease of the honeybees. Veterinary Medicine, series 6, issue 2: 11-21.

Colins, E; Ducos de Lahitte, J; Larribau, E. and Boue, T. (1989): Activity of some essential oils of Labiaceae on *Ascosphera apis* and treatment of an apiary. Apidologie, 20,3:221-228.

Curry, R. (1951): Mycosis of honeybees and fungi beehives. Biologico (Brazil). 17: 214-220.In: ----Roger Morse (19800): `Honeybee pests, predators and diseases`, 2nd ed. Cornell University press Ltd. , UK.

Difco, Manual (1984). Difco Manual of Dehydrated Cultural Media and Reagents for Microbiological and Chemical Laboratory. Procedures. 9th Edition.

Gilliam, M; Taper, S; Richardson, G. V. (1983): Hygienic behavior of honeybees in relation to chalk-brood disease. Apidologie 14: 29–39.

Gilliam, M; Lorenz, B. J. and Richardson, G. V. (1988) Digestive enzymes and microorganisms in honey bees, *Apis mellifera*. Influence of streptomycin, age, season and pollen. Microbios, 55: 95–114.

Gilliam, M. and Lorenz, B. J (1993) enzymatic activity of strains of *Ascosphera apis* an entomopathogenic fungus of honeybee larvae, *Apis millifera*. Apidologie, 24: 19-23 .

Harbo, J, (1995): Observation on hygienic behaviour and resistance to chalk-brood. Am. Bee J; 135,828.

Heath, L. A. (1982): Chalk-brood pathogen: A review. Bee world, 63, 3:130-135.

Higes, M; Rojo, N; Sanz, A; Perez, M; Martin, R. and Cruz, M. (2000): Use of essential oils in controlling ascospherosis in *Apis mellifera*. Efficiency *in vitro* Apiacta, 35, 4:183-191.

Hornitzky, M (2001): Literature review of chalk-brood, a fungal disease of honeybee. A report for the Rural Industries Research and Development Corporation. RIRDC Publication No 01/150

Hussein, M. H. (2000): A review of beekeeping in Arab countries. Bee World. 81, 2:56-71.

Jendrejak, R. and Kopernicky, J. (1998): Evaluation on therapeutic efficiency of the preparation Apiform for Varroa and chalk-brood disease control Journal of Farm Animal Sciences: 31: 209-214.

Johonson, R; Tauheedzman, M; Decelle, M; Siegel, A; Tarpy, D; Siegel, E. and Starks, P. (2005): Multiple microorganisms in chalk-brood mummies: evidence and implications. Journal of Apicultural Research, 44(1): 29–32 (2005).

Koenig, J. P; Boush, G. M; Erickson, E. H. (1986): Effect of type of brood comb on chalk brood disease in honeybee colonies. Journal of Apicultural Research, 25:58–62.

Lapidge, K. L; Oldroyd, B. P; Spivak, M. (2002): Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. Naturwissenschaften, 89: 565–568.

Matheson, A. (1995): World bee health update. Bee world. 76, 1:31-39.

Mohamed, A. A; Hassan, A.R; Elbana, A. A.(2001): Using antioxidant compounds for controlling chalk-brood of honeybee colonies in Middle Egypt. In the proceeding of conference `Safe alternatives of pesticides for pest management`. Assiut Univ., Egypt (October 28-29, 2001)

Nabil N. Youssef,a and Joyce Knoblettaa (1998): Culture filtrate of Bacillus atrophaeus induced abnormalities in *Ascosphaera apis*. Mycologia, 90, 6: 937–946.

Omar, M. O. M; Abd-Aal, Y. A. L; Kalil, F. M. and Khodairy, M.M.(2001): Effect of naturally occurring substances on Vorroa mites. The 1st Conf. on safe alternatives of pesticides for pest management, Oct. 28-29, Assiut Univ., Egypt.

Owayss, A.A.(2002): Ecological and control studies on certain diseases of honeybee with special reference to integrated management of Varroa Mite. PhD thesis, fac. of Agric., El-Fayoum, Cairo Univ.

Ruffinengo, S; Pena, N. I; Clemente, G; Palacio, M. A; Escande, A. (2000): Suitability of culture media for the production of asco-spores and maintenance of *Ascosphaera apis*. Journal of Apicultural Research 39: 143–148.

Sleigh, J. D. and Timburg, M.C.(1981): Notes on medical bacteriology. Churchill Livingstone, London.

Spivak, M. and Downey, D. L. (1998): Field assay for hygienic behaviour in honeybees(*Hymnoptera, Apidae*). J. Econ. Entomol. 91, 64-70.

Tarpy, D. R. (2003): Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proceedings of the Royal Society of London, Series B, 270: 99–103.

دراسات ميكروبيولوجية علىمرض الحضنة الطباشيري فى خلايا نحل العسل فى مصر

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لم يكن مرض تحجر الحضنة الطباشيرى ليرقات نحل العسل والذى يسببه فطر *Ascosphaera apis* معروفا كأحد أمراض نحل العسل فى مصر الى أن تم تسجيله حديثا عام ١٩٩٥، ويعتبر الآن من أهم الأخطار اللتى تهدد تربية نحل العسل. ولا توجد طريقة فعالة لمكافحة هذا المرض فى جميع أنحاء العالم غير أستبدال الطوائف المصابة بأخرى لها صفة المقاومة الوراثية لهذا المرض. وقد تم فى هذا البحث دراسة بعض الصفات المزرعية والمورفولوجية والفسيولوجية لفطر أسكوسفيرا ابيس المعزول من يرقات نحل العسل المصابة بمرض الحضنة الطباشيرى واللتى تم جمعها من مناحل مصرية مختلفة. أيضا تم دراسة الميكروبات المختلفة المصاحبة ليرقات نحل العسل المصابة بالمرض حيث أظهرت الدراسة أن فطر *Ascosphaera apis* هو الغالب ويمثل نسبة٣٣,٣ % من أجمالى عدد الميكروبات المعزولة من اليرقات يليه فطر اسبرجيلس فيوميجيتاس (27.4%) وفطر اسبرجيلس فلافس(14.1%) . وكانت نسبة عزلات البكتريا والخمائر 12.6 و 10.6% على التوالى. كانت بيئة أجار البطاطس ومستخلص الخميرة هى أفضل البيئات لعزل وتنمية الفطر الممرض ليرقات النحل تلاها فى ذلك بيئة أجار المولت و بيئة أجار Saboruad . و قد تم دراسة التأثير المثبط للزيوت العطرية المستخلصة من نباتات البردقوش و النعناع الفلفلى والنعناع البلدى و الريحان على نمو الفطر الممرض للنحل فى المعمل و فى المنحل ووجد أن كل من زيت البردقوش وزيت النعناع الفلفلى لهما فعالية كبيرة فى تثبيط نمو الفطر على البيئات الصناعية. وقد أدى تغذية النحل على شراب سكرى يحتوى على ١% أو 0.5% من زيت البردقوش أو زيت النعناع الفلفلى ألى أنخفاض فى نسبة أعداد اليرقات المصابة بمرض الحضنة الطباشيرى يقارب الأنخفاض الناتج عن استخدام محلول 40% من حامض الفورميك. ثم أجرى أختبار التضاد بين الفطر الممرض للنحل ومجموعة من الميكروبات المعروفة حيث ظهر أن العزلة *Bacillus spp.*(CHBr). المعزولة من اليرقات المصابة بالمرض هى أكثر الميكروبات المختبرة من ناحية أظهار التضاد للفطر المنمى على البيئات الصناعية. وأظهر الأختبار المبدئى لهذة العزلة في المنحل أنه يمكن أدراج هذه البكتريا بجانب زيت البردقوش العطرى فى برنامج شامل لمكافحة مرض الحضنة الطباشيرى فى المناحل المصرية.