# ADDIS ABABA UNIVERSITY

# SCHOOL OF GRADUATE STUDIES
# DEPARTMENT OF BIOLOGY

## CHARACTERIZATION OF THE CAUSATIVE AGENT OF CHALKBROOD DISEASE OF HONEYBEE BROOD (*APIS MELLIFERA* L.) IN ETHIOPIA

**BY TESHOME GEREMEW**

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOLOGY

*JULY 2006*

## ACKNOWLEDGEMENT

I would like to thank my supervisor Dr. Dawit Abate for his excellent constructive advice, encouragement, supervision, and facilitating field trips despite the heavy load of work and responsibilities in the Department.

I would also like to express my heart felt thanks to my co-supervisor Dr. Aster Johannes (Ethiopian Agricultural Research Institute) for her warm welcoming in to her laboratories and provide me with samples for this study.

I am highly indebted to Dr. Amsalu Bezabih (Ethiopian Agricultural Research Institute) for providing laboratory facilities, experimental honeybee colonies and transport the hives to the research center.

Special thanks also go to W/O Enani Beshawred, Shewanesh Negera, Ato Hiwot Birhanu and Ato Nigusse for their help during the filed work in evenings and weekends at Holleta Bee Research Center.

I am highly grateful to Dr. Seyoum Leta for allowing me to use his computer fitted microscope equipped with reliable software

I thank ARF secretariat, EARI and AAU for financial support.

I'm also highly grateful to Addis Ababa Education Bureau, Nifas Silk Lafto Sub-city and Higher 23 S.S. School for sponsoring me.

I can't find words to thank my fiancée Yetayesh Maru and my sister Zinash Geremew for their special encouragement and assistance during my study.

Lastly but not certainly least, I want to thank my friends in Applied Microbiology Stream in 2005/6 at AAU.

# Table of contents

**Page**

List of tables

# List of figures

**Figures** **Pages**

## List of abbreviations

AAU – Addis Ababa University.

AI – *Ascosphaera* isolate.

EARI – Ethiopian Agricultural Research Institute.

MY20 – Malt extract, Yeast extract with 20% glucose.

PDA – Potato Dextrose Agar.

SDA – Sabouraud's Dextrose Agar.

## Abstract

Chalkbrood disease is an invasive mycosis of honeybee larvae (*Apis mellifera* L) caused by *Ascosphaera apis (*Maassen ex Claussen) Spiltoir and Olive (1955). The causative agent of this disease occurs in most bee keeping countries of the world. This study aims to characterize the causative agent of the disease and compares it with reference strain (MUCL 34668). The mummies were surface disinfected and inoculated onto Sabouards Dextrose Agar for macroscopic and microscopic identification. The local isolates were also compared with the reference strain, assayed for proteolytic ability, toxicity, and virulence on honeybee larvae in the laboratory and in the hive. Microbiological analysis of 45 samples of dead honeybee larvae collected from the regions during 2004-2006 yielded 28 positive cases for chalkbrood disease incidence (62.22%). Spore cyst, spore ball, and ascospores length to width ratios measured with phase contrast inverted computer fitted microscope were 66.15 –97.66μm, 11.00 –19.27μm and 2.00-2.95μm, respectively. The measurements obtained and examination results enabled us to confirm that the local isolates belongs to the genus *Ascosphaera* and to the species *Ascosphaera apis.*



**Key words:** *Chalkbrood disease, Ascosphaera apis, spore cyst, spore ball, Ascospores, Apis mellifera.*

# 1. INTRODUCTION

Honeybees (*Apis mellifera*) are the most intensely studied of all insects. Their economic and ecological importance has driven the need for scientific research (Bailey and Ball 1991). The economic importance of honeybee is due to the products they produce and services they provide. Hundreds of million of dollars are generated through the sale of honeybee products such as honey, wax, pollen, propolis, royal jelly and venom in USA. It is estimated that an average of 30,000 tons of honey are produced each year in Australia. The gross value of production over all sectors of the honeybee industry is estimated between 60 and 65 million Australian dollars per annum, of which 49 million comprises honey production (Gibbs and Muirhead, 1998). In Africa; Ethiopia, Kenya, Angola and Tanzania are known for honey production. Ethiopia, which is the leader in honey production in Africa and fourth in wax production in the world, produces 32.5 metric tones of honey and 3.02 thousand tones of wax per annum (Amsalu, personal communication).

Honeybees (*Apis mellifera*) are absolutely essential for the production of agricultural food crops. In USA, honeybees pollinate more than 50 different agricultural crops valued at more than 20 billion dollars (Heath, 1982). They commonly pollinate agricultural crops such as apples, cherries, melons, almonds, oil seeds, etc (Vandenberg *et al*., 1980). In fact, in USA, many farmers hire beekeepers to raise and maintain bee colonies on their farms entirely for this purpose. It is estimated that 80% of all crop insect pollinations are accomplished by honeybees (Bendek, 1985). This economically and ecologically important insect has been attacked by various pathogens (such as fungi, bacteria, protozoa), parasites, and also affected by pesticides. Fungi are common saprophytes of

dead bees and combs. Majority of the fungi collected by bees are unable to become established within the bee colony and beehive. However, some of the fungal species such as, *Ascosphaera apis, Aspergillus* spp., *Aureobasidium pullulans, Trichoderma lignorum, Mucor hiemalis, Rhizopus* and yeast *Torulopisis* are considered to be honey bee fungal pathogens (Glinski and Buczek, 2003). Most entomopathogenic fungi have similar mechanism of attack on insects (Shah and Pell, 2003). In most insects, fungal infection is initiated by spore germination. The developing invasive hyphae penetrate the cuticle mechanically and enzymatically and enter the bee body cavity where they rapidly develop and overgrow in the internal organs. The invasion may some times start by ingested fungal spores that germinate in the intestine (Bailey, 1967; Chorbinski, 2004). Physical, chemical and biological stress factors, mainly temperature, high humidity, environmental pollution, pesticide poisoning, parasite invasion, attack of predators, are the factors predisposing the development of fungal infection in insects (Heath, 1982). These all can reduce the resistance pattern of insects to mycosis by compromising the immune system and by impairment the protective barriers of the body coverings, alimentary tract and tracheae (Glinski and Buczek, 2003).

Fungal toxins released by some fungi, for example, aflatoxin produced by *Aspergillus flavus,* act directly on the central nervous system of insects. By affecting the endocrine system and most probably the internal defense system of the invaded individual, they decrease the resistance in the bees to mycotic infection (Minho, *et al*., 2005). The outcome of the infection depend up on the genetic potential of the pathogen to grow rapidly, utilize host body constituents for nutrition, production of cuticle degrading

enzymes to penetrate anatomical barriers, and resist the host immune mechanism (Minho, *et al*., 2005). Death of an insect may result from mechanical and enzymatic damage to tissue affected by mycelium, abnormal function of organs, mechanical disturbance of blood circulation, and toxic action on the host bee. Competition for food between growing fungus and infected bee organs cannot be excluded in the pathogenesis of mycosis (Jarosz and Glinski, 2000).

This research focused on isolation and characterization of the causative agent of chalkbrood disease from mummies collected from nine administrative zones of Ethiopia.

## 2. Objectives of the study

The objectives of this study are

-To isolate, characterize and identify the causative agent of chalkbrood disease of honeybee larvae from mummies collected from various areas of the country.

-Compare the reproductive structures of the isolates among themselves, with literature and reference stains.

-Investigate virulence factors of the isolates.

- To confirm that the isolates are honeybee pathogens.

# 3. Literature review

## 3.1. Chalkbrood disease

Chalkbrood disease of honeybees *(Apis mellifera* L.) is an invasive mycosis produced by the fungus *Ascosphaera apis,* affecting stretched larvae. At first, a fluffy white mould covers dead larvae inside recently capped cells, and later they dry and become black or white mummies (Figs **l).** At the peak of the disease, mummies are easily detected at the entrance to the hive as nurse bees remove them from their cells (Floyd and Paul, 1976)**.**

Fig.1. Symptom of chalkbrood disease, x 4 **(**Paweł and Krzysztof, 2003)

### 3.2. History

The disease and its causal fungus were first described by Maassen from Germany in 1913. Reports of chalkbrood disease from Switzerland, Poland, France, Russia Czechoslovakia and Greece followed that of Maassen. Maassen proposed the name *Pericistis apis,*which was retained by Claussen who made a short description of the fungus. The life cycle was first described by Spiltoir (1955). Spiltoir and Olive (1955) introduced the name of the genus, *Ascosphaera*. However, the taxonomic position of *Ascosphaera* sp. was given by Skou (1 972):

Subdivision: *Ascomycotina.*

Class: *Plectomycetes*

Order: *Ascosphaerales*

Family: *Ascosphaeraceae*

Genus: Ascospharea

The chalkbrood disease of honeybees (*Apis mellifera*) has been recognized since the early 1900s and has been studied over the years (Heath, 1982). Chalkbrood is common in most beekeeping countries. It occurs widely in the temperate regions of the Northern hemisphere and has long been known in Europe, Scandinavia and Russia (Betts, 1932)

and in New Zealand (Seal, 1957). It was officially recognized in America and Canada in about 1970, and has subsequently been detected in Argentina, Japan, the Philippines, Central America and Mexico (Heath, 1985). Chalkbrood was first reported in Southeast Queens Land in 1993 and by 1995 it had spread to New South Wales, Victoria and South Australia. More recently, it has been confirmed in Tasmania, Western Australia and the Northern Territory (Hornitzky, 2001). Different strains of the fungus may have become established, perhaps in solitary bees, which have been cultivated and distributed on a large scale in America for many years, particularly for the pollination of alfalfa. Solitary bees have suffered greatly from their own species of *Ascosphaera*, probably as a result of the increasingly industrial style of their management (Vandenberg *et al.*, 1980). *A. apis* has been isolated from some species of solitary bee and there may be strains with increased virulence for honey bees among them. Newly emerging healthy adult leafcutter bees have to chew their way through any larvae killed by the fungus, or through contaminated nest material in the tunnel ahead of them. Each young solitary bee can carry many spores on their bodies (Vandenberg *et al.*, 1980), and some of these spores may find their way to honey bees foraging on alfalfa. Although Heath (1985) found no cultural differences of any significance between American and British strains of *A. apis*, Glinski (1982) reported up to 20-fold differences between the virulence of some of the 40 strains they tested on young honeybee larvae (Bailey and Ball, 1991).

Nixon (1982) indicated the presence of chalkbrood disease in North Africa (Tunisia). In Ethiopia the disease, Chalk brood, was detected in Holleta Bee Research Center apiary in 2000. Based on this detection, a survey was made around Holleta area on individual farmer beekeeper's hives and at Gedo demonstration site (150 km away from Holeta).

From a total of 88 honeybee colonies inspected, 15 of them were infected with this disease. Reports coming from different apiaries of the center and from the beekeeping farmers suspected that this chalkbrood disease of honeybee is becoming a threatening problem on the development of the sector (Dessalegn, 2006). Chalk brood disease has also detected in South Wollo, North Gonder, West Gojam zones of Amhara regional State and West Showa, East Wellega, Illubabor, Jimma, Arsi and East Showa zones of Oromia regional State. According to the reports obtained from Holleta Bee Research Center, 27% of honeybee colonies diagnosed in East Showa and Arsi zones are infected with chalk brood disease of which 21% dwindled, 44% died, and 35% were absconded (Amsalu, personal communication).

3.3**.** Multiplication and spread

In nature, fungal spores are present in the honey and pollen stored in the hive. The fungus has also been isolated from the surface of combs, water sources, and from the digestive tract of adult bees (Koenig ***et al***., 1987). The continuous food sharing in a colony provides a mechanism by which spores can be spread amongst adult bees, including those feeding brood. In this way, the spores can easily reach the digestive tract of bee larvae from stored food, contaminated surfaces, or nurse bees. Larvae ingest spores of ***A. apis*** with their food (Chorbinski, 2004; Purta ***et al***., 1994; Flores**, *et al*,**. 1996). The spores germinate in the lumen of the gut, probably activated by $CO_2$ from the tissues (Heath and Gaze, 1987), and the mycelium begins to grow there, particularly at the hind end. The mycelium then penetrates the gut wall and eventually breaks out of the hind end of the larva's body, often leaving the head unaffected. When they occur, fruiting bodies form on

the outside of the dead larvae. *A. apis* grows best in slightly chilled larvae as its optimal temperature for growth and formation of fruiting bodies is about $30^0$C (Bailey, 1967). Experiments have shown that brood is most susceptible when chilled immediately after it has been capped. The chilling need only a slight reduction of temperature, from the normal $35^0$C, for a few hours; and it can easily occur, even in warm climates, in colonies that temporarily have insufficient adult bees to incubate their brood adequately. Larvae are most likely to be chilled in warm and moist seasons when colonies are growing, and drone larvae often suffer most as they are generally on the periphery of brood nests (Hornitzky, 2001). The smallest colonies are at the greatest risk of becoming chilled because they have the lowest capacity for heat and relatively large surface areas. Koenig *et al.* (1986) and Heath (1982), in their reviews, quoted several observations that chalkbrood is aggravated when colonies are rapidly expanding in spring. When the ratio of brood to adult bees is high, or when it is increased experimentally in observation hives are very susceptible. Pederson (1976) showed that artificially heating of hives in spring diminished the incidence of the disease. Other non-lethal factors, such as slight infections by viruses or bacteria, or poisoning, or inadequate food from diseased nurse bees may well cause the same effect as chilling by slowing the rate of development of larvae (Bailey and Ball, 1991). Heath (1982) makes it evident that *A. apis* is widespread throughout Britain and states that in South West England "it has been possible to detect *A. apis* in any colony of bees if the search is diligent enough". Clearly then, spread of the disease within colonies is almost entirely suppressed spontaneously. The migratory practice of many commercial beekeepers within Australia is likely to have contributed to its rapid spread within the continent. Each larva that is killed by chalkbrood produces

cysts that can intern form about $10^8$-$10^9$ spores (Nelson and Gochnauer, 1982). Most of these are ejected from the colony by the house-cleaning bees that remove dead larvae from their cells, but many will inevitably find their way to healthy larvae via mechanical contamination on nurse bees or become lodged in food stores, and especially in brood comb (Koenig *et al*., 1986), where they stay infective for many years. Transmission of spores may be by wind from mummies carried to the exterior of the hives contends that *A. apis* is rarely lethal unless larvae are chilled for a brief period shortly after sealing. Spores could he picked up by foraging bees at nectar, pollen, or water sources and passed on to larvae in their food; or infection could be spread by adult bees with contaminated mouth and body parts.

The spread of chalkbrood within colonies is probably much suppressed by the normal temperature of the brood-nest or the absence of possible predisposing factors; but some further limitation would seem to be imposed by the need for at least two spores of different strains to germinate and mate within a larva to form fruiting bodies. Many chalky-white mummies often larvae indicate limitation with fruiting bodies in typical outbreaks of disease (Puerta *et al*., 1994). Indeed, combs have been found with all the dead larvae apparently infected with one strain of the fungus. This implies infection stemming from a single larva that had been infected with only one strain, and that larvae can become infected with mycelium as well as spores, which seem improbable (Gochnauer and Margett, 1979). A more likely answer may lie in the observations of Christensen and Gilliam (1983) that isolated both strains from larvae that nevertheless appeared chalky-white. They suggested that unknown conditions within the gut sometimes favour the proliferation of one strain over the other. Consequently, larval

remains may become overgrown and depleted by one strain before the other has time to mate and form cysts. According to Floyd and Paul (1976), at Madison, colonies in outyards not showing chalkbrood in 1974 were requeened with queens reared in colonies at the homeyard that contained chalkbrood. In 1975, all these outyards showed some colonies with chalkbrood, an indication that the infection had spread to them via queens. In the spring of 1975, colonies clean in 1974 were requeened with queens purchased from a breeder who had chalkbrood in his outyard. During the summer of 1975, these colonies showed some chalkhrood, further evidence that queens may disseminate the disease. Spread via queens could account for the rapid spread throughout the country. Floyd and Paul, (1976) also stated that spore remain viable for 15 years on the comb.

### 3.4. Life cycle of *Ascosphaera apis*

The life cycle of the causative organism of chalkbrood disease is not clearly defined in nature (Heath 1982). In culture, *A. apis* exists as dense grayish-white mycelium containing aerial, surface, and subsurface hyphae. Surface hyphae are 4 to 8 microns wide, and vegetative nuclei are very small. It is morphologically heterothallic, that is, it has + and - strains (or male and female strains) that, when inoculated onto the same agar plate, will show a black line of spore cysts where the mycelia of the two strains make sexual union (Floyd and Paul 1976). No apparent morphological differences exist between the two types when seen in culture. When the hyphae of compatible types come close together, some of the female hyphae produce large protuberances (ascogonial primordia) that elongate and grow toward the male hyphae. The formation of the

ascogonium and its contact with the male hypha take only 15 to 20 minutes. The nutriocyte reaches maximum size in about one day and then begins a complex series of cell divisions and delineations that terminate in the formation of ascospores contained in numerous spore balls enclosed in a spore cyst that appears black because of the thick, dark colored wall. The spores of *A. apis* require a nearly anaerobic environment for germination, but the mycelium requires an aerobic environment for growth (Bailey 1967). Thus, optimum temperature for growth is about 30ºC (86ºF), and spores germinate best at 35ºC (95ºF). However, when the temperature is lowered to 25ºC (77ºF), oxygen can move more readily into the agar, which allows the mycelium to grow. Bailey (1967) indicated that infection of the honeybee larva begins when chalkbrood spores are ingested with the larval food. However, the germinated spores are generally voided harmlessly with the feces because the mycelium cannot grow in the anaerobic environment of the gut. Spores apparently germinate in the lumen of the gut, especially at the hind end that does not communicate with the rectum until after the larva is sealed in the cell to pupate (Bailey 1967).

### 3.5. Pathogenesis

Several histological studies have been conducted on larvae infected with chalkbrood (Carrera *et al.,* 1987; Bamford and Heath, 1989; Puerta *et al.,* 1994). Nevertheless, there is still controversy about the route of invasion of the fungus into larvae. The most widely accepted theory is that spores are ingested by the uncapped larvae and germinate inside the gut lumen, eventually growing through the gut wall. The spores germinate in the lumen of the gut, probably activated by $CO_2$ from the tissues and the mycelium begins to

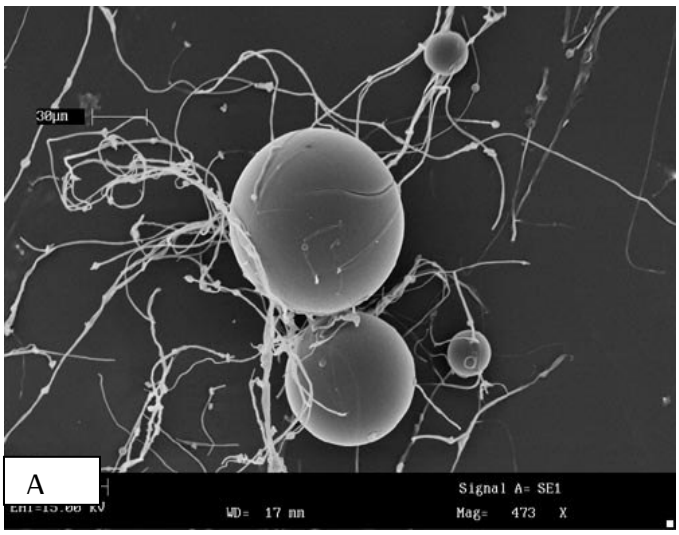
grow there, particularly at the hind end (Heath and Gaze, 1987). The mycelium then penetrates the gut wall and eventually breaks out of the hind end of the larva's body, often leaving the head unaffected. When they occur, fruiting bodies form on the outside of the dead larvae. Although the fungus lacks many of the lytic enzymes usually found in other fungal pathogens (Gochnauer and Margetts, 1979), *A. apis* can emerge through the larval cuticle by a combination of mechanical (pressure of hyphae produced from the invaded larval tissues) and enzymatic action (proteolytic, lipolytic and N-acetyl-glucosaminidase). Infection seems to be initiated by ascospores (Heath, 1982), although some authors suggest that infection is directly produced by invading hyphae (Gilliam, 1978). Larvae can ingest the fungus at an early stage, but only stretched larvae, inside capped cells, present symptoms of the disease.

### 3.6. Laboratory diagnosis

The diagnosis of chalkbrood is based on the recognition of signs of disease and the identification of *A. apis* in diseased material. The following signs characterize clinical evidence of the disease (Gilliam, 1990). Larvae usually die of chalkbrood after their cells have been capped. Infection is more commonly found in worker and drone larvae than in queen larvae. When uncapped, dead larvae at first are somewhat fluffy white, swollen and sponge-like and may take on the hexagonal shape of the cell. Later they become hard and chalk-like in appearance and are called "mummies" which will either remain whitish or, if the fungus develops fruiting bodies, turn grey or black (Puerta, *et al.*, 1994). The mummies remain whitish if they are infected with only one strain of the fungus but will turn grey or black when infected with both strains of the fungus as a result of the

production of fruiting bodies. By this stage, the cappings have frequently been removed by the bees (Gilliam, 1990; Puerta, *et al.,* 1994).

The laboratory diagnosis is based on the demonstration of the causative agent (*A. apis*) in diseased material (Puerta, *et al.,* 1994). This is achieved by mounting some diseased material, preferably 'mummies' which have turned grey or black, on a microscope slide, adding water or a dye to the material and mixing thoroughly. The resultant suspension is then examined under the microscope. The presence of spore cyst is usually sufficient to make the diagnosis. The sporocyst, which is about 80µm in diameter (Fig.2A), contains a smaller round bodies called spore balls (average 12µm in diameter, Fig. 2B) and it is in these spore balls that the ascospores (average 2.9 x 1.4µm, Fig. 2C) the most infective stage of the fungus is found (Gilliam, 1990). In samples where only white 'mummies' have been submitted and spore-producing bodies cannot be detected when examined under the microscope it may be necessary to grow the fungus on potato dextrose agar, Sabourads dextrose agar or MY20 medium (200g glucose, 5g peptone, 3g yeast extract, 3g malt extract, 20g agar and 1liter water).



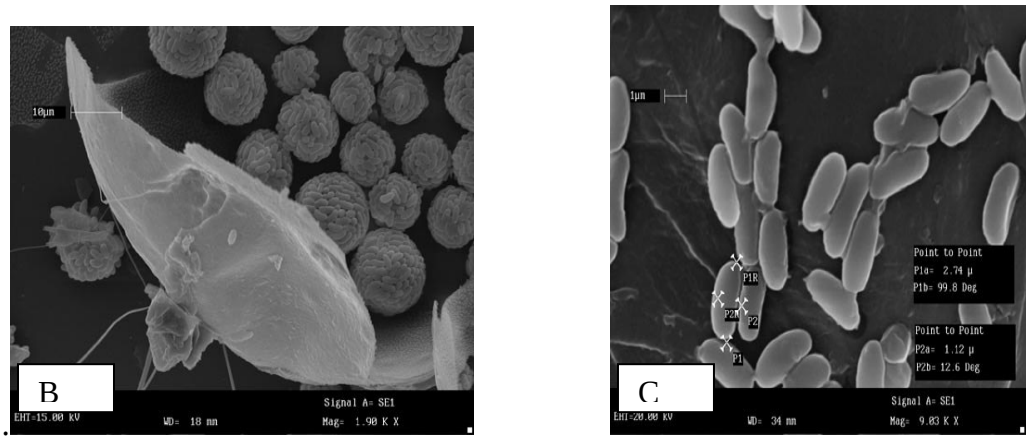



Fig. 2. A. Young formed spore cyst (SEM x 2260). B. Spore balls (SEM x 3460). C. Ascospores (SEM x 10400) of ***A. apis*** (Paweł and, Krzysztof, 2003).

3.7. Culture of *Ascosphaera apis.*

Many media supporting the growth of ***A. apis*** have been described (reviewed by Heath, 1982). The most frequently used are Potato Dextrose Agar, Saborauds Dextros Agar and MY20 (Skou, 1972, Takatori and Tanaka, 1982). The latter is composed of 5 g peptone, 3 g yeast extract, 3 g malt extract, 200 g glucose, 20 g agar and 1 L of water. ***A. apis*** grows luxuriously on potato dextrose agar fortified with 4 g yeast extract per liter. Growth and sporulation also occur on malt agar but less profusely and with no aerial hyphae. These characteristics facilitate subculturing and microscopic examination. Cultures have a characteristic fruity odor similar to that of fermenting peaches. The optimum temperature for growth is $30^{o}$ C. For spore germination the plates should be incubated at $35^{0}$C in glass jar with flame and transferred to $30^{0}$C for mycelium growth.

Fungal colonies grow moderately slowly and are 5 to 7 cm in diameter after 10 days; they produce aerial mycelia deeply floccose or matted, white to pale buff and may

become coral to pale reddish brown with age (Bissett, 1987). To confirm the identity as *A. apis,* it is necessary to mate it with one of two known mating strains of *A. apis* (Anderson *et al.*, 1998). This is achieved by inoculating the culture medium with the suspected *A. apis* culture and inoculating the two known mating strains on either side of the test isolate about 1 cm away. The fungus is confirmed as *A. apis* if it mates with one of the two mating strains by producing spore cysts. These appear as a brown line at the junction where the test fungus and control fungus meet and mate. Mating usually takes from 5 to 10 days, however, the production of spore balls and the spores within make take up to 15 days.

# 4. Materials and methods

## 4.1. Study area

Chalkbrood disease surveys were undertaken by the Ethiopian Agricultural Research Institute (EARI), Holleta to assess the occurrence and distribution of the disease in nine administrative Zones of Ethiopia during the years 2004-2006 were the basis for this study. The mummified larvae were collected from South Wollo, North Gondar and West Gojam, Jimma, Illubabor, East Wellega, West Showa, Arsi and Asosa areas (fig. 3).

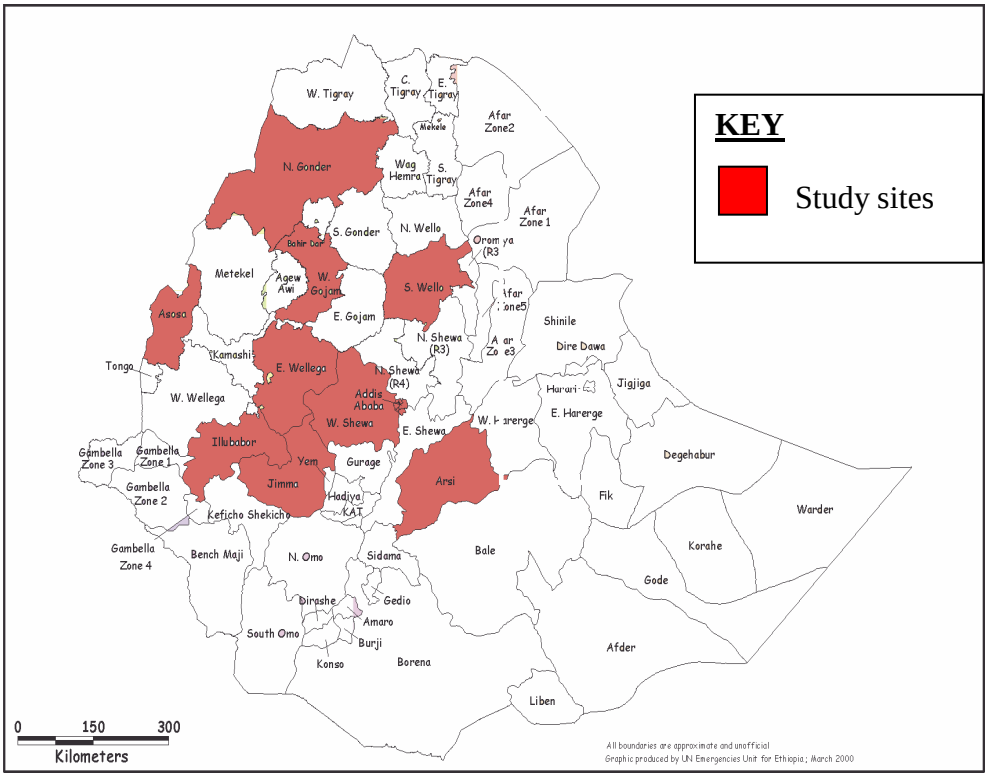



Fig. 3. Study sites for disease incidence.

### 4.2. Sample Collection

The samples of brood that had the sign of chalkbrood (dead infected larvae left uncapped by nurse bees on the comb, and mummies at the entrance of infected colonies or on the hive floor) were colleted from all selected sites by Holleta Bee Research Center, Holleta Animal Health Research Division and Sebeta National Animal Heath Center. Mummies were collected either from the combs by opening the hives or from entrance of the hives or from the ground immediately in front of the hives. The specimen used in this study was kindly obtained from EARI. Further collections were made from the research center apiaries.

### 4.3. Cultivation of the pathogen (*Ascosphaera* spp.)

Black mummies were surface sterilized with 70% ethanol for one minute and rinsed three times in sterilized distilled water. Surface sterilized mummies were dubbed onto Sabouaurd Dextrose Agar (SDA) (Oxoide) and incubated in microaerophilic condition in glass jar with a flame at $35^0$C for 48h. After this period, the plates were removed from the jar and transferred to $30^0$C incubation. Fungal colonies developing after five to seven days were transferred to the same medium to obtain pure cultures according to Francisco *et al.* (2003). White mummies were surface sterilized in similar way, but the plates were incubated at $30^0$C aerobically and the mycelia were purified by successive sub culturing.

### 4.4. Maintenances of isolates

All the isolates were maintained in four different ways for short-term storage. Mature spores from 10 day old culture were aseptically scraped and mixed with 10ml sterile distilled water, melted filtered and sterile honey, SDA (Oxoide) slant and MY20 slant (200g glucose, 5g peptone, 3g yeast extract, 3g malt extract, 20g agar and 1liter water). Each maintenance mechanism was made in four replicas and viability was examined every month until the completion of this research. All the isolates were maintained in 20% glycerol at $-80^0$C for long-term storage according to Francisco *et al*. (2003). All the cultures used in this study are maintained in the fungal collection of the mycology laboratory (AAU).

### 4.5. Morphological characteristics of the isolates

#### 4.5.1. Cultural characteristics

Ranges of cultural variations among the isolates were assessed according to Pawel and Krzysztof, (2003). Colony characteristics include aerial growth of mycelia (appraised, slightly raised and raised), density (spare, slightly dense and dense) and radial growth rate will be measured using 1mm plastic ruler. The color of mycelia (obverse side) and chromogenic pigmentation diffused in the agar media (reverse side) of the plate, and ascospore production were examined.

#### 4.5.2. Spore cyst, spore ball and ascospore measurements

Morphological studies of the characteristic reproductive structures of each isolate was made on the sporulating cultures by measuring the diameter of spore cyst, diameter of spore ball and the length and width of ascospores. Ascospore length to width ratios of each isolate was also calculated. The preparations were mounted in lactophenol cotton blue and observed at X400 for spore cyst and spore ball under camera fitted inverted phase contrast microscope connected to computer. Ascospores were measured using the same apparatus at X1500. The means of 6-replica measurement for every reproductive structure were taken for all the isolates and analysis of variance was computed. The grand means of spore cyst diameter, spore ball diameter and ascospore length to width ratios of

all the isolates were calculated and compared with the species model after Anderson and Gibson (1998), Bissett (1987), and Pawel and Krzysztof (2003) using Student's T-test.

### 4.6. Compatibility test

Single spore colonies were transferred to SDA media and used for mating test. The two mating types of the fungus, monokaryons (+ and - strains) were separated from the spores of the heterothallic isolate. Using 5mm diameter sterilized iron cork borer mycelium disk was taken from sporulating area of 10 day old culture and placed in 2mmX16mm Pyrex test tubes with 10ml of sterilized distilled water, vortex mixed and diluted serially up to 5 fold. Using sterile tips, 100µl of each dilution were aseptically spread over solidified SDA media using sterile glass road and incubated at $35^0$C for 48h as indicated earlier. Then the plates were transferred to $30^0$C and checked daily until the appearance of the first single spore colony. Mating tests were made to confirm the identity of each isolates as described by Francisco, *et al.* (2003).

### 4.7. Bioassay design

The general bioassay method was designed in the hive and in the laboratory condition at $30^0$C using spores from AI.2 and AI.37. Three colonies of honeybee, which had no prior history of chalk brood disease was selected from Holleta Bee Research Center apiaries. The first hive was used as a control. The remaining two hives were maintained 100m away from other hives in the research center. Frames consisting of honeybee larvae at $3^{rd}$ and $4^{th}$ instar level were taken from the two experimental hives. 60 larvae from the first experimental hive were fed with 10µl of $10^4$ spores/ml of *Ascosphaera* species, from local isolates prepared in 40% sterile honey solution. The same numbers of larvae were

allowed to feed on 10µl of $10^5$, $10^7$ and $10^{10}$spores/ml. 60 other larvae were feed with 40% honey solution with out spores using micropipette with sterile tips.

The spore suspension was prepared with melted, filtered, and sterilized 40% honey solution. The suspensions were always plated on SDA to test for viability and purity according to Gilliam *et al*. (1983). This procedure was repeated in the second experimental hive in the same way. Six 10cm X 10cm combs containing 3rd to 4th instar larvae were cut from frames of the experimental hives and rapidly transferred and incubated at $30^0$C in incubator. The same numbers of larvae as in the case of hive condition from experimental hives No. 1 and 2 were fed with $10^4$, $10^5$, $10^7$ and $10^{10}$ spores/ml prepared as described before. Two pieces of the combs with 60 larvae on each were fed with 40% honey solution and used as control.

## 5. Enzymatic activities of the isolates

100ml of malt extract broth in five 500 ml Erlenmeyer flasks were sterilized at 121 $^0$C for 15 minutes and aseptically inoculated with 10 mycelia blocks (5mm X 5mm) from 7-day old cultures of the isolates (AI. 2 and AI. 37). The cultures were grown on a rotary shaker at 120rpm for 8 days at room temperature. At the end of incubation period, the mycelium biomass was separated from the supernatant by filtration using Watman filter paper number 1. The biomass was discarded and the supernatant was used for enzyme assay.

**Protease assay**.

0.5ml of the supernatant (enzyme solution) was mixed with 1.5 ml of 0.5% casein substrate in 0.1M phosphates buffer pH 6, 7 and 8 and in 0.1M Glycin-NaOH buffer pH 9

and 10. The mixtures were incubated at $30^0$C, $40^0$c, $50^0$C, $60^0$C, $70^0$C, and $80^0$C for 30 min in water bath. After the incubation period 2.5ml TCA (trichloroacetic acid) was added to stop the reactions. The mixtures were centrifuged in an ependrhoff tubes at 10,000 rpm for 5min. 0.5 ml of the supernatant was mixed with 0.5M $Na_2CO_3$. The content was vortexed immediately by adding 1:10 phenolciocaltus reagent and incubated in dark at room temperature for 20 min. The absorbency was measured spectrometrically at 660nm and the optimum temperature and pH were determined. One unit of enzyme activity was defined, as the amount of enzyme required to solubilize 1 mg of trichloroacetic acid-soluble material, calculated as tyrosine, per 30 min at $35^0$C (Mcdonald and Chen, 1965).

## 6. Toxic assay

100ml malt extract broth in five 500 ml Erlenmeyer flasks were sterilized at 121 $^0$C for 15 minutes and aseptically inoculated with 10 mycelia blocks of 5mm X 5mm from 7 day old culture of the isolates (AI. 2 and AI. 37). The cultures were grown on shaker at 120rpm for 15 days at room temperature. At the end of incubation period, the mycelium biomass was separated from the supernatant by using Whatman filter paper number 1 and divided in to two parts. The first proportion of the supernatant was tested at 10%, 20%, 30%, 40% and 50% (v/v) on mosquito larvae collected from a bitch. Toxin was extracted from the second proportion of the culture supernatant with equal volume of ethyl acetate and separated from the solvent by vacuum pump rotary evaporator at $60^0$C and 138 rpm. Crude extract was transferred to vials and allowed to dry in refrigerator at $4^0$C. The crude extract was also tested on mosquito larvae at 0.005, 0.0125, 0.025, and 0.05mg/ml.

# 7. Results

## 7.1. Cultural characteristics

In cultures grown on PDA at 30 °C a moderately slow development was observed between 2 days and 9 days after inoculation. The mycelium was cottony white to pink, fluffy or slightly floccose, aerial, mostly glossy, covered with droplets of "exudates" on the hyphae. This indicates the characteristic colony morphology of *A. apis* which is the same as the description of Andrson and Gibson (1998). Spore cyst growth for some isolates was visible on 3 days after inoculation (fig. 4). As the spore cysts were produced in the center of the colony dark green or complete darkening of the cultures were observed (Fig. 4). A characteristic fruity odor similar to that of fermenting peaches was noticed while opening the culture plates.

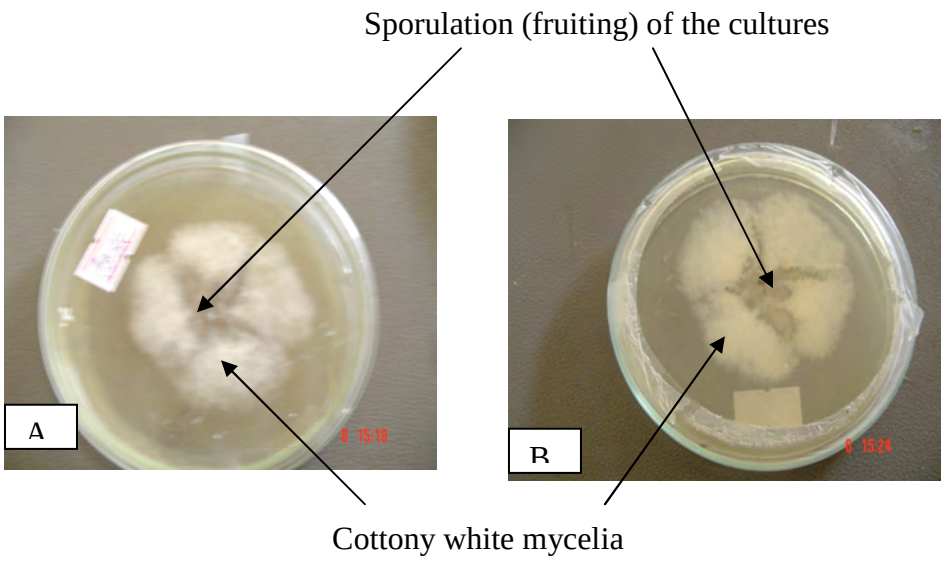



Fig. 4. A sporulating 5-day-old culture of AI. 2 on PDA. A/ upper side.

B. Reverse side

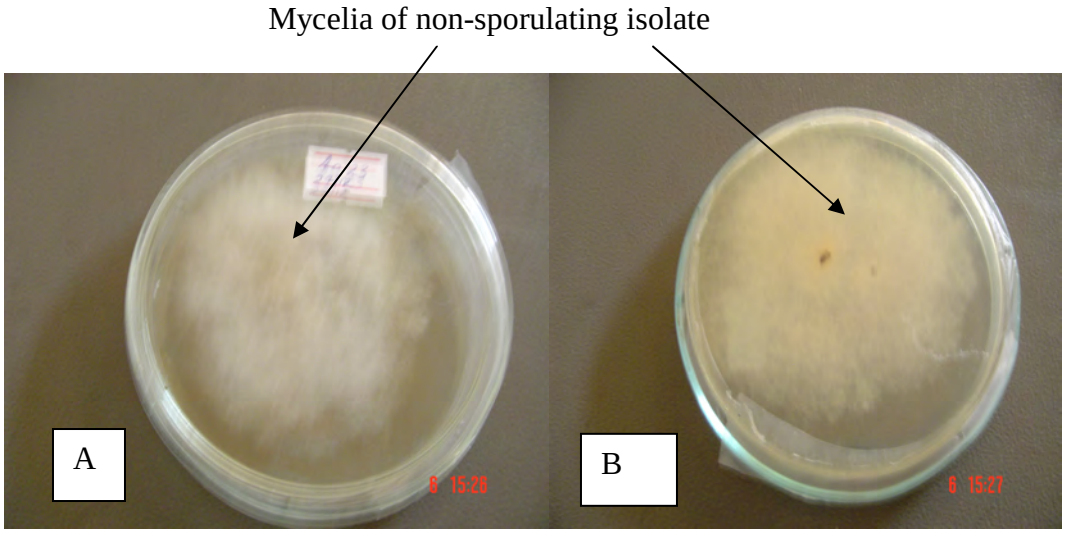



Fig. 5. Non-sporulating 5$^{th}$ day culture of AI. 3 on PDA.

A. Upper side. B. Reverse side

The colony diameter of the isolates after 8-day ascospores germination was 4-8 cm. The respective measurement obtained for each isolate with mycelium type and sporulation patterns are presented in (Table 1).

***Table 1****. Origin, year of sample collection, and mycelium type of* Ascosphaera *isolates from Ethiopia in this study.*

| Isolates | Geographic Origin | Source of colony | Mycelium type | Diameter after 8 days (cm) |
|---|---|---|---|---|
| AI.1 | S/Wello (Assgedo) | Mu | D | 4*** |
| AI1.1 | S/Wello(Assgedo) | Mu | M | $6^0$ |
| AI.2 | W/Showa(Holleta) | Mu | D | 8** |
| AI.3 | S/Wello(Ambassel) | Mu | D | 6* |
| AI3.3 | S/Wello( Ambassel) | Mu | M | $8^0$ |
| AI.4 | E/Wellega(Yachi) | Mu | D | 8* |
| AI6 | W/Showa(Holleta) | Mu | D | 8** |
| AI7 | S/Wello(Kembolcha ) | Mu | D | 8** |
| AI8 | N/Gondar (Chilga) | Mu | D | 7* |

N.B. AI = *Ascosphaera* isolate, D = dikaryon, M= monokaryon, Mu= mummy

0 = non-sporulating, * =few spore cyst, ** medium sporrulation, *** abundant in spore cyst

**Table 1**. *Continued*

| Isolates Code | Geographic origin | Source colony | Mycelium type | Diameter of after 8 days (cm) |
|---|---|---|---|---|
| AI.10 | W/Gojam(And Asa) | Mu | D | 7*** |
| AI.11 | W/Gojam(And Asa) | Mu | D | 5** |
| AI.17 | W/Gojam(Bure) | Mu | D | 8** |
| AI.26 | W/Gojam (Bure) | Mu | D | 6** |
| AI.28 | S/Wello(Dessie) | Mu | D | 7* |
| AI.30 | Arsi(Robe) | Mu | D | 6*** |
| AI.3 | Arsi(Limmubilbilo) | Mu | D | 4** |
| AI.37 | Arsi(Ameyya) | Mu | D | 5** |
| AI.38 | Arsi(Dimma) | Mu | D | 7** |

N.B. AI = *Ascosphaera* isolate, D = dikaryon, M= monokaryon, Mu= mummy

0 = non-sporulating, * =few spore cyst, ** medium sporrulation, *** abundant in spore cyst

### 7.2. Spore cyst, spore ball and ascospores measurements

Results of the morphological studies of reproductive structures are summarized in table 2. Comparison of morphometric measurements of the reproductive structures of *A. apis* isolates form different countries by different investigators is also summarized in table 3. The result of microscopic observation indicates that the local isolates are similar in morphometric characteristics with previous findings of Pawel and Krzystof, (2003). The spore cysts mounted with lacto phenol cotton blue observed under 430X phase contrast inverted microscope were globes, dark-brown containing numerous spore balls (fig. 6). Spore balls are rounded clusters consists of one celled ascospores and are with out membrane. This observation was in agreements with the findings of Anderson and Gibson (1998), Rose *et al*. (1984), Skou (1972), and Christensen and Gilliam (1993)**.** Ascospores were one celled and slightly curved.

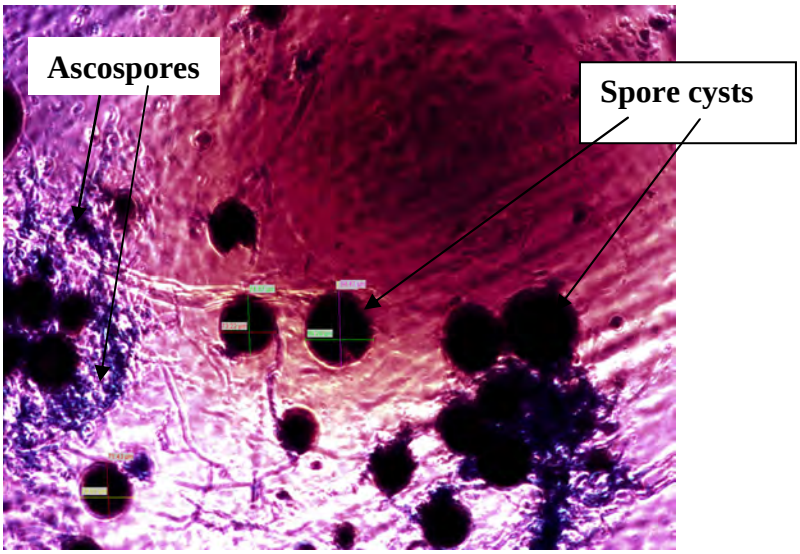


Fig. 6. Spore cyst of *A. apis*, 400X phase contrast microscope.

**Table.2.** Morphometric characteristics of *Ascosphaera* species isolated from Ethiopian after 12 days of growth at $30^0$C on PDA media.

| **Isolate** | **Spore cyst** | **Spore ball** | **Ascospore** | **Ascospore** | **Ascospore** |
|---|---|---|---|---|---|
| Code | Diameter(μm) | Diameter(μm) | length)(L)(μm) | width)(W)(μm) | L:W ratio |
| AI.1 | 78.35±3.91 | 14.59±1.55 | 2.87±0.33 | 1.32± 0.13 | 2.17 |
| AI1.1 | 86.72±11.49 | 14.72±0.93 | 3.08±0.36 | 1.47±0.21 | 2.09 |
| AI.2 | 97.66±11.31 | 14.24±1.33 | 3.05±0.46 | 1.37±0.19 | 2.22 |
| AI.3 | 94.70±8.40 | 13.22±0.46 | 3.13±0.26 | 1.30±0.12 | 2.4 |
| AI.3.5 | 72.47±2.44 | 14.49±1.20 | 2.90±0.29 | 1.36±0.14 | 2.13 |
| AI.4 | 89.17±10.89 | 13.36±0.76 | 2.80±0.18 | 1.42±0.11 | 2.00 |
| AI.6 | 76.99±4.39 | 15.15±1.20 | 2.73±0.25 | 1.27±0.24 | 2.15 |
| AI.7 | 85.32±9.86 | 14.91±0.57 | 3.00±0.20 | 1.23±0.21 | 2.43 |
| AI.8 | 91.09±13.40 | 12.40±0.8 | 2.74±0.62 | 1.30±0.30 | 2.11 |
| AI.10 | 86.71±12.24 | 14.75±2.08 | 2.95±0.45 | 1.43±0.13 | 2.06 |
| AI.11 | 66.15±10.88 | 15.52±0.68 | 3.03±0.63 | 1.30±0.25 | 2.33 |
| AI. 17 | 74.87±11.83 | 11.67±1.09 | 3.05±0.11 | 1.40±0.08 | 2.18 |

| | | | | | |
|---|---|---|---|---|---|
| AI.26 | 78.83±9.45 | 11.22±0.95 | 3.18±0.15 | 1.45±0.08 | 2.19 |

Table 2. Continued

| Isolate Code | Spore cyst Diameter(μm) | Spore ball Diameter(μm) | Ascospore length)(L)(μm) | Ascospore width)(W)(μm) | Ascospore L:W ratio |
|---|---|---|---|---|---|
| AI.28 | 82.00±12.59 | 11.72±1.98 | 2.58±0.49 | 1.20±0.20 | 2.15 |
| AI.30 | 81.92±7.42 | 19.27±0.27 | 2.40±0.34 | 1.07±0.04 | 2.24 |
| AI.32 | 79.64±7.08 | 15.62±1.76 | 2.98±0.23 | 1.23±0.27 | 2.42 |
| AI.37 | 78.50±5.20 | 14.24±1.20 | 3.12±0.20 | 1.32±0.16 | 2.36 |
| AI.38 | 74.82±7.26 | 11.00±1.50 | 3.23±0.31 | 1.43±0.20 | 2.26 |

N.B. Data are for the mean ± standard deviation of 6 spore cyst, 6 spore ball and 6 ascospores per isolates.

**Table 3**. Comparison of morphometric measurements of the reproductive structures of Ascosphaera isolates with results of different studies.

| Ascosphaera Isolate (According to) | Spore cyst average diameter (μm) | Spore ball average diameter (μm) | Ascospore | | |
|---|---|---|---|---|---|
| | | | Length(L) average(μm) | Width(W) average(μm) | L**:**W ratio |
| This study (Ethiopia) | 80.88* | 14.01* | 2.94* | 1.3* | 2.3 |
| Reynadi *et al.* (2003) **a** | 88.5 | 14.9 | 3.4 | 1.5 | 2.3 |
| Reynadi *et al*. (2003) **b** | 91.6 | 15.1 | 3.5 | 1.5 | 2.3 |
| Alonso *et al*. (1993) **c** | 74.6 | 14.2 | 3.1 | 1.6 | 1.9 |
| Anderson and Gibson (1988) **d** | 82.0 | 13.0 | 2.9 | 1.4 | 2.1 |
| Christensen and Gilliam (1993) **e** | 80.2 | ND | 3.1 | 1.6 | 1.9 |
| Rose *et al.* (1984) **f** | 81.0 | 14.5 | 2.8 | 1.4 | 2.0 |
| Skou (1972) **g** | 80.2 | ND | 2.7 | 1.4 | 1.9 |
| Bisset (1987) **h** | 70.80 | 12.0 | 2.9 | 1.4 | 2.04 |

(*) Data from mean of 108 measurements

**N.B**. Letters indicate ***A. apis*** isolates from (a) Argentina,(b)Chile, (c)Spain,(d)Australia, (e) USA, (f) North America, (g)Denmark, (h) Canada.

*7.3. Compatibility test*

*Ascosphaera* is a fungus, which has a perfect stage. Therefore hybridization capacity can be tested in order to verify if the isolates from different regions are sexually compatible. Single spore colonies obtained from AI. 2 were tested among themselves to identify the + (plus) and – (minus) strains. The + (plus) and – (minus) strains of AI. 2 were separated according to the method described before and labeled as AI. $2^+$ and AI. $2^-$. All the non-sporulating isolates were tested against AI.$2^+$ and AI. $2^-$. Based on this test, the non-sporulating isolates (monokaryon) gave the characteristic dark band of spore cyst and confirmed as *A. apis* (fig. 7*)*. Out 8 non-sporulating isolates, 6 mated with AI.2+ and confirmed as AI.$^-$ strains. The remaining 2 isolates mated with AI.$2^-$, produce the characteristic spore cyst band and confirmed as $AI^+$. Based on this mating experiment eight AI [six minus (-) and 2 plus (+)] were identified and their hybrid spores were preserved as mentioned earlier. Non-sporulating isolate obtained from various areas in this study are compatible and thus belong to *Ascosphaera apis* population.

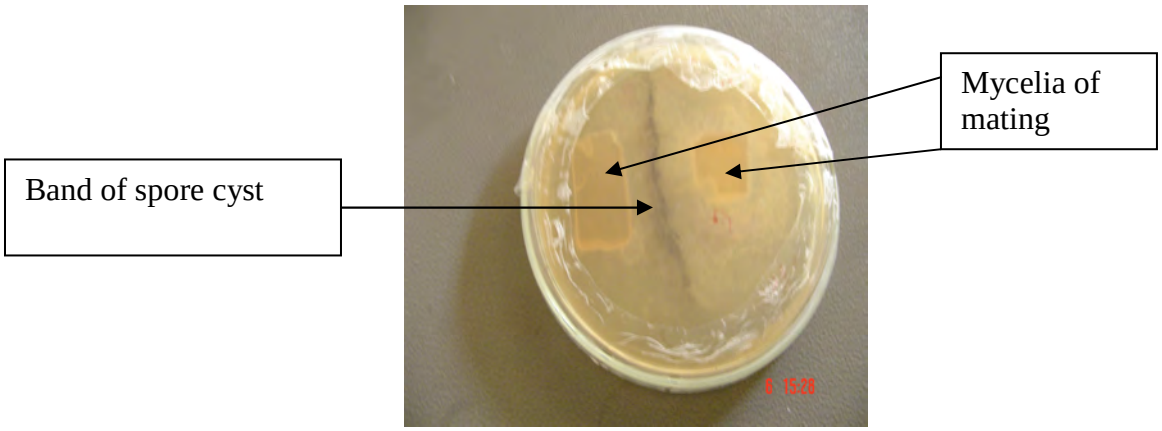
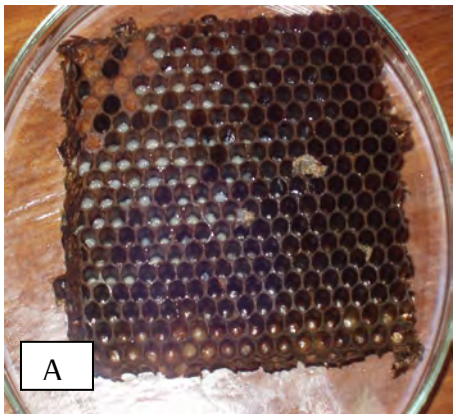


**Fig. 7**. Typical spore cyst bands confirm sexual compatibility between ***A. apis*** isolates.

*7.4. Bioassay results*

All larvae fed with spore-contaminated feed, $10^7$ spores/ml, in the incubator at $30^0$C were fully invaded by the mycelia of fungus. The mycelium was fluffy white (fig. 9B an C) with abundant spore cysts on the dead larvae after $7^{th}$ day. The spore cysts were visible on the comb when observed using 40X dissecting microscope (fig. 10). The death of honeybee larvae due to the infection of $10^4$ spores/ml in the incubator was 53.33% (Table 4). This result indicates that only 10-spores/μl is sufficient to cause infection on the chilled and nutritionally stressed larvae. Under normal hive condition symptoms of the disease was not produced at $10^4$ and $10^5$spores/ml. However, only 8.33% of the larvae had developed the symptom of the disease among the larvae fed with $10^7$ spores/ml. As the spore load increased to $10^{10}$/ml, the number of mummified larvae increased to 10 %( Table 4).

**Fig. 8**. The two selected experimental honeybee colonies hived in Zander hives.



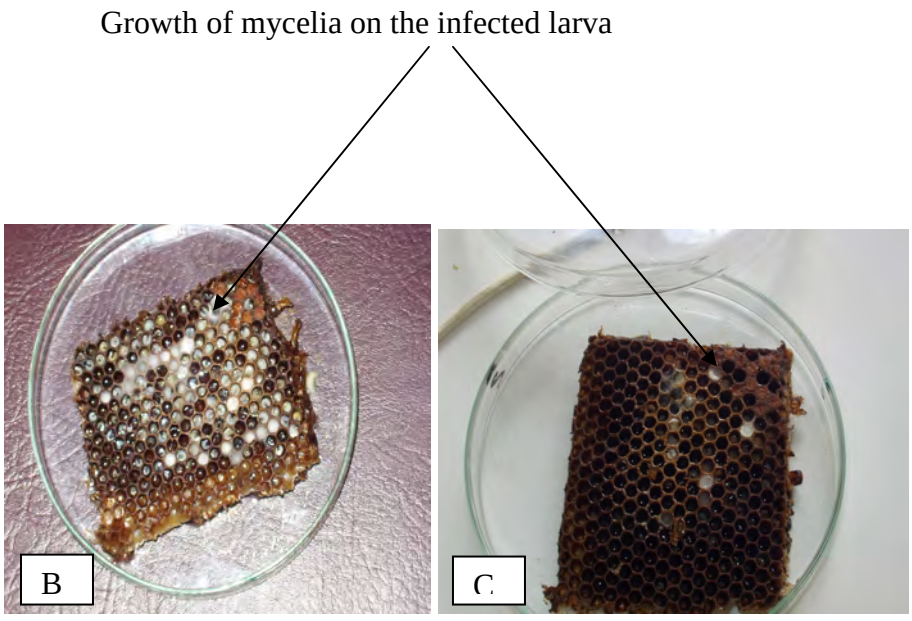



**Fig. 9**. Honeybee larvae: A/ before infection B/ After infection with $10^7$ spores/ml from the local isolates ($5^{th}$ day). C/ After infection with $10^4$ spores/ml from AI.2 among the local isolates ($5^{th}$ day).

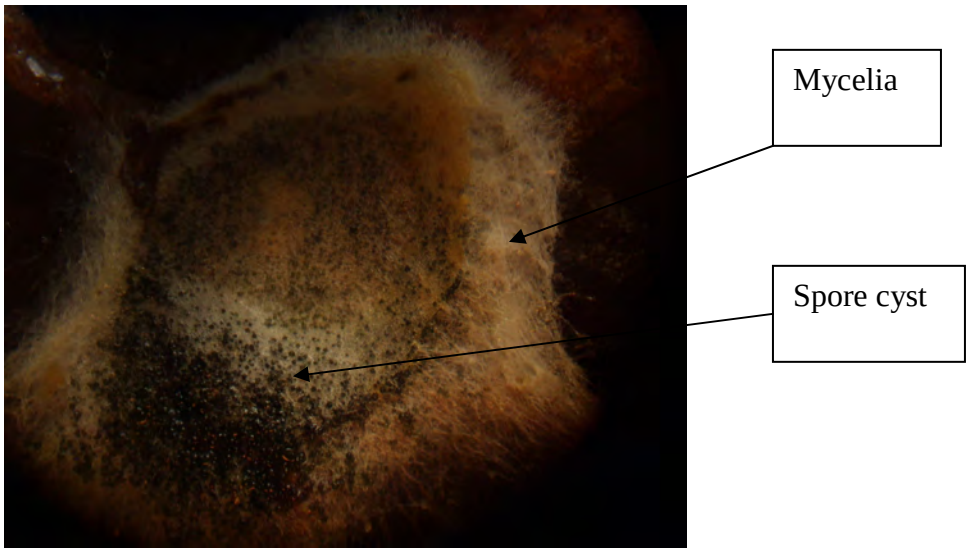



**Fig. 10**. Mycelium producing spore cyst on the infected larvae in the comb cells (100X)

**Table 4**. Bioassay on honeybee larvae

| Spore Load | Number of infected larvae | | | |
|---|---|---|---|---|
| | In the incubator ($30^0$C) | **Hive 1** | **Hive 2** | **Average** |
| **$10^4$** | **53.33%** | Nil | Nil | Nil |

| | | | | |
|---|---|---|---|---|
| **$10^5$** | **70%** | **Nil** | Nil | Nil |
| **$10^7$** | **100%** | **8.33%** | **5.0%** | **6.66%** |
| **$10^{10}$** | **100%** | **11.66%** | **8.33%** | **10.0%** |

The slight variation in the degree of virulence observed in the two hives might be either due to the genetic variation of the two honeybee colonies or higher brood to worker ration in the first hive.

### 7.5. Enzyme assay results

#### 7.5.1. Effect of temperature on the proteolytic activity of the isolates.

All of the isolates in this study were found to be highly proteolytic. The result shows that enzyme activity is affected by temperature and that the optimum protease activity was achieved at $40^0$C (fig. 11). There was sharp apparent decline above and below this value.

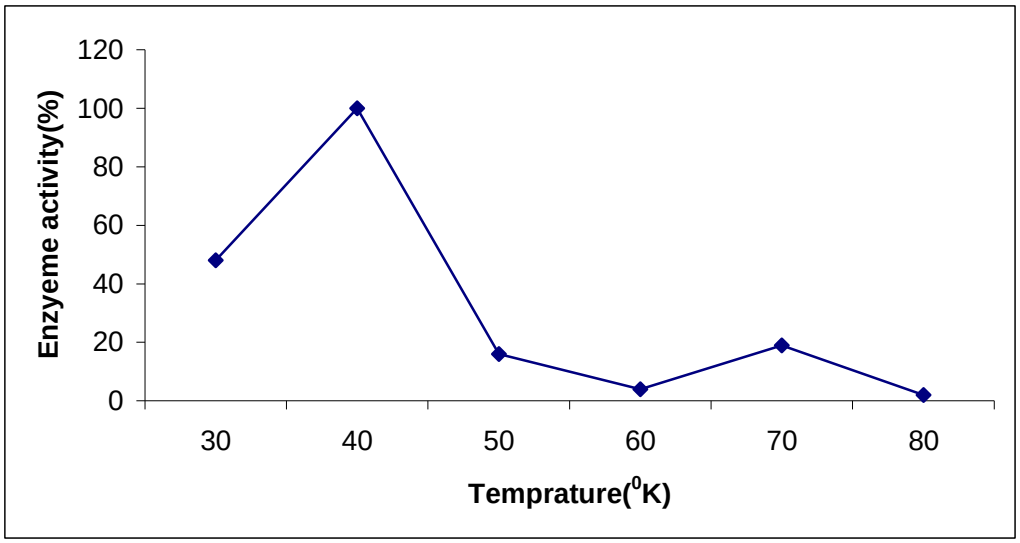


**Fig 11**. Effect of temperature on the activity of protease

### 7.5.2. Effect of pH on the activity of protease

Optimum pH of protease activity from the culture supernatant was determined by using 50Mm phosphate buffer (pH 6-8) and glycin-NaOH buffer (pH 9-10) in the assay system. Maximum enzyme activity was observed at pH 7. Protease activity declined sharply below and above pH 7. The result indicates that protease from the local isolate is active at neutral pH (fig. 12)

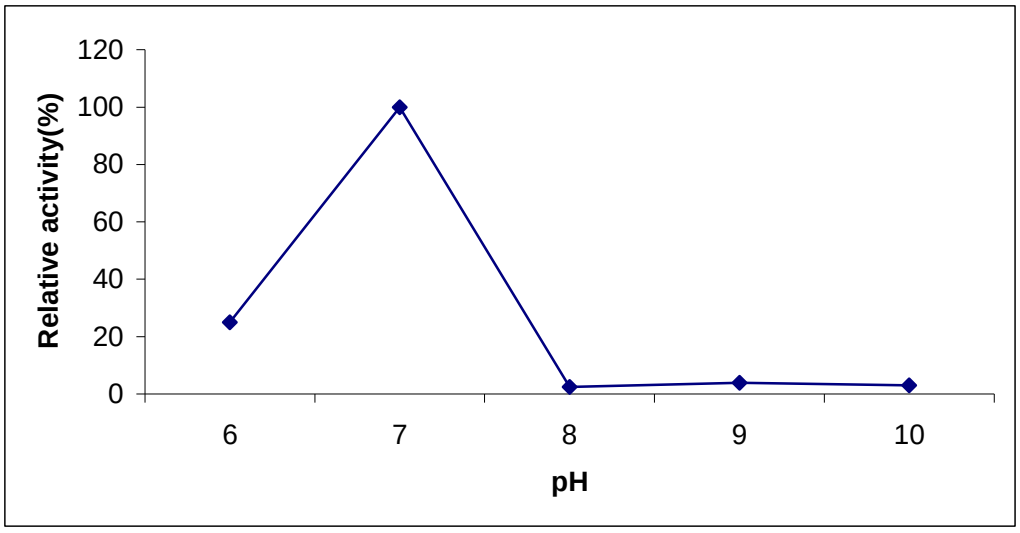



**Fig 12**. Effect of pH on the activity of protease

# 8. Discussion

Chalkbrood disease of honeybee (*Apis mellifera* L) is caused by the fungus *Ascosphaera apis* (Maassen ex Clausen) Olive and Spiltoir, and can seriously affect colony build up (Baily and Ball, 1991; Heath, 1982). In this study the causative agent of chalkbrood disease was isolated from mummies in the laboratory. The isolates were morphologically characterized according to their colony appearance, colony size, reproductive structures morphometery, sexual compatibility with reference strain (MUCL 34668), proteolytic activity, toxicity and virulence ability. Isolation of the causative agent from dead honeybee larvae (mummies) using surface disinfection technique was carried out in this study. Beside white cottony colonies of *Ascosphaera* various fungi, molds and bacteria were observed in the culture plates. Some infrequently appeared fungi were the black fungi with head like spore bearing structure (*Aspergillus* spp.) and the blue-green powdery colonies (*Penicillium* spp.). Among bacteria gram-positive spore formers were common.

The result obtained in this study from culture examination agreed with cultural characteristic of *Ascosphaera apis* described by Chorbinski and Rypula, (2003). Particularly, culture grown on SDA at $30^0$C (Fig. 4, 5, and 6) was cottony white to light pink, compact or slightly flucose and raised, which are the characteristics of *A. apis.* While spore cysts were produced, darkening which starts from the center were noticed (fig. 4). This characteristic of our local isolates is in line with the study of Chorbinski and Rypula, (2003) that described the mycelium of the fungus as cottony white or light pink,flucose snd raised. There was no any cultural or mycelial difference between the sporulating and non-sporulating isolates (fig. 4 and 5). Gilliam (1978), Heath (1982) and

Glinski, (1982) also observed no mycelial difference between the two mating strains. Upon sporulation, the development of spore cysts results in the formation of blanking which characteristically distinguishes the sporulating isolates from the non-sporulating once.

Baily (1967) described that germination of *A. apis* spores requires nearly anaerobic environment, but the mycelium requires an aerobic environment for growth. Floyde and Paul, (1976) found out that the optimum temperature for spore germination and mycelium development for *A. apis* were $35^0$C and $30^0$C, respectively. In this study spore germination was tested at $25^0$C, $30^0$C and $35^0$C. At $35^0$C spores germinated with in 48h showing that the characteristic of *A. apis*. However, growth of mycelia was very poor. Cultures of *A. apis* incubated at $35^0$C for 48h and transferred to 26-$30^0$C showed luxurious mycelial growth. This finding coincides with the findings of the Baily (1967) that suggest movement of Oxygen in to the medium enhanced at lower temperature and facilitate mycelial development. This idea also substantiates the fact that germinated spores are generally void harmlessly with the faces because the mycelial growth suppressed in anaerobic environment of the larval gut. The plates were frequently sealed with parafilm through out this study to reduce contamination and decrease $O_2$ concentration. This finding confirms the work of previous studies conducted on *Ascosphaera apis* isolated from different countries (Floyde and Paul, 1976; Heath and Gaze, 1987).

The reproductive structures of *Ascosphaera* include the sexual spore cyst with transparent membrane filled with spore balls consisting of ascospores. The spore cyst dimension of

the isolates obtained in this study has a range of 66.15 - 97.66μm with mean of 80.88μm.This value falls with in the range of spore cyst diameter obtained by Anderson and Gibson, (1998) (45-119μm) and Chobinski and Rypula (2003) (35-118μm) and strongly suggest that the isolates under this study are *Ascosphaera apis*. There is a significant variation in spore cyst diameter among the local isolates at F (17, 90) =1.737; P< 0.05. Isolates coded as AI.2 (W/Showa) and AI.11 (W/Gojam) have the largest and smallest spore cyst diameter respectively. The grand mean of spore cyst (80.88μm) obtained in this study is significantly higher than the result reported by Bisset (1987)(70.00μm) and Alonso *et al.* (1993) (74.60μm) (Table. 3). However, this value is similar with the measurement obtained by Rose *et al.* (1984) (81.00μm)(Table. 3). On the other hand this value is significantly smaller than the measurements of Francisco *et al*. (2003)(88.50μm and 91.60μm) **t**(17)=2.383, p=0.025).

Spore balls were full of ascospores, oval, dark brown with diameter of 11.00 – 19.27μm (14.01μm on average). The local isolates differ significantly in spore ball diameter (table 3) at F (17, 90)= 1.737; P<0.05. AI.38 (Arsi/Dimma) and AI.30 (Arsi/Robe) have the smallest and the largest spore balls, respectively among the local isolates. There is no linear correlation between the diameter of spore cyst and spore ball diameter. The grand mean of spore ball diameter obtained in this study (14,0μm) is bigger than Canadian isolates (12μm) (Bisset, 1987) and Australian isolates (13.00μm)(Anderson and Gibson, 1998)(Table 3). On the other hand this value is smaller than 14.20, 14.50, 14.90, and 15.10 obtained from Spain, North America, Argentina and Chile respectively (table 3). Variations in the size of spore cyst and spore ball among the isolates studied by different investigators from different countries might be due to the use of different culture media.

Ruffinengo *et al*. (2003) studied suitability of culture media for the production of ascospores of *A. apis* and found out that the spore cyst size was the most morphometric feature affected while ascospores were the least affected by culture medium variation of the three structures.

Unlike variation in spore cyst and spore ball diameter, there is no significance difference in the size of ascospores examined in this study $F(17, 90)=1.737$; $P<0.05$. The Length to width (L: W) ratio obtained from the local isolates was 2.30µm. Similar values have reported by Reynaldi *et al.* (2003) and Ruffinengo *et al.* (2000). However, as can be seen from table 3, ascospore L:W ratio of the local isolates was larger than Spain, USA, Denmark, Canada, North America and Australian isolates. This result suggests that there might be variations between the local isolates and isolates from Spain, USA, Denmark, Canada and Australia.

Bioassay designs conducted in the lab ($30^0$C incubator) and in the hive (field condition) were aimed to demonstrate Koch's postulate and to evaluate the virulence of local isolates on honeybee larvae. Under laboratory condition at $30^0$C the larvae were highly susceptible to infection and the fungal mycelia appeared on the larvae on the third day. Numerous spore cysts were visible (40X) on the fifth day in the larvae comb cells (Fig.10). Spore cysts, spore balls and ascospores were also taken from artificially inoculated larvae and observed from slide preparations under the microscope on the same day (400X), thus retrieving the pathogen from artificially inoculated host reveals the success of proving Koch's postulate. These attempts also confirm that the isolates are pathogens of honeybee larvae. More importantly the method used in this study, feeding

honeybee larvae with a meal contaminated with spore using micropipe is safer than spraying spores on the frame containing honeybee larvae as it is non-selective.

The result of feeding larvae with a diet contaminated with *A. apis* spores in the hive indicates that spores from our local isolates can cause infection on honeybee larvae regardless of the different stress factors described by many authors. In the initial experiment conducted in the hive, $10^7$spores/ml could only cause 6.66% infection. In the second repeated experiment in the hive with higher spore load ($10^{10}$) than the previous, 10.0% of the targeted larvae were infected with the fungus. Majority of the larvae fed with spore-contaminated diet in the hive were continued their development to pupation. However, a few of them were not found in the comb cells or on the floor of the hive or around the entrance of the hive. It was suspected that hive-cleaning bees might have taken the dead larvae far away from the hive. This could be one factor which contributed to the spread of the pathogen from infected hive to the non-infected once through wind. From the field experiment we came to know that our local isolates could infect unstressed (normal) honeybee larvae and are highly virulent types.

In the laboratory at $30^0$C as low as 100 spores could cause infection. This infection by few numbers of spores might be due to the fact that the larvae were subjected to various stress factors such as low temperature ($30^0$C as compared with the hive temprature$35^0$C), lack of nurse bee supervision, excessive manipulation, inappropriate feeding, etc. These stress factors, mainly temperature, inevitably reduce the resistance of the larvae to fungal infection. The decline of temperature from $35^0$C to $30^0$C (slight chilling) on one hand

negatively affects the physiological activity of the larvae and on the other hand it favors the rapid fungal growth.

However, the attempt to establish $LD_{50}$ value for the isolates under normal hive condition was not successful due to the extraordinary behaviors of honeybee. First, it was suspected that the nurse bees that regularly visit the larvae might screen the spores from the contaminated diet before the larvae ingest the spores and reduce the danger to be caused on the larvae. Second, adult bees may share the feed introduced in to the larval cells and might reduce the appropriate dosage of the spore needed to cause the infection. Third, the larvae might ingest the spores and got the infection, however the infected and dead larvae might be discarded as early as it died and made the study difficult.

It is well known that fungal pathogens of insects are often efficient producers of protease and other lytic enzymes. The role of protease in the invasion process has been shown by Chorbinski (2004) and Chorbinski and Rypula (2003). The results of protease assay (figs. 11-12) in this study indicate that protease from the local isolates has optimum temperature between around $40^0$C and optimum pH of 7. This property of the enzyme indicates that the organism is adapted to hive conditions and thus the enzyme might be one of the important virulence factors of the pathogen. In their study Bmford and Heath (1989) indicated that high proteolytic activity of *A. apis* strains provide high adaptation of this fungus to develop and colonize larval body.

In this study it was also suspected that toxin produced by the fungus might play a role in the infection process. However, the cultures of local isolates were easily overgrown by other moulds and bacterial contaminants. Hence, they were unable to clean their

immediate environment of competing microorganisms during growth. There was no indication of toxin production, which has antimicrobial activity in the cultures. The assay conducted on mosquito larvae with culture supernatant up to 50% v/v and extract up to 0.05mg/ml had no effect on mosquito larvae. This finding suggests that the fungus under study dose not produce insecticidal secondary metabolites and thus secondary metabolites do not contribute to pathogenesis. Literatures were not available on toxic activity of *Ascosphaera* species to substantiate this finding with previous studies. However both the supernatant up to 50% v/v and the extract up to 0.05mg/ml had no effect on mosquito larvae. Therefore, it is suggested that the mortality of honeybee larvae infected with our isolates were more related to the proteolytic enzyme used in the degradation of larval body and mechanical damage of host by the invasive hyphae.

## 9. Conclusion

1. Examination of the results and obtained values of colony characteristics, reproductive structures morphometry, mating test, and comparison of homothallic isolates with reference strain (MUCL 34668) reveals that the fungal pathogen of honeybee in Ethiopia belongs to the genus Ascosphaera and to the species *Ascosphaera apis*
2. Unlike ascospores size, there is significant variation in spore cyst and spore ball dimensions among the local isolates. The length to width ratios of ascospores best describe *Ascosphaera apis* than the spore cyst and spore balls which tend to vary with culture medium.
3. All of the Ethiopian isolates of *A. apis* are highly proteolytic and hence the protease is likely to contribute to pathogenesis of *A. apis.*

4. Ethiopian isolates of *A. apis* are relatively more virulent but do not produce insecticidal secondary metabolites in vitro.

## 10. Recommendation

**1. This study was conducted based on morphological characteristics and virulence of the isolates and hence corresponding molecular**

**characterization of homokaryon is a matter of highly recommended aspect of study for further analysis of the isolates from Ethiopia.**
**2. The disease has a wide spread incidence in many beekeeping countries including some part of Africa and recently in Ethiopia so development of method for control is a matter of highly practical importance.**

3. It has been observed that no efficacious chemical treatment for chalkbrood disease exists. Even if such methods were developed, problems with chemical residues in honey, wax, and other hive products might preclude the use of such substances in commercial sector. Therefore, control strategies should focus on:

A. Management practices that reduce the stress on hives also reduce the number of chalkbrood spores. Maintaining strong healthy colonies has to be demonstrated to reduce the effects of chalkbrood
B. Some colonies are affected with chalkbrood disease more than others. Most of this variation in susceptibility is due to differences in the ability of bees to uncap and remove diseased brood. Selecting queen bees or obtaining queen bees from colonies that show resistance to this disease can reduce the effects of chalkbrood.
C. Biological methods of control are needed.

## 10. References

Alonso, J., Rey, J., Puerta, F., Hermoso, M., and Flores, J. (1993). Enzymatic equipment of *Ascosphaera apis* and the development of infection by this fungus in *Apis mellifera. Apidologie.* **24**: 383-390.

Anderson, D. and Gibson, N. (1998). New species and isolation of spore cyst fungi (*Plectomycets: Ascosphaerales*) from Australia. *Australian Systemic Botany*. **11**: 53-72.

Anderson, D., Gibson, N. and Gibbs, A. (1998). Identification and phylogeny of the spore-cyst fungi (*Ascosphaerales*: Ascomycetes). *Mycol. Res*. **102:** 541-547.

Bailey, L. (1967). The effect of temperature on the pathogenicity of the fungus, *Ascosphaera apis*, for larvae of the honeybee, *Apis mellifera. Insect Pathol. Microb*. **6:** 162-167.

Bailey, L. and Ball, B. (1991). *Honey Bee Pathology*. Academic Press. London. Pp. 53-63.

Bamford, S. and Heath, L. (1989). The effects of temperature and pH on the germination of spores of the Chalkbrood fungus, *Ascosphaera apis. J. Apic. Res.* **28:** 36-40.

Bendek, P. (1985). Economic importance of honeybee pollination of crop at the national level in Hungary. Proceedings of the 29th International Beekeeping Congress. *Apimondia.* **1:** 286-289.

Betts, A. (1932). Chalkbrood. *Bee World.* **13**(7): 78-80.

Bissett, J. (1987). Contribution towards a monograph of the genus *Ascosphaera*. *Can. J. Bot*. **66:** 2541- 2560.

Carrera, P., Sommaruga, A. and Vailati, G. (1987). The development of *Ascosphaera apis* within larvae of *Apis mellifera ligustica*. *J. Apic.* **26**: 59-63.

Chorbinski, P. (2004). The development of the infection of *Apis mellifera* larvae by *Ascosphaera apis*. *Veterinary Medicine*. **7(2):** 1-5

Chorbinski, P. and Rypula, K. (2003). Study on the morphology of strains of *Ascosphaera apis* isolated from chalkbrood disease of honeybees. *Veterinary Medicine*. **6:** 10-18.

Christensen, M. and Gilliam, M. (1983). Notes on the *Ascosphaera* species inciting chalkbrood in honeybees. *Apidologie.* **14:** 291-297.

Dessalegn Begna (2006). Chalkbrood disease in Ethiopia. *Bees for development.* **78:** 8-9

Flores, J., Ruiz, J.,Puerta, F., Bustos, M., Padilla, F. and Campano, F. (1996). Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. *Apidologie,* **27:** 185-1 92.

Floyd, E. and Paul, H**. (**1976). Life cycle of *Ascosphaera apis*. *American Bee Journal*. **116** (10): 484

Francisco, J., Claudis, L., Gracieila, N. and Adriana, M. (2003). Differenciation of *Ascosphaera apis* isolâtes by rep-PCR fingerprinting and détermination of chalkbrood incidence in Argentinean honey samples. *IBRA.* 65 : 69-76.

Gibbs, D. and Muirhead, I. (1998)**.** The economic value and environmental impact of the Australian bee keeping industry. Report prepared for the Australian Bee Keeping Industry. P. 47

Glinski, Z. (1982). Studies on pathogenicity of *Ascosphaera apis* for larvae of the honeybee *Apis mellifera* L. Part II. Relationships between biochemical types and virulence of *A. apis*. *Annales Universitatis Marie Curie – Sklodowska*. **37(8**): 69-77.

Glinski, Z. and Buczek, K. (2003). Response of the apoidae to fungal infection. *APIACTA.* **38**: 183-189.

Gilliam, M. (1978). Chalkbrood status today and hopes for control. *American. Bee J.* **118:** 468-471.

Gilliam, M. (1990). Chalkbrood disease of honeybees, *Apis mellifera,* caused by the fungus, *Ascosphaera apis*: Review of past and current research. **In:** *Fifth International Colloquium on Invertebrate Pathology and Microbial Control.*

Gilliam, M., Taber, S. and Richardson, G. (1983). Hygienic behavior of honeybee in relation to chalkbrood disease. *Apidologie.* 14: 29-30.

Gochnauer, T.and Margetts, V. (1979). Properties of honeybee larvae killed by Chalkbrood disease. *J. Apic.* **18**: 212-216.

Heath, L. (1982). Development of chalk brood in a honeybee colony: A review. *Bee World.* **63**: 119-130.

Heath L. (1985). Occurrence and distribution of chalkbrood disease of honeybees. *Bee World.* **66**: 9-15.

Heath, L. and Gaze, B. (1987). Carbon dioxide activation of spores of the chalkbrood fungus *Ascosphaera apis*. *Journal of Apicultural Research*. **26**: 243-246.

Hornitzky, M. (2001). Literature review of chalkbrood – A fungal disease of honeybees. Rural Industries Research and Development Corporation. Publication No. 01/150.

Jarosz, J. and Glinski, Z. (2000). The honeybee defense in mycotic disease. *Honeybee Science*. **21:** 69-74.

Koenig, J., Boush, G., Erickson, M. and Jr, E. (1986). Effect of spore introduction and ratio of adult bees to brood on chalkbrood in honeybee colonies. *Journal of Apicultural Research.* **26**: 191-195.

Koenig, J., Boush, G. and Grocksa, M. (1987). Isolation of chalkbrood pathogen *Ascosphaera apis* from honeybee (*Apis mellifer*) surface, pollen loads and water sources. *American Bee .J.* **13:** 581- 583.

Mcdonald, C. and Chen, L. (1965). The Lowry modification of the Folin reagent for determination of proteinase activity. *Anal. Biochem*. **10**: 175-177.

Minho, L., Cheol, S., Jinsoo, Y., Jum, R. and Hong, S. (2005). Cellular immune response and FAD glucose dehydrogenase activity of *Mamestra brassica* challenged with entomopathogenic fungi. *Physiologycal Entomology*. **30**: 287-292.

Nelson, D. and Gochnauer, T. (1982). Field and laboratory studies on Chalkbrood disease of honeybees. *American. Bee J.* 122: 29-32.

Nixon, M. (1982). Priliminary world map of honeybee disease and parasite. *Bee World.* **63(1):** 23-42.

Paweł, C. and Krzysztof, R. (2003). Studies on the morphology of strains of *Ascosphaera apis* isolated from chalkbrood disease of honeybee. *Electronic J. Polish Agricultural University*. **6(2):** 1-10

Pederson, K. (1976). Chalk brood: Possible methods of control and the effect of additional heat. *Birkteren.* **92**: 18-22.

Puerta, F., Flores, J., Bustos, M., Padilla, F. and Campano, F. (1994). Chalkbrood development in honeybee brood under controlled conditions. *Apidologie.* **25**: 540-546.

Reynaldi, J., Claudia, L., Graciela, N. and Adriana, M. (2003). Differentiation of *Ascosphaera apis* isolates by rep-PCR finger printing and determination of chalkbrood incidence in Argentina honey samples. *IBRA.* **5:** 68-76.

Rose, J. Christensen, M. and Wilson, W. (1984). Ascosphaera species inciting chalk brood disease in N. America and taxonomic key. *Mycotaxon.* **19**: 41-55.

Ruffinengo, S., Pena, I., Clemente, G., Palacio, A. and Escande, A. (2000). Suitability of culture media for the production of ascospores and maintenance of *Ascosphaera apis*. *J. Apicultural Reasearch*. **39(3):** 143-148.

Seal, D. (1957). Chalk brood disease of bee. *N. Z. J. Agric*. **96(6):** 562

Shaha, P. and Pell, J. (2003). Entomophatogenic fungi as biological control agents. *App. Microbiol. Biotech.* **61**: 413-423.

Skou, J. (1972). Notes on habitats, morphology and taxonomy of spore cyst fungi *(Ascosphaerales). Apimondi.* **30**: 260-264.

Spiltoir, C. (1955). Life cycle of *Ascosphaera apis (Pericystis apis). Amer. J. Botany,* **42**: 501-508.

Spiltoir, C. and Olive, L. (1955). A re-classification of the genus *Pericystis betts. Mycologia*, **47**: 238-244.

Takatori, K. and Tanaka, I. (1982). *Ascosphaera apis* isolated from Chalkbrood in honey bees. *Jap. J. Zootech. Sci.* **53**: 89-92.

Vandenberg, J. Fichter, B. and Stephen, W. (1980). Spore load of Ascosphaera species on emerging adults of alfalfa leafcutter bee, *Megachile rotundata. Appl. Envir. Microbiol*. **39(3):** 650-655.

DECLARATION

I the undersigned declare that this thesis is my original work. It has not been presented for a degree in this or any other University and all the source materials duly acknowledged.

Name of the candidate_____

Signature_____

Addis Ababa

July 2006