BEESWAX BASED FILMS AS ALTERNATIVE SUBSTRATES FOR REARING PARASITOIDS OF THE COTTON BOLL WEEVIL, *ANTHONOMUS GRANDIS* BOHEMAN (COLEOPTERA: CURCULIONIDAE)

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BEESWAX BASED FILMS AS ALTERNATIVE SUBSTRATES

FOR REARING PARASITOIDS OF THE COTTON BOLL

WEEVIL, *ANTHONOMUS GRANDIS* BOHEMAN

(COLEOPTERA: CURCULIONIDAE)

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I would like to dedicate this work to the Creator of all things, "Our Father in heaven...", for the magnitude of His unconditional love through the "Wonderful Counselor, Mighty God, Everlasting Father, Prince of Peace". "Through Him all things were made; without him nothing was made that has been made".

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ABBREVIATIONS USED

°C	Celsius Degree
CAPES	Federal Agency For Pos-Graduate Education
CNPA	National Center of Cotton Research
CNPq	National Council For Research
CRD	Completely Randomized Design
EHB	European Honey Bee
EMBRAPA	Brazilian Company of Agriculture and Stock Raising Research
IPM	Integrated Pest Management
LSD	Fisher's Protected Least Significant Difference
OSU	Oklahoma State University
PAT. PEND.	Patent Pending
RCBD	Randomized Complete Block Design
RH	Relative Humidity
UFPB	Universidade Federal da Paraiba

PREFACE

This dissertation is comprised of five manuscripts formatted for submission to the Southwestern Entomologist. This preface introduces the rest of the dissertation. The five manuscripts are complete as written and do not need supporting material. The manuscripts include: Chapter I, Waxfilm (Pat. Pend.): An alternative film for rearing parasitoids; Chapter II, Use of colored beeswax sheets in the production of films for rearing parasitoids; Chapter III, Use of carnauba (*Copernicia cerifera* Arruda Camara) wax in rearing parasitic hymenoptera for biological control; Chapter IV, Emergence behavior of *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae) on host containment cells in the laboratory; and Chapter V, Managing humidity and drinking water in acrylic cages for rearing parasitoid.

CHAPTER I

WAXFILM (PAT. PENDING): AN ALTERNATIVE FILM FOR REARING PARASITOIDS

ABSTRACT

Reducing the impact of insecticides on the environment is a concern of researchers. The use of parasitoids for controlling pests is ecologically preferred. One of the methods used in the mass rearing of parasitoids requires the use of Parafilm® "M". This film, however, is inconvenient to use in Brazil because of import restrictions and duties. Waxfilm (Pat. Pend.) is a film made from beeswax (*Apis mellifera* L.) that does not depend on importation, is 100% natural, 100% recyclable, and 100% biodegradable. This film is used in the laboratory as an artificial flower bud, in which boll weevil *Anthonomus grandis* Boheman larvae are placed and then exposed to parasitoids. Research was conducted at a temperature of 21±2°C and a relative humidity of 70±5%. *Bracon* spp. and *Catollacus grandis* Burks parasitoids were tested for rearing using both films. The results show that Parafilm® "M" (control) and Waxfilm (Pat. Pend.) do not differ from each other statistically with respect to percentage of parasitoid emergence. This new film could have a large impact on biological control programs in developing countries, where the use of cheap, locally available materials is very important to the successful implementation of new techniques. This would also provide increased income to indigenous beekeepers.

RESUMEN

Reducir el impacto de insecticidas en el ambiente és una preocupación de los investigadores. El uso de parasitóides para control de plagas es ecologicamente preferido. Un de los métodos usados en la producción masiva de parasitóides require del uso de Parafilm® "M". Esa película, todavia, es incoveniente para su uso en Brasil por causa de las restricciones de importación e impuestos. Waxfilm (Pat. Pend.) es una película hecha de la cera de abeja (*Apis mellifera* L.) y que no depende de importación y es 100% natural, 100% reciclavle y 100% biodegradavle. Esta película es usada en laboratorios como un botón floral artificial en que la larvas del pícudo *Anthonomus grandis* Boheman son colocadas y entoces expuestas a los parasitoides. Esta investigación fue realizada en temperaturas de 21±2°C y una humedad relativa de 70±5%. La producción de los parasitoides *Bracon* sp. y *Catolaccus grandis* fueron probados usando ambas películas. Los resultados muestran que Parafilm® "M" (controle) y Waxfilm (Pat. Pend.) no diferen estadísticamente una de la otra con respecto al porcentage de parasitóides emergidos. Esta nueva puede tener un grande impacto en programas de control biológico en paises en vías de desarrollo, donde el uso de materiales baratos y localmente disponibles es muy importante para lograr una implementación exitosa de nuevas técnicas. Esto también provería un aumento salarial para los apicultores de la región.

INTRODUCTION

After the appearance of the cotton boll weevil (*Anthonomus grandis* Boheman) in Brazil in the early 1980's, cotton (*Gossypium hirsutum L. var. latifolium and G. hirsutum var. marie galante Hutch.)* production declined, especially in Northeastern Brazil. *A. grandis* infestation resulted in an 80% decline in cotton yield production in this region (Matthews 1988). The introduction of the cotton boll weevil into Brazil is still mysterious, and whether it was accidental or intentional is unknown (Aquino 1983). Geographically, Brazil would be naturally protected against the entrance of the boll weevil. Natural barriers against boll weevil migration include the Amazon rain forest in the North, cold Argentina in the South, the Atlantic ocean in the East, and the icy Andes mountains in the West.

Biological control has been the most efficient tool in integrated pest management (IPM) in Northeastern Brazil, mainly because of the rich complex of naturally occurring enthomophagous arthropods in this tropical region of the country (Ramalho 1994). In Brazilian cotton agrosystems, *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae) and *Bracon* spp. (Hymenoptera: Braconidae) are some of the predominant parasitoids of *A. grandis* (Ramalho & Gonzaga 1990; Ramalho et al. 1986; and Pierozzi et al. 1984). Rearing of *C. grandis* has been reported by Cate (1987), USDA/APHIS (personal communication), Guerra (1992), and Morales-Ramos, Cate (1992), and Aquino et al. (1996 and 1997). Their conditions and results are summarized in Table 1.

Great progress has been made in rearing these parasitoids in the laboratory using

the method proposed by Cate (1987) in which Parafilm® "M" is used for host containment cells. However, Parafilm® "M" is costly to import. A roll of Parafilm® "M" in Brazil costs almost 5 times as much it does in the United States. Besides the cost of the film, the import bureaucracy is time consuming and causes several problems: delay of research, delay in releasing the parasitoids, and delay in mass production of the parasitoids and sometimes loss of the laboratory culture. Even in the United States, Parafilm® "M" is considered expensive for rearing *C. grandis* (Guerra et al. 1994).

A study conducted by Aquino et al. (1993) suggests that beeswax may be a plausible alternative for rearing parasitoids. The use of bees in biological control is a reality today. Bees have been used indirectly as both delivery agent of bacteria and as producer of beeswax for making films for parasitoid production. Bees have been reported to be effective agents in controlling a severe pest in apple trees, *Erwinia amylovora*, a bacteria that causes fire blight (Southwich 1992). The honey bee delivers fire-blight-fighting bacteria to apple blossoms. Wax sales also could be a potential expanded market for beekeeping, and could improve the economic status of beekeepers in Brazil. Currently, the majority of beekeepers in Northeast Brazil only use wax for the production of new sheets of comb foundation. Therefore, we propose to test the hypothesis that beeswax can provide an alternative film for rearing parasitoids. The objective of this study is to determine the costs of production for this alternative film as well as its efficiency in mass production of *C. grandis* in the laboratory.

MATERIALS AND METHODS

This study used third instar larvae of boll weevil *A. grandis* collected from cotton squares in a commercial cotton field. These larvae were encapsulated in both Parafilm® "M" and Waxfilm (Pat. Pend.) and exposed to the parasitoids *Bracon* sp and *C. grandis*.

Procedure - The procedures proposed by Cate (1987) and by Aquino et al. (1993) were used. The cells are formed by using one 15 x 7.5 x 1.1 cm aluminum plate. This plate has a 5 x 8 matrix of 0.8 cm holes drilled through it (Fig. 1). The Parafilm® "M" (10 cm long sheet) is placed on this and then a No. 2 pencil eraser is pressed on top of the Parafilm® "M" and aluminum plate. For Waxfilm (Pat. Pend.) the method used was the same, except the pencil eraser was replaced by the ball of the thumb. Third instar boll weevil larvae

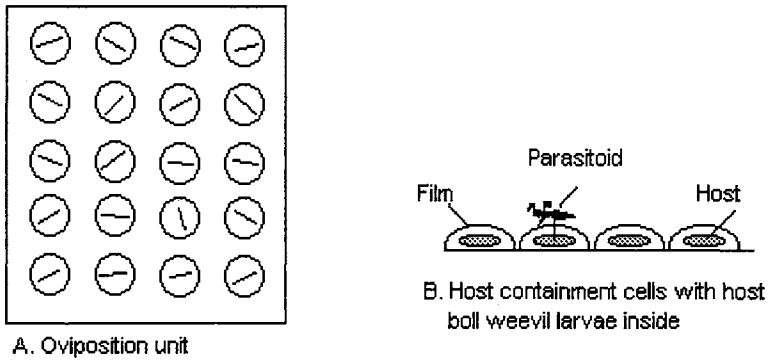

Fig. 1 - A schematic diagram showing the host containment cells in Waxfilm (Pat. Pend.). A. Waxfilm (Pat. Pend.) oviposition unit; B. Transversal view of a complete oviposition Waxfilm (Pat. Pend.) unit with host larvae enclosed.

were placed by hand inside the cells, shaped so as to enclose host larvae. Four larvae were encapsulated inside the cells, placed inside a petri dish with one gravid female parasitoid for oviposition, and left for 24 h. After the oviposition period, the female parasitoid was removed and the encapsulated-parasitized larvae were held until the emergence of adult parasitoids. The number of emerging parasitoids was recorded.

The experimental design used was a Randomized Complete Block Design (RCBD), with the six days as the blocks and the two films (Parafilm® "M" and Waxfilm - Pat. Pend.) as the treatments. The total emergence of *Catolaccus grandis* was measured and a square root transformation was applied to alleviate problems associated with count data (Steel and Torrie 1980, p. 234). The level of significance used was 0.05.

How To Produce Waxfilm (Pat. Pend.): A Brief Description - Comb wax from honeybee (*Apis mellifera* L.) is collected from beekeepers and placed in a wax melter at 80°C. When the wax gets to its melting point, it is filtered through a metal screen for purification. Then, in a second wax melter at the same temperature, the melted wax is kept fluid for 30 minutes. A sheet of wood, measuring 30 x 40 x 0.5 cm, is soaked in water for 24h (the day before), and then submerged into the hot melted wax. The sheet is removed from the saturated wood surface. The sheets are pressed using a home mill in order to get the wax sheet thinner. In order to have a smooth pressing process, a mixture of honey-alcohol (50%-50% v/v) is spread on the mill cylinders constantly. The second step, in order to get the wax sheet even thinner, is to use a industrial bakery mill. In this case, vegetable oil is used on the surface of the wax sheet which is then passed through the cylinders until a sheet as thick as Parafilm® "M" is obtained. The excess vegetable oil on the surface is removed with a cotton cloth, and Waxfilm (Pat. Pend.) is ready.

RESULTS AND DISCUSSION

The honey bee (*Apis mellifera* L.) could be seen as an indirect biological control agent against the boll weevil (*Anthonomus grandis* Boheman) [Fig. 2]. The results of this study show that Waxfilm (Pat. Pend.) is as efficient as Parafilm® "M" with respect to the number of parasitoids that emerged. For instance, in Table 2, the means were 2.152 and 2.047 for *Bracon* sp., which differ by only one fourth of the smallest standard deviation. In case of *Catolaccus grandis*, the means were 1.542 and 1.980, which differ by less than the smallest standard deviation. Waxfilm (Pat. Pend.) compares favorably to Parafilm® "M" in its other properties as well (Table 3). We noticed that the sequence of parasitism of *C. grandis* on boll weevil larvae when using Waxfilm (Pat. Pend.) was very similar to the sequence when using Parafilm® "M" (Figs. 3 and 4).

When superparasitism occurred during the mass production of *C. grandis*, we noticed that this parasitoid oviposited excess eggs on the Parafilm® "M" itself, in the flat surface between the cells. The number of these wasted eggs was much less on Waxfilm (Pat. Pend.). We noticed that aproximately 48h after laying, *C. grandis* larvae started migrating underneath (between) the sheets of Parafilm® "M". We believe that these larvae were potentially viable to parasitize boll weevil larvae which had not been directly oviposited on.

A useful property of Waxfilm (Pat. Pend.) is its fungus resistance. *Aspergillus*

niger van Teighem was a problem with Parafilm® "M" cells (Fig. 5), but we did not find *A. niger* in Waxfilm (Pat. Pend.). This may be the result of beeswax having anti-fungal properties like propolis. Propolis contains a multitude of anti-microbial flavolenoids and phenolics (Johnson et al. 1994). Propolis has been demonstrated to inhibit *Saccharomyces cerevisae*, *Kluyveromyces fragilis* and *Rhodotorula rubra* (Grzybowski and Szewczyk 1987), as well as the *Aspergillus* species *ochraceus*, *sulphureus* (Pepeljnjak and Jalsenjak 1984), and *niger* (Grzybowski and Szewczyk 1987). Considering that unrefined beeswax contains flavolenoids and phenolics due to the small amount of propolis incorporated in the wax, it can be assumed that this may be the reason that Waxfilm (Pat. Pend.) inhibits the growth of *A. niger* in culture. The fungus *Aspergillus niger* can be easily spread through the colony, especially during parasitism and feeding. *Aspergillus* spores can be passed on through an infected boll weevil larvae and/or by manipulation of the film with forceps. If there is a focus of infection in the beginning of the process, later this can become a problem for the rearing process. We have noticed healthy boll weevil larvae that have been infected after parasitism, which leads us to assume that the parasitoid ovipositor may be an agent of fungus contamination. Also, we have seen 'nuclei' of *Aspergillus niger* develop in some drops of honey placed on plastic cards for feeding the parasitoids. *A. niger* is an opportunistic fungi that may cause Aspergillosis of the lungs, a serious disease prevalent in birds and several mammals as well as in humans (Alexopoulos and Mims 1979). Also, *A. niger* may cause diseases such as bronchopulmonary aspergillosis, aspergilloma, invasive aspergillosis, and colonization and invasion on abnormal tissue such as burned skin and injured eyes (Rose and Barron 1983). Therefore, the presence of *A.*

niger is not good for workers in a mass rearing facility. *A. niger* propagation was not visible inside the Waxfilm (Pat. Pend.) cells, but was on the plastic sheets holding honey for adult parasitoid feeding. Approximately 7 days after placing the feeding sheet containing drops of pure honey in the cage, the presence of dark spots in the honey drops was noticed. By the distribution pattern, we assume that this was due to the presence of *A. niger* spores in the mouth parts of the parasitoids. In order to verify that this was a mouth contamination, we placed several sheets containing drops of honey outside the cage and no visible sign of *A. niger* was found. To prevent any breathing of *A. niger* in the laboratory while handling Parafilm® "M" cells contaminated by *A. niger*, it is important to use dust masks (e.g. maxi-mask® Flents Products Co., Inc., Norwalk, CT) that will filters out the fungus nuclei. When opening Parafilm® "M" cells for a close observation of reasons why parasitoids did not emerge it is important to use a hood as well as a mask because *A. niger* nuclei are very easily dispersed in the air. A potential source of *A. niger* contamination is larvae reared on artificial media. We observed that some trays of first instar boll weevil we received from the supplier had colonies of *A. niger*. Guerra et al. (1994) note that bacterial and fungal contamination severely affect *C. grandis* yield in artificial diet. They suggest inclusion of antimicrobial into the diet, such as penicillin, streptomycin, amphotericin, and phosphoric acids.

We analyzed the cost of parasitoid production in both films. The costs of film, labor, and larvae were calculated (Fig. 6). Taking Brazil as an example for calculation of costs (considering that this alternative film is primarily designed for developing countries that cannot afford buying Parafilm® "M"), labor was estimated at $ 0.70/hour. Boll weevil

larvae are obtained by collecting infected cotton squares from local cotton fields. Infested squares abort and are picked up by hand when they have changed from yellow to brown, indicating the presence of 3rd instar larvae. Using a temporary worker, it is possible to pay $ 2.10 per 1,000 of cotton squares collected (~3 hours). Considering a production of 1,000 parasitoids, we can estimate 2 hours/person to set up 1,000 cells in any film. The cost of 1 kilo of beeswax is $ 6.00. To make Waxfilm (Pat. Pend.) that holds 1,000 parasitoids, we need 200 grams of beeswax, which is $ 1.20. Parafilm® "M" costs $ 65.00 in some parts of Brazil (five times more than in the US, for instance). To produce 1,000 parasitoids we usually consume 1/3 of a roll of Parafilm® "M", which would cost around $ 21.60.

Besides being 100% natural and 100% biodegradable, we noticed that Waxfilm (Pat. Pend.) is 100% recyclable. We tried to recycle both films during our study. Pieces of Waxfilm (Pat. Pend.) and Parafilm® "M" were placed in jars, 50% water/50% films, and placed in a laboratory oven at 100°C for about 15 minutes. Recycling Waxfilm (Pat. Pend.) was possible. It melts as beeswax and any impurity is distinctly separated from the wax as an easily removed layer on the bottom of the wax. However, Parafilm® "M" did not melt but fused to a non-separating mass that could not be recycled.

Beekeepers in Northeastern Brazil, the region this project was designed to help, derive their main income from honey sales. Processing beeswax is a secondary activity. The production of Waxfilm (Pat. Pend.) could provide a profitable market for beeswax, which presently is used only for making comb foundation. Because beeswax is easily

available in Brazil, it seems that Waxfilm (Pat. Pend.) may be adopted as an alternative film for rearing parasioids.

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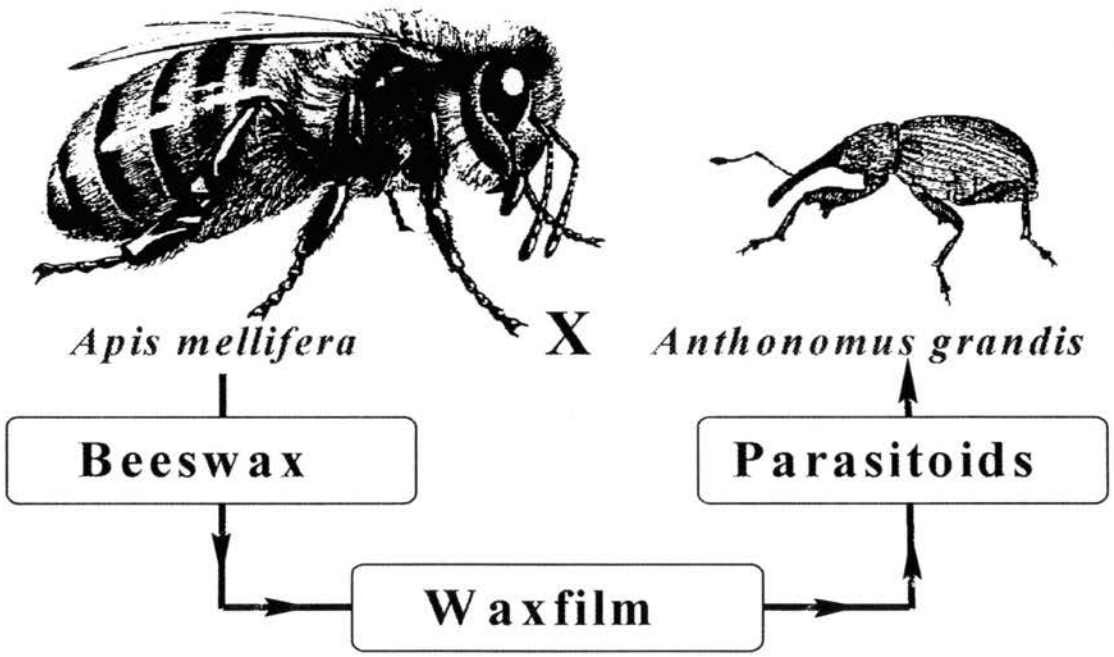

Fig. 2 - A schematic diagram showing the indirect biological control of the cotton boll weevil (*Anthonomus grandis* Boheman) by a by-product of the honey bee (*Apis mellifera* L.). Beeswax: provided from honey combs; Waxfilm (Pat. Pend.): used as a sheet for host containment cells for parasitism on boll weevil larvae; and Parasitoids: *Catolaccus grandis* Burks and *Bracon* sp. reared and mass released to control the cotton boll weevil.

Fig 3 - Sequence of *Catolaccus grandis* parasitizing boll weevil larvae encapsulated in Waxfilm (Pat. Pend.); (A) Antennating containment cell; (B) Positioning ovipositor. (Photography by Vladimir Beregovoy, Senior Agriculturist, Dept. of Entomology, Oklahoma State University)

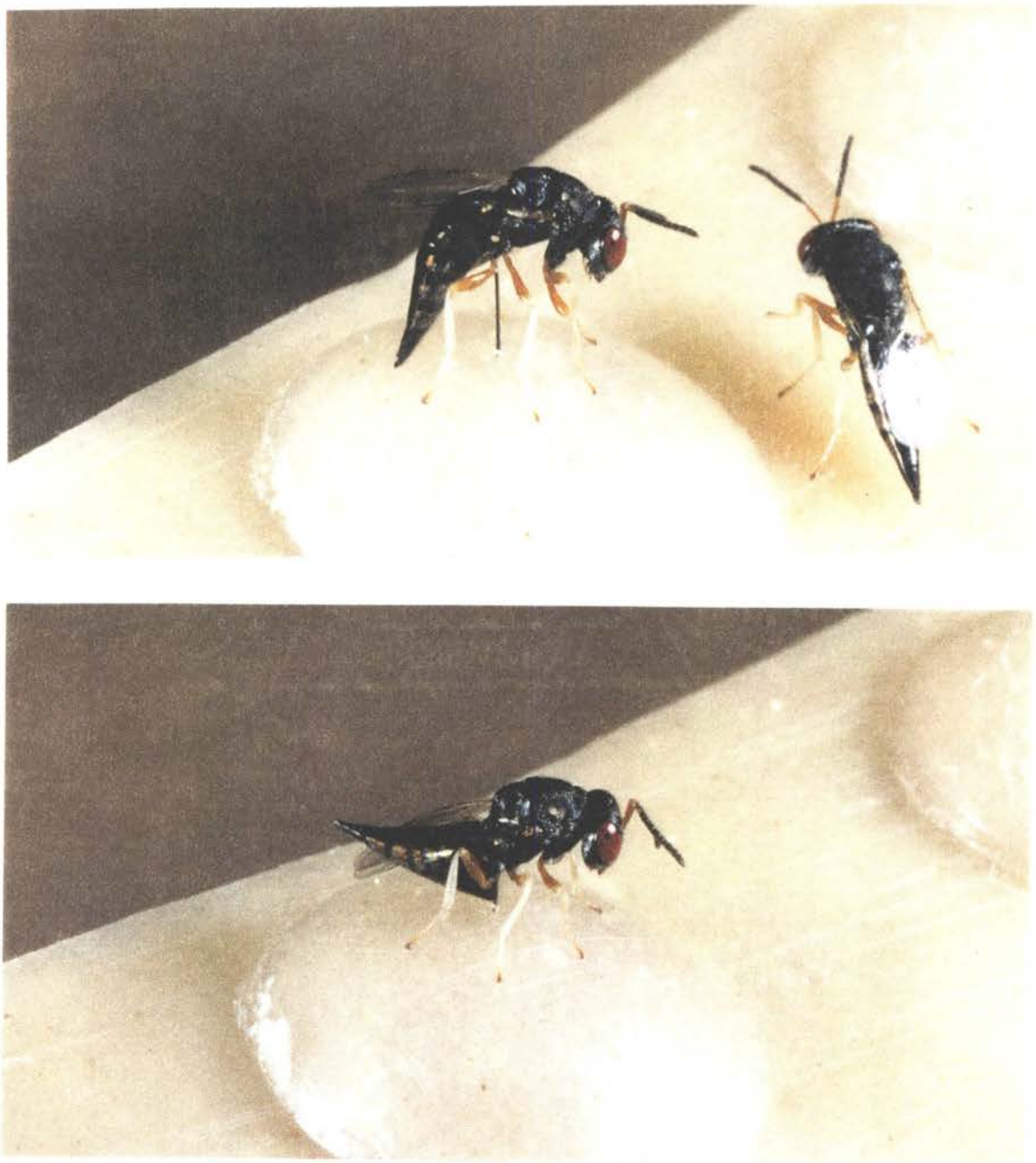

Fig 4 - Sequence of *Catolaccus grandis* parasitizing boll weevil larvae encapsulated in Waxfilm (Pat. Pend.); (C) Inserting ovipositor; (D) Ovipositor fully inserted. (Photography by Vladimir Beregovoy, Senior Agriculturist, Dept. of Entomology, Oklahoma State University)

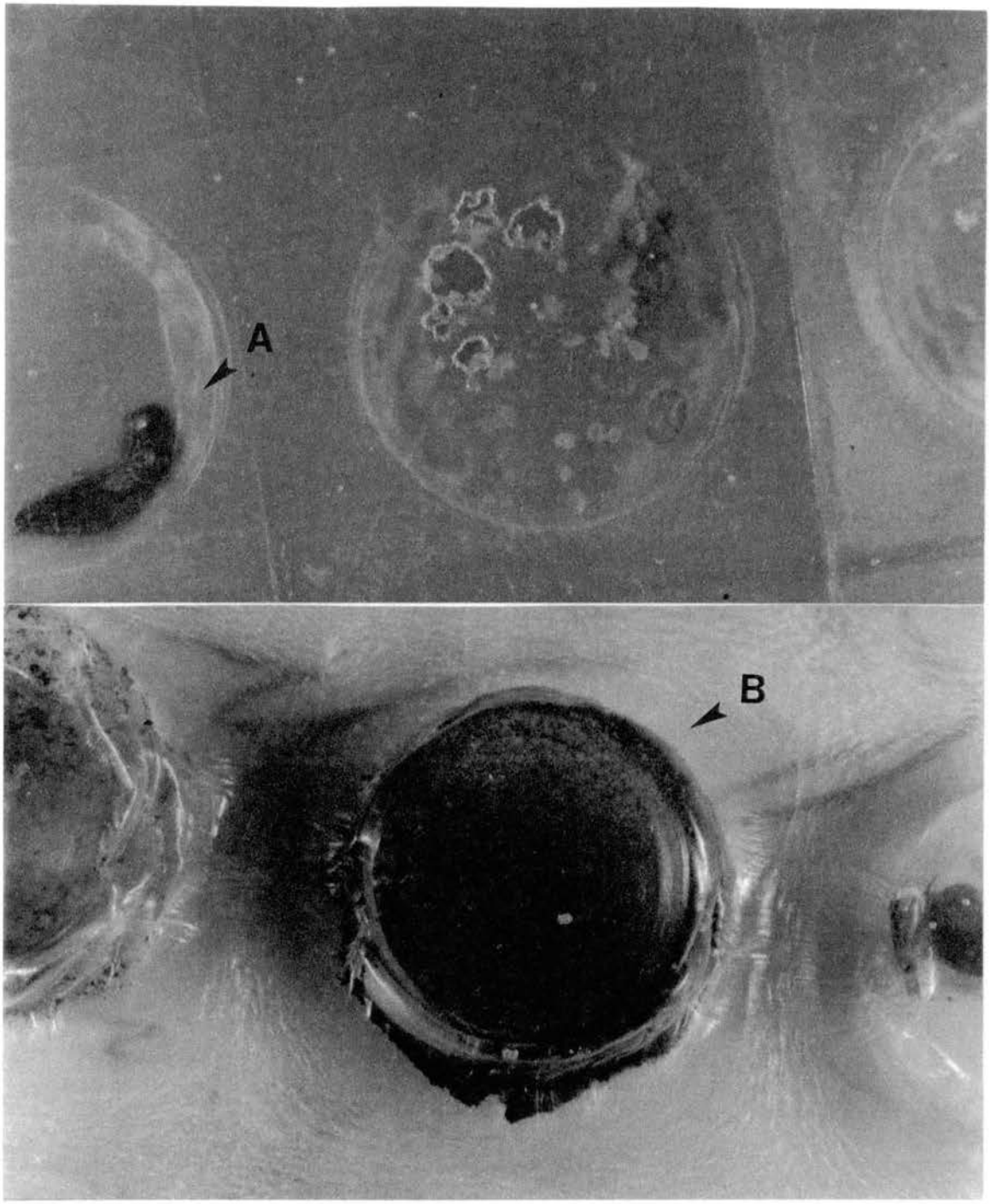

Fig 5 - *Catolaccus grandis* pupae infected by *Aspergillus niger* in Parafilm® "M" sheet (A); typical Parafilm® "M" cell completely infected by *A. niger* (B). (Photography by Vladimir Beregovoy, Senior Agriculturist, Dept. of Entomology, Oklahoma State University)

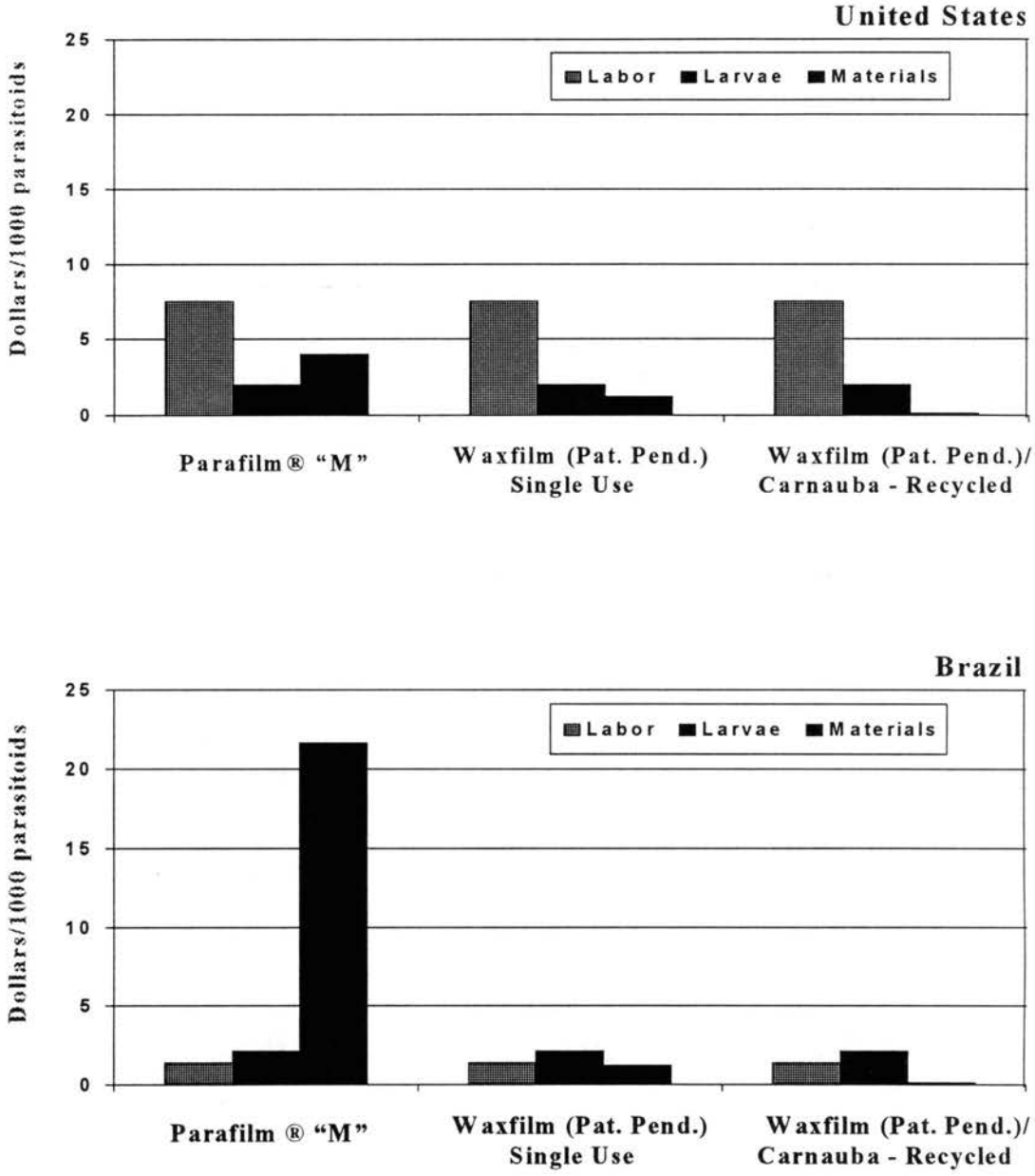

Fig. 6 - Relative costs of production of 1000 *Catolaccus grandis* in the United States and in Brazil.

TABLE 1

IN VIVO REARING CONDITIONS FOR *CATOLACUS GRANDIS* REPORTED BY VARIOUS RESEARCHERS.

	Temperature	Relative Humidity	Photoperiod	Parasitism (%)		Honey
Cate (1987)	(22-24°C)	50%	16L:8D	Field	Lab	15%
USDA (personal communication)	-	-	-	41.24	26.00(low)	100%
Guerra (1992)	28°C	60-65%	14L:10D	-	-	-
Morales-Ramos and Cate (1992)	25°C	HIGH	14L:10D	-	-	100%
Aquino et al. 1993	21±2°C	70±5%	~12L:12D	-	~30	~50%
Aquino et al. 1997	26°C	80%	12L:12D	-	~35	100%

TABLE 2

NUMBER OF PARASITOIDS EMERGING FROM DIFFERENT FILMS AFTER 24 H OF PARASITISM ON *ANTHONOMUS GRANDIS* LARVAE

	Bracon sp.		*C. grandis*	
Treatment	Mean	S.D.	Mean	S.D.
Parafilm® "M"	2.152a	0.97	1.542a	0.74
Waxfilm (Pat. Pend.)	2.047a	0.42	1.980a	0.54

Means within columns followed by same letter are not significantly different (P=0.05; ANOVA procedure, MYSTAT for Windows 1994; N=6, T=24); LSD performed on square root - transformed data. Means and S.D. displayed are raw values.

TABLE 3

SOME COMPARISONS BETWEEN PARAFILM® "M" AND WAXFILM (PAT. PEND.)

Characteristics	Parafilm® " M"	Waxfilm (Pat. Pend.)
Flexible	Yes	Yes
Moldable	Yes	Yes
Self-sealing	Yes	Yes
Odor	No	Typical (beeswax)
Moisture-resistant	Yes	Yes
Transparency	Semi	Semi
Color	Colorless	Light white-yellow
Fungus resistant	No	Yes (*Aspergillus niger*)

CHAPTER II

USE OF COLORED BEESWAX SHEETS IN THE PRODUCTION OF FILMS FOR REARING PARASITOIDS

ABSTRACT

Third instar boll weevil (*Anthonomus grandis* Boheman) were placed in artificial flower buds made out of commercial colored beeswax for the mass production of the parasitoids *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae) and *Bracon thurberiphagae* Mueseback (Hymenoptera: Braconidae). Twenty boll weevil larvae were exposed to a 24 h parasitism, during six days, by 50-70 *C. grandis* gravid females (5 days and older) at 26±2°C, 80±3% relative humidity.

The data indicate that some colors of beeswax (red, green, and white) can be used as an alternative film for rearing both *C. grandis* and *B. thurberiphagae*. Results indicate that red and green do not differ from Parafilm® "M" (control) with respect to the number of emerged parasitoids.

The Parafilm® "M" and Waxfilm (Pat. Pend.) combination was the best film for rearing *C. grandis* and *B. thurberiphagae*. However, when *C. grandis* had a choice of where to oviposit it showed no preference for Parafilm® "M" and its combination with Waxfilm (Pat. Pend.). A LSD test showed that these two films did not differ statistically from each other with respect to parasitoid emergence. *C. grandis* preferred the green wax film more than the other colors. *B. thurberiphagae* showed similar preference. The use of these films for other parasitoids is discussed.

RESUMEN

Larvas del pícudo (*Anthonomus grandis* Boheman) en el tercer instar larval fueron colocadas en botones florales artificiales de cera de abeja colorida para la producción masiva de los parasitoides *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae) y *Bracon thurberiphagae* Mueseback (Hymenoptera: Braconidae). Veinte larvas huéped fueron expuestas a 24 h de parasitismo, durante seis dias, por 50-70 hembras de *C. grandis* (5 dias y más vieja) en 26+2°C, 80+3% de humedad relativa.

Los datos indican que algunos colores de cera de abeja (rojo, verde y blanco) pueden ser usados como una película alternativa para producción de *C. grandis* y *B. thurberiphagae*. Los resultados indican que rojo y verde no diferen de Parafilm® "M" (controle) con respecto al número de parasitóides emergidos.

La combinación de Parafilm® "M" y Waxfilm (Pat. Pend.) fue la mejor película para la creación de *C. grandis* y *B. thurberiphagae*. Todavía, cuando *C. grandis* tuvo la oportunidad de escojer adonde hacer la oviposición, este no mostró diferencia con respecto a Parafilm® "M" y su combinación con Waxfilm (Pat. Pend.). La prueba LSD mostró que estas dos películas no diferencian estadisticamente una de la otra con respecto a la emergencia del parasitoide. De las películas coloradas, *C. grandis* prefiere la película de cera verde más que cualquier otro color. *B. thurberiphagae* mostró resultados similares. El uso de estas películas para otros parasitóides es discutido.

INTRODUCTION

Cotton producers have faced a great challenge in controlling the cotton boll weevil, *Anthonomus grandis* Boheman, (Coleoptera: Curculionidae), since its first appearance in August 1983 in Northeastern Brazil, one of the poorest regions in the country. Cotton production decreased 80% (Matthews 1988) in the first five years after its first infestation, both due to yield loss and from abandonment (Ramalho and Wanderley 1996). More than a decade has passed since the boll weevil's first appearence in Brazil and not much has since changed. Unable to afford insecticides, farmers are appealing for alternative methods to control this pest. One of the main goals of the National Center for Cotton Research (CNPA/EMBRAPA) in Brazil is to study alternative methods for controlling the boll weevil that are both cheap and effective.

Augmentation of predators and parasioids of the cotton boll weevil is desirable for control when environmental conditions prevent stable populations of biocontrol agents from establishing (Robinson et al. 1995). In many cases augmentative releases of parasitoids have estabblished control without the application of pesticides (Morales-Ramos et al. 1994). In the entomological world, Parafilm® "M" plays a very important role as a feeding and/or rearing system in the laboratory. There are several investigations on feeding insects by the use of Parafilm® "M", such as northern fowl mites (Carrol et al. 1992), horseflies *Tabanus nigrovittatus* (Friend & Stoffolano 1991), greenbugs *Schizaphis graminum* (Gildow & D'Arcy 1990; Ma-Runlin et al. 1990), mites *Tetranychus urticae* (van der Geest et al. 1983), and the carambola fruit borer *Eucosma notanthes* Meyrick

(Hung & Hwang 1991). For rearing arthropods in the laboratory Parafilm® "M" has also been used by Kainoh & Tatsuki (1988) as an artificial egg for the oviposition of *Ascogaster reticulatus*, an egg parasitoid of the smaller tea tortrix *Adoxophyes* sp. Also, Parafilm® "M" has been used successfully in the study of arthropods that cause infection in humans and animals. In these studies, Parafilm® "M" was used as a feeding membrane for blood feeding arthropods, such as the fly *Hippobosca equina* (Fouda 1984), mosquitoes (Collins et al. 1986; Failloux et al. 1991), and ticks (Klunker & Kieskow 1981; Kirch et al. 1991; Schwan et al. 1991). After Cate (1987) proposed the use of Parafilm® "M" for rearing parasitoids of the cotton boll weevil (*Anthonomus grandis* Boheman), Ramalho and Gonzaga (1991) proposed an adaptation of the method using a wood press instead of metal to form the cells. Recently, Aquino et al. (1993, 1996, and 1997) have proposed the use of alternative films such as Waxfilm (Pat. Pend) - a film derived from beeswax - and other films made out bees wax and carnauba (*Copernicia cerifera* Arruda Camara).

Besides honey, silk, and shellac, beeswax is probably the most common insect product for the 'entomological industry'. Its use goes back to ancient times (Krochmal 1987), and is used both at home (artistic painting, candles) and in specialized industry (cosmetics, lubricants, etc.).

Insects can perceive colors (Wigglesworth 1964) and, moreover, display attractiveness to different spectral ranges (Borror and DeLong 1971; Chapman 1971). Homopterans, for instance, are attracted to yellow (Ramalho and Albuquerque 1979), fruit flies to yellow and green (Robacker et al. 1990), and *Bracon hebetor* to ultraviolet (UV) (Cline 1989). Therefore, colored films may affect attraction of parasitoids. Additionally, it

may be a cheap substitute for when Waxfilm (Pat. Pend.) and/or Parafilm® "M" is/are in temporary short supply. Besides that, because colored beeswax has a seasonal market, making films out of colored beeswax may assist its manufactures. From these reasons, the purpose of this study was to evaluate the use of colored beeswax sheets in the production of films for rearing parasitoids.

MATERIALS AND METHODS

All studies were conducted in the laboratory at 26°C, 80±3% relative humidity, and photoperiod 9L:15D.

Parasitoids: The parasitoids *Bracon thurberiphagae* Mueseback (Hymenoptera: Braconidae) and *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae) were obtained from Integrated Bio-Control Systems (IBCS), Lawrenceburg, IN, and were cultured in the laboratory using third instar boll weevil larvae encapsulated in Parafilm® "M" according to the technique proposed by Cate (1987).

Host: Third instar boll weevil (*A. grandis*) larvae were used as hosts. The larvae were provided by the USDA/ARS, Mississippi State, Mississippi, and had been cultured in artificial diet as described by Sikorowski et al. (1984). IBCS obtained their initial colony of parasitoids from the USDA/APHIS in Mission, Texas, and had cultured them according to the method proposed by Cate (1987). Parasitoids were shipped as larvae and/or pupae encapsulated in Parafilm® "M", and kept in in a growth chamber for emergence.

Host sheet: The host sheets were made of Parafilm® "M" and Waxfilm (Pat. Pend.) according to the methods proposed by Cate (1987) and Aquino et al. (1997), respectively. Waxfilm (Pat. Pend.) was made in different colors. The colored waxes (containing wax soluble dyes) used to make Waxfilm (Pat. Pend.) were provided by Mann Lake Ltd., Hackensack, Minnesota, and The Walter T. Kelly Co., Clarkson, Kentucky. The treatments used for *B. thurberiphagae* were: 1. Parafilm® "M" (control), 2. Green Wax, 3.

Red Wax; 4. Yellow Wax; and 5. White Wax. The treatments used for *C. grandis* were: 1. Parafilm® "M" (control), 2. Green Wax, 3. Red Wax; 4. Yellow Wax; 5. White Wax; and 6. Parafilm® "M"-Waxfilm mixture. Pieces of colored beeswax were cut into squares measuring 6.5 x 7.5 cm. Then, with the back of a tablespoon, the sheets were smoothed to flatten the film in order to be used as a host containment cell for the cotton boll weevil larvae.

Parasitism with choice: The parasitoids *B. thurberiphagae* and *C. grandis* were used. The host sheets were placed in an acrylic cage (45.8 cm X 25.5 cm X 48.5 cm), with 40-70 gravid female parasitoids (5 days old and older). Two cages were used, each containing one species of parasitoid. Each sheet contained 20 cells with one boll weevil larvae in each cell. The sheets were exposed to parasitoids for 24 h. After that, the cells were removed from the parasitoids and placed in plastic petri dishes (100 x 15mm) for emergence observation.

Parasitism with no-choice: Only the parasitoid *C. grandis* was used. The host sheets were placed in a 100 x 15 mm petri dish with one male and 10 gravid females (5-10 days old). Each sheet contained 20 cells with one boll weevil larvae in each. The sheets were exposed to parasitoids for 24 h. parasitism.

The experimental design was a Randomized Complete Block Design (RCBD), with six days as the blocks and the films (colored beeswax sheets) as the treatments, five films for *B. thurberiphagae* and 6 films for *C. grandis*. The emergence of parasitoids from the films was monitored. When there was a statistical difference determined by the F test (alpha=0.05), then Fisher's Protected Least Significance Difference (LSD) procedure was

used to make pairwise comparissons among the films - the best film having the higher total emergence of parasitoids. The total emergence of *Catolaccus grandis* was measured and a square root transformation was applied to alleviate problems associated with count data (Steel and Torrie 1980, p. 234). The level of significance used for Fisher's LSD was 0.05. Each film contained twenty cells. The films were placed in cages for parasitism for 24 hours, for each of the six 6 days. Each day was considered as a block. Then, after parasitism, the sheets were transfered to petri dishes for emergence.

RESULTS AND DISCUSSION

Results show that *B. thurberiphagae* and *C. grandis* oviposited in colored beeswax as well as Parafilm® "M" (Fig. 1 and 2). However, in a choice trial, *C. grandis* showed a preference for Parafilm® "M" and its combination with Waxfilm (Pat. Pend.) [Table 1]. The differences were not statistically significant at 0.05, but there was a trend. *C. grandis* showed indistinguishable attractiveness to green, white, red, and yellow films, but yellow wax had lower emergence levels than the Parafilm® "M" and Parafilm® "M"-Waxfilm (Pat. Pend.) combination. When *C. grandis* was tested with a no-choice trial, Parafilm® "M"-Waxfilm (Pat. Pend.) combination was the best film (Table 2). The control Parafilm® "M" was not different from red wax film. Possibly, *C. grandis* may have a similar attractiveness to red color as certain butterflies (Romoser 1981). White and green films did not differ from each other. Yellow had no response. LSD results for the emergence of *B. thurberiphagae* are in Table 3. *B. thurberiphagae* showed better parasitism in Parafilm®"M", its combination, and green wax. However, the raised areas formed in the film to place the host larvae inside received more probing. The use of both Parafilm®"M" and Waxfilm (Pat. Pend.) proved to be effective "M" and green wax. However, the colored waxes were not significantly different ($P>0.05$).

Probing behavior was observed on all colors of film (sheets). Observations showed that both *C. grandis* and *B. thurberiphagae* would probe, besides the cell containing the host, the flat surface between the cells in both Parafilm® "M" and Waxfilm (Pat. Pend.).

However, the raised areas formed in the film to place the host larvae inside received more probing. The use of both Parafilm®"M" and Waxfilm (Pat. Pend.) proved to be effective in rearing a large number of parasitoids. Waxfilm (Pat. Pend.) has advantages of being inexpensive, and being made from only one, readily available material. Since other species have been reared in artificial films, probably other species of *Catolaccus* and *Bracon* may have similar responses to colored beeswax films.

Preliminary tests on alternative substrates: During preliminary research, other substrates were tried to see if other modes of containment would prove superior to the Cate's (1987) method. *C. grandis* was reared in Glad® cling wrap, a crystal clear polyethylene, and in Kix®, a round crispy corn cereal. The Glad® wrap was cut into squares identical to Parafilm® "M". Larvae were placed on one layer and a second layer was placed on top of it. Sealing occurred automatically because of inherent self-adhesiveness of the film. The Kix® cereal was cut in half, and boll weevil larvae was placed inside. Then, melted beeswax was used to seal the cut. Water was applied to the cereal to soften it. In a high humidity condition (80%), it remained soft throughout the trial. Successful emergence occurred with both substrates. However, extensive *A. niger* contamination prevented definitive determination of the actual parasitism levels. Kix® was tried unmoistened and much less *A. niger* occured with equivalent emergence. *A. niger* was found between the two layers of Glad® wrap and on the outside of the corn cereal. Further investigations would involve treatment with fungicides. Another alternative film that could be tested is filo dough, a commercially available unbaked pastry, in very thin moldable sheets, and it

could provide a breathable containment cell. Saran Wrap® is still another possibility. It is regenerated cellulose and should, therefore, provide breathable film as well as be chewable by the emerging parasitoids. Films that allow air exchange and/or can be treated with fungicide may greatly reduce mortality due to *A. niger*.

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TABLE 1

LSD RESULTS FOR THE EMERGENCE OF *CATOLACCUS GRANDIS* ADULTS WHEN FEMALES ARE GIVEN CHOICE TO OVIPOSIT ON CONTROL AND COLORED BEESWAX FILMS

Films	Means	Grouping	S.D.	S.E.
Parafilm® "M"	4.167	a	4.215	1.721
Para-Wax	3.333	ab	5.086	2.076
Green	2.167	abc	3.488	1.424
White	1.667	bc	2.875	1.740
Red	1.667	bc	2.658	1.085
Yellow	0.167	c	0.408	0.167

Means followed by the same letter are not significantly different (P>0.05; SAS® for Windows™ 95, SAS PROC GLM; N=6, T=36); LSD performed on square root - transformed data. Means and S.D. displayed are raw values.

TABLE 2

LSD RESULTS FOR THE EMERGENCE OF *CATOLACCUS GRANDIS* ADULTS WHEN FEMALES ARE GIVEN NO-CHOICE TO OVIPOSIT ON CONTROL AND COLORED BEESWAX FILMS

Films	Means	Grouping	S.D.	S.E.
Para-Wax	12.60	a	3.647	1.631
Parafilm® "M"	6.00	b	4.301	1.924
Red	5.00	b	4.690	3.098
White	3.40	bc	3.209	1.435
Green	2.80	bc	3.564	1.594
Yellow	0.00	c	0.000	0.000

Means followed by the same letter are not significantly different (P>0.05; SAS® for Windows™ 95, SAS PROC GLM; N=5, T=30); LSD performed on square root - transformed data. Means and S.D. displayed are raw values.

TABLE 3

LSD RESULTS FOR THE EMERGENCE OF *BRACON THURBERIPHAGAE* ADULTS WHEN FEMALES ARE GIVEN CHOICE TO OVIPOSIT ON CONTROL AND COLORED BEESWAX FILMS

Films	Means	Grouping	S.D.	S.E.
Parafilm® "M"	11.167	a	3.167	5.375
Green	4.500	ab	7.369	3.008
Red	3.333	ab	4.227	1.736
White	2.667	ab	2.503	1.022
Yellow	0.333	b	0.816	0.333

Means followed by the same letter are not significantly different (P>0.05; SAS® for Windows™ 95, SAS PROC GLM,; N=6, T=30); LSD performed on square root - transformed data. Means and S.D. displayed are raw values.

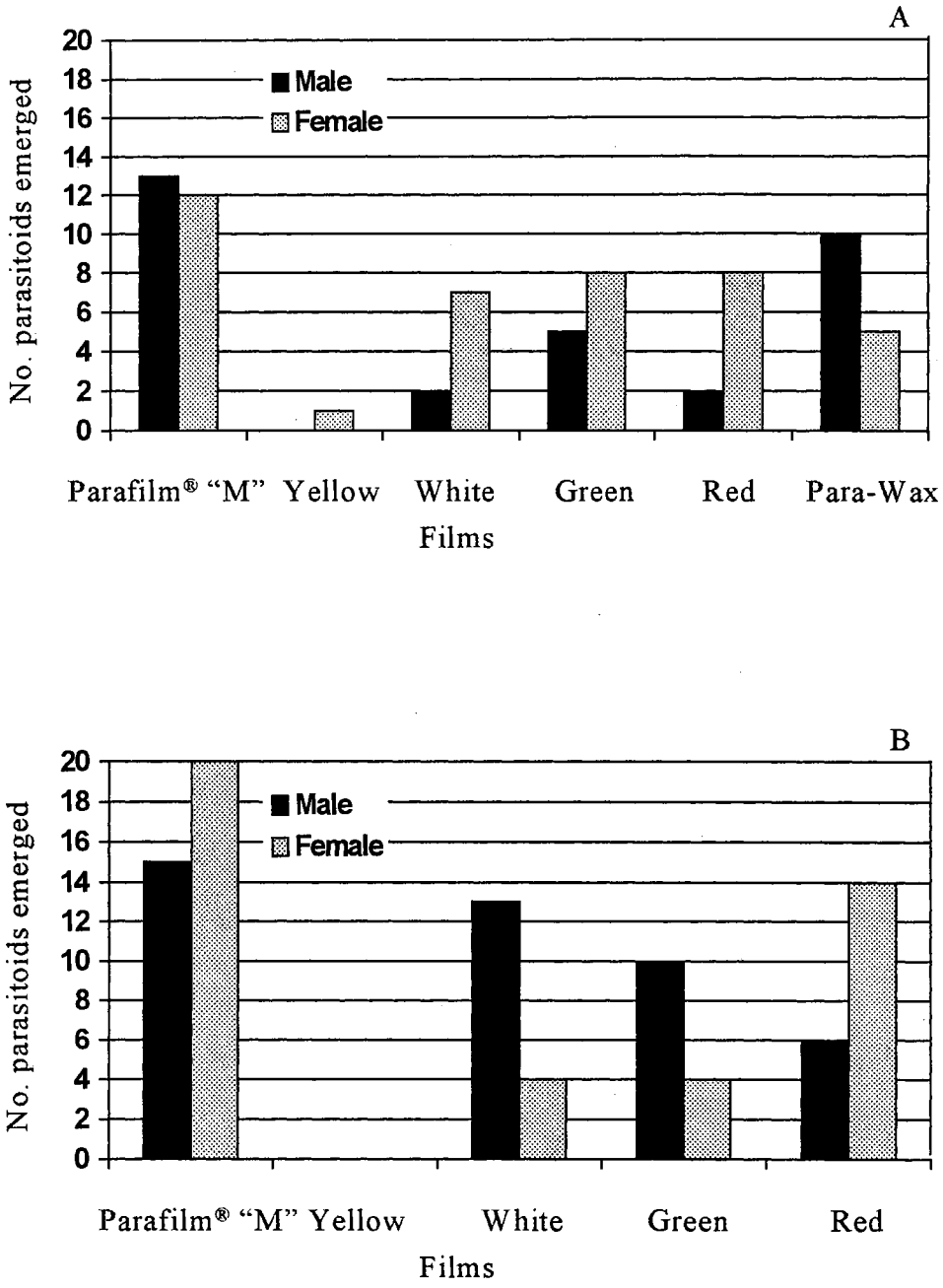

Fig. 1 - Emergence of *Catolaccus grandis* adults from colored beeswax films: (A) choice and (B) no-choice trials.

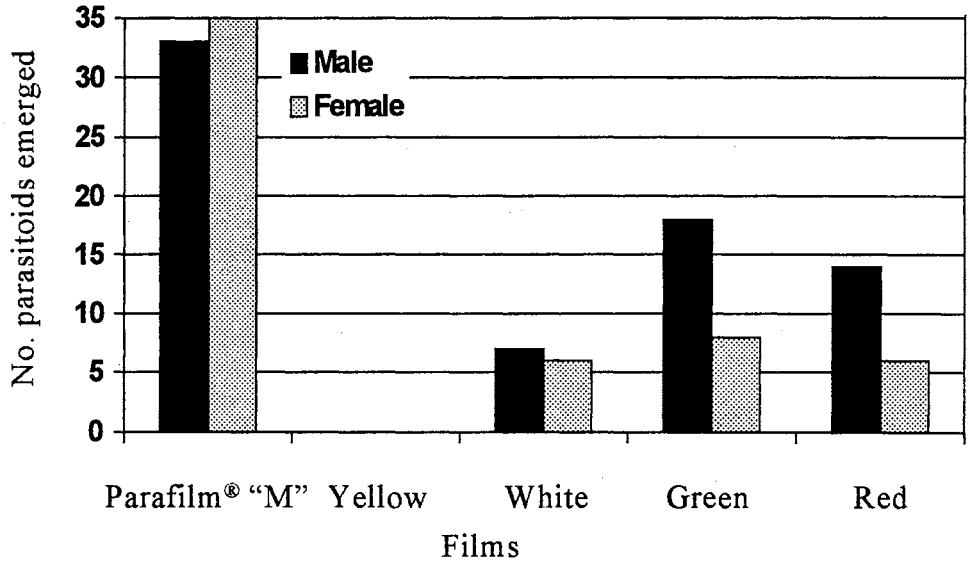

Fig. 2 - Emergence of *Bracon thurberiphagae* adults from colored beeswax films: choice trial.

CHAPTER III

USE OF CARNAUBA (*COPERNICIA CERIFERA* ARRUDA CAMARA) WAX IN REARING PARASITIC HYMENOPTERA FOR BIOLOGICAL CONTROL

ABSTRACT

This is the first report of rearing the parasitoid *Catolaccus grandis* Burks on *Anthonomus grandis* Boheman larvae using carnauba (*Copernicia cerifera* Arruda Camara) wax as a host containment cell. It is concluded that the combination of carnauba wax and beeswax in the Waxfilm (Pat. Pend.) process is suitable for rearing *C. grandis* in the laboratory for biological control purposes. This study reveals that beeswax can be mixed with up to 20% carnauba wax for an effective film. About 23% of the cells in Parafilm® "M" become contaminated by *Aspergillus niger*. This study shows that these new films are not good substrates for such contamination. New films could have a large impact on biological control programs in developing countries, where the availability of techniques using cheap, locally available materials is very important to successful implementation. This would also provide increased income for indigenous beekeepers and carnauba growers. Use of carnauba film in the rearing of other parasitoids is discussed.

RESUMEN

Este es el primer reporte de creación del parasitoide *Catolaccus grandis* Burks en larvas de *Anthonomus grandis* Boheman usando cera de carnauba (*Copernicia cerifera* Arruda Camara) como una celda huépede de acomodación. Se concluye que la combinación de cera de carnauba y cera de abeja en el processo de Waxfilm (Pat. Pend.) es apropiado para la creación de *C. grandis* en laboratorio con el objectivo de control biológico. Este estudio revela que la cera de abeja puede ser mezclada hasta un 20% con cera de carnauba para la obtención de una película eficiente. Aproximadamente 23% de las celdas de Parafilm® "M" se contaminan de *Aspergillus niger*. Esta investigación muestra que estas nuevas películas no son buenos sustratos para tal contaminación. Nuevas películas poden tener un grand impacto en programas de control biológico en paises en vías de desarrollo, donde la disponibilidad de técnicas que usan materiales materiais locales y baratos es muy importante para una implementación eficiente. Esto también promovería el aumento del salario para los apicultores de la región y para los plantadores de carnauba. El uso de películas con carnauba para la producción de otro parasitoides es discutido.

INTRODUCTION

One of the great challenges for cotton growers is controlling the cotton boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae). This pest is responsible for almost 40% of the insecticide used in the United States (Davich 1984). Besides chemical control, other actions such as cultivar selection, cultivar practices, planting date, and biological control (Ramalho and Wanderley 1996) are taken based on the principles of Integrated Pest management (IPM).

The use of parasitoids has been reported as an efficient tool in controlling the boll weevil (Cate 1985), especially *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae), which is the most promising (Morales-Ramos and Cate 1992a; 1992b). Several methods for rearing *Catolaccus grandis* in the laboratory have been proposed (Table 1). Araujo et al. (1993) found that *C. grandis* caused 72% parasitism in laboratory conditions, using 3rd instar boll weevil larvae encapsulated in Parafilm® "M", according to the method proposed by Cate (1987). Guerra (1992) and Guerra et al. (1993; 1994) studied *in vitro* rearing of *Bracon mellitor* and *Catolaccus grandis* with artificial diets containing insect haemolymph. The percentage of adult emergence obtained with parasitoids reared *in vivo* on *A. grandis* larvae, on semi-artificial diet retained with cotton fabric pads, and on diets with 0.7% agar were 50, 28 and 60%, respectively. The higher emergence from agar diet is not enough to justify the higher cost. Also, the parasitoid needs host exposure to maintain genetic vigor.

The ectoparasitoid *C. grandis* (Burks) has the ability to repress and maintain the cotton boll weevil infestations at sub-economic levels when properly augmented in sufficient quantities, particularly during times in which the first and second host generations develop in cultivated cotton fields (Summy et al. 1995). Inundative releases of *C. grandis* (500 to 1,000 females per acre per week) to control the boll weevil has been recorded as an effective method in the United States (Summy et al. 1994). Morales-Ramos et al. (1995) found that augmentative releases of *C. grandis* suppressed boll weevil populations during 1992 and 1993 in cotton fields in the Lower Rio Grande Valley, Texas. They found that boll weevil survival (from egg to adult) in the control cotton fields ranged from 72.8 to 78.2%, while in the cotton fields tested with augmentative releases of *C. grandis*, the boll weevil survival was only 0.5 - 11.8%.

Carnauba wax is a plant wax obtained from carnauba wax palm (*Copernicia cerifera* Arruda Camara), which is native to Northeastern Brazil. Carnauba wax has been used as a natural preservative (reducing weight loss) in fruit coatings for post-harvest storage of 'Arkin' carambola (Miller et al. 1993), 'Ankara' pears (Sumnu & Bayindirli 1994), oranges and grapefruits (Hagenmaier & Baker 1994), coconuts (Bruton 1982), and mature-green guavas (McGuire & Hallman 1995). A carnauba wax and beeswax combination have been reported by Raghuvanshi et al. (1992) to be a suitable coating material for controlling the *in vitro* release of the drug salbutanol sulphate. Carnauba wax, combined with beeswax, has also been reported to be safe for use in cosmetics (CIREP 1984). However, it was not known if such combinations were acceptable for biological control purposes. This project was designed to test the hypothesis that beeswax (animal wax) and carnauba wax (plant wax), waxes readily available in Brazil, can provide

alternative films for rearing parasitoids. It is important to note that carnauba wax is available when beeswax may be scarce. Also, the consistency of carnauba is much harder, and mixtures may have beneficial effects. Additionally, the secondary chemicals in carnauba may have parasitoid attracting or fungus inhibiting properties. The objective of this study was to evaluate the use of carnauba wax as substrate for mass rearing of parasitic hymenoptera for biological control purposes.

MATERIAL AND METHODS

All studies were conducted in the laboratory at 26°C and 80% relative humidity.

Parasitoids: The parasitoids *Catolaccus grandis* were obtained from the USDA/APHIS in Mission, Texas, and had been cultured in the laboratory by using third instar boll weevil larvae encapsulated in Parafilm® "M" according to the technique proposed by Cate (1987).

Host: Third instar boll weevils (*Anthonomus grandis* Boheman) were used as hosts. The boll weevil larvae were provided by the USDA/ARS in Mississippi State, Mississippi, and had been cultured on artificial diet as described by Sikorowski et al. (1984). Larvae were received and placed in a chamber at 22°C and 73% RH until the larvae reached the 3rd instar. Those larvae not used immediately were kept in a cold chamber at 10°C to be used the next day.

Host sheet: The host sheets were made of Parafilm® "M" and Waxfilm (Pat. Pend.) according to the methods proposed by Cate (1987) and Aquino et al. (1997) respectively. The Waxfilm (Pat. Pend.) was made of beeswax with 0, 10 and 20% carnauba wax. The treatments used were: 1. Parafilm® "M" (control), 2. Waxfilm (Pat. Pend.); 3. Waxfilm - 10% carnauba; and 4. Waxfilm - 20% carnauba.

Parasitism: The host sheets were placed in a 100 x 15 mm petri dish with one male and 6, 8 or 10 gravid female parasitoids (5-10 day old). Each sheet contained 20 cells with one boll weevil larvae in each. The sheets were exposed to a 24 h parasitism.

The petri dishes were kept in a growth chamber (Percival, model # 1-35 LVL, 115 volts, 60 Hz) at 26°C, 80% RH, with a 12L:12D, for 24h parasitism. After parasitism, the sheets were removed and placed into new plastic petri dishes (100 x 15mm) for emergence at the same temperature, relative humidy and photoperiod.

The experimental design used was a Randomized Complete Block Design (RCBD), with 9, 7, and 6 days as the blocks with 6, 8, and 10 females, respectively. The 4 films were the treatments. Three RCBD's were used for the 3 different female-day combinations. The total emergence of *Catolaccus grandis* was measured and a square root transformation was applied to alleviate problems associated with count data (Steel and Torrie 1980, p. 234). If there was a statistical difference among the four films, then Fisher's Protected Least Significant Difference (LSD) procedure was used to make pairwise comparisons - the best film having the higher emergence average. SAS® for Windows™ (SAS Institute 1996) was used to analyze the data.

RESULTS AND DISCUSSION

Some characteristics (comparisons) between beeswax, carnauba wax, and mix films are presented in Table 2. We noticed during our study that a mixture of beeswax and carnauba wax is suitable when carnauba wax does not exceed 20%. Films that have more than 20% of carnauba breaks easily when handled at room temperature (20-25°C) and parasitoids have difficulty in both parasitizing and emerging.

It was found that the positive parasitoid reaction to the carnauba wax incorporated in Waxfilm (Pat. Pend.), as shown by increased searching and probing behavior, is clearly seen on both Waxfilm (Pat. Pend.) and its mixtures. Parafilm® "M" did not differ statistically from Waxfilm (Pat. Pend.) and its carnauba mixtures (10 and 20%) in terms of parasitoid emergence when fewer than 8 females were used (Table 3). When films were exposed to 8 or 10 females (Table 4 and 5), there was a statistical difference in the number of parasitoids emerged, with the higher parasitism in Parafilm® "M" (Fig. 1 shows a graph of the means of the parasitoid emergence for the different numbers of females per trial). As the number of parasitoids was increased from 6 to 8 and 10, a trend of higher emergence on Parafilm® "M" became evident. This may be due to the observed trait of parasitoid larvae crawiling between layers of Parafilm® "M" and reaching unparasitized boll weevil larvae. The oviposition caused by the excess of females would provide a large pool of mobile larvae. This inter cell migration was not observed for Waxfilm (Pat. Pend.) and its derivatives. layers to an unocupied host. Plans are underway to redesign Waxfilm

(Pat. Pend.) sealing techniques to allow such migration. Superparasitism was not investigated in this research, but Morales-Ramos and Cate (1992a; 1992b) report an average of 1.9 eggs per larvae. Because *C. grandis* females have a tendency of superparasitize boll weevil in laboratory conditions, even though not all larvae are used, either host searching was inefficient, or some larvae were unavailable or unattractive to the female parasitoids. This may be due to the developmental stage of the host larvae. Even though Parafilm® "M" gives similar results to the alternative films, it is still an expensive film in comparison to those wax based presented in this study. Carnauba wax makes the Waxfilm (Pat. Pend.) better by retaining adult weevils. This will reduce the number of adult boll weevils released to the cotton fields when this system is implemented. In case beeswax availability becomes scarce for making Waxfilm (Pat. Pend.), carnauba wax can be added (up to 20%) and parasitoids can be reared. Considering that carnauba wax and beeswax have similar prices in the Brazilian market (around $ 6.00 a kilo), the addition of carnauba wax into Waxfilm (Pat. Pend.) would not alter its final cost. The results of this study indicate that carnauba now has a new purpose for use which consequently will aid the carnauba growers in Northeastern Brazil. It must be pointed out that the favorable response of parasitoids to plant and animal waxes constitute evidence that this parasitoid, and others as well, should be tested by using other natural waxes and oils available in nature. Thus, the efficiency of artificial rearing *C. grandis* and other beneficial insects might be increased by the use of alternative waxes. For this reason, additional investigations are needed.

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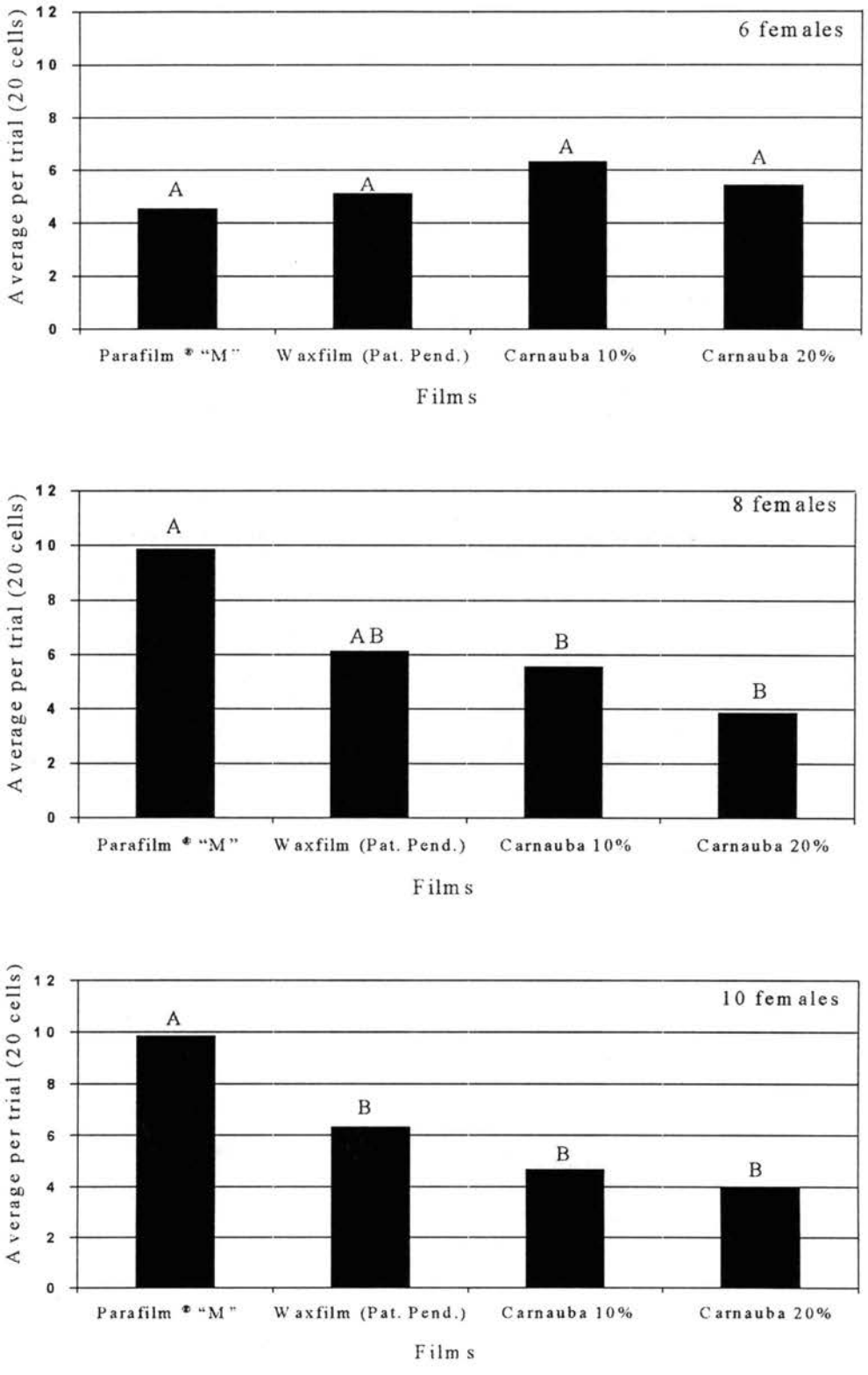

Fig. 1 - Emergence of *Catolaccus grandis* adults from alternative films with 6, 8, and 10 ovipositing females per trial.

TABLE 1

TECHNIQUES FOR REARING *CATOLACCUS GRANDIS* AND OTHER PARASITOIDS ARTIFICIALLY IN LABORATORY FOR BIOLOGICAL CONTROL PURPOSES

Technique	Developed by
Parafilm® "M"	Cate, 1987
Diet	Guerra, 1992; Guerra et al. 1993 and 1994; Guerra and Martinez 1994
Parafilm® "M" (adaptation)	Ramalho and Gonzaga, 1991
Waxfilm (Pat. Pend.)	Aquino et al., 1993
Colored beeswax	Aquino et al., 1997

TABLE 2

SOME COMPARISONS BETWEEN BEESWAX, CARNAUBA WAX, AND MIXED FILM

Characteristics	Beeswax	Carnauba wax	Mixture (10-20%)
Source	Animal	Plant	Animal and Plant
Flexible	Yes	No	Semi flexible
Moldable	Yes	No	Semi moldable
Self-sealing	Yes	No	Yes
Odor	Typical (beeswax)	No	Typical (beeswax)
Moisture-resistant	Yes	Yes	Yes
Transparency	Semi	No	No
Color	Colorless	White-yellow	Light white-yellow
Melting point	60°C	80°C	70°C
Hardness	Soft	Hard	Semi hard

TABLE 3

LSD RESULTS FOR THE EMERGENCE OF *CATOLACCUS GRANDIS* ADULTS ON ALTERNATIVE FILMS WITH 6 OVIPOSITING FEMALES PER TRIAL

Films	Means	Grouping	S.D
Parafilm® "M"	4.556	a	2.6034
Waxfilm (Pat. Pend.)	5.111	a	3.4075
Carnauba 10%	6.333	a	1.8028
Carnauba 20%	5.444	a	1.8780

Means followed by the same letter are not significantly different (P>0.05; SAS® for Windows™ 95, SAS PROC GLM,; N=9, T=36); LSD performed on square root - transformed data. Means and S.D. displayed are raw values.

TABLE 4

LSD RESULTS FOR THE EMERGENCE OF *CATOLACCUS GRANDIS* ADULTS ON ALTERNATIVE FILMS WITH 8 OVIPOSITING FEMALES PER TRIAL

Films	Means	Grouping	S.D
Parafilm® "M"	9.857	a	2.478
Waxfilm (Pat. Pend.)	6.143	ab	2.116
Carnauba 10%	5.571	b	4.791
Carnauba 20%	3.857	b	2.193

Means followed by the same letter are not significantly different (P>0.05; SAS® for Windows™ 95, SAS PROC GLM,; N=7, T=28); LSD performed on square root - transformed data. Means and S.D. displayed are raw values.

TABLE 5

LSD RESULTS FOR THE EMERGENCE OF *CATOLACCUS GRANDIS* ADULTS ON ALTERNATIVE FILMS WITH 10 OVIPOSITING FEMALES PER TRIAL.

Films	Means	Grouping	S.D
Parafilm® "M"	9.833	a	1.722
Waxfilm (Pat. Pend.)	6.333	b	1.033
Carnauba 10%	4.667	b	3.724
Carnauba 20%	4.000	b	2.191

Means followed by the same letter are not significantly different (P>0.05; SAS® for Windows™ 95, SAS PROC GLM,; N=8, T=32); LSD performed on square root - transformed data. Means and S.D. displayed are raw values.

CHAPTER IV

EMERGENCE BEHAVIOR OF *CATOLACCUS GRANDIS* BURKS (HYMENOPTERA: PTEROMALIDAE) FROM HOST CONTAINMENT CELLS IN THE LABORATORY

ABSTRACT

Emergence behavior of the ectoparasitic wasp *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae) from larvae of the cotton boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) was assessed by using artificial rearing procedures. The methods proposed by Cate (1987) for Parafilm® "M", and by Aquino et al. (1993) for Waxfilm (Pat. Pend.) were used. The length of time to emergence from both cells was determined at 26°C and 80% RH. After eclosion, the emergence time of *C. grandis* from Waxfilm (Pat. Pend.) was 1/6 of the time from Parafilm® "M". Also, the emergence hole on Waxfilm (Pat. Pend.) is a round single hole, very similar to the natural escape hole from cotton squares. However, the emergence holes on Parafilm® "M" were very diverse, varying in shapes and in numbers (1 to 7 holes per cell). Determination of morphological differences in emergence holes was made. A key for identifying *C. grandis* and *A. grandis* emergence from Waxfilm (Pat. Pend.) is provided.

RESUMEN

El comportamiento de emergencia de la vespa ectoparasítica *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae) de larvas del picudo del algodonero *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) fue evaluada usando processos de creación artificiales. Los métodos propuestos por Cate (1987) para Parafilm® "M", y por Aquino et al. (1993) para Waxfilm (Pat. Pend.) fueron usados. El lapso de tiempo para emergencia de ambas las celdas fue determinado con 26°C y 80% RH. Después de la eclosión, el tiempo de emergencia de *C. grandis* del Waxfilm (Pat. Pend.) fue 1/6 del tiempo de emergencia del Parafilm® "M". También, el orificio de emergencia en el Waxfilm (Pat. Pend.) es único y circular, muy parecido con el orificio natural de emergencia en botones florales de algodón. Además, los orificios del emergencia en Parafilm® "M" fueron muy diversos, variando en forma y en número (1 hasta 7 orificios por celda). Determinaciones de las diferencias morfológicas en los orificios de emergencia fueron investigados. Un elemento clave para identificar la emergência de *C. grandis* y *A. grandis* de Waxfilm (Pat. Pend.) es providenciado.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) production in Brazil was adversely affected soon after the appearance of the cotton boll weevil, *Anthonoums grandis* Boheman (Coleoptera: Curculionidae) in 1983 (Sobrinho & Lukefahr 1983). The appearance of the boll weevil caused many farmers in this economically deprived region to become bankrupt, a fact revealed by an 80% decline in cotton production in the Northeastern region (Matthews 1988).

Emphasis has been placed on Integrated Pest Management (IPM), especially in the use of electrodynamics sprayers (Electrodyn® system) and use of parasitoids for augmentation in cotton fields. The parasitoids used by the National Center for Cotton Research (CNPA-EMBRAPA), have been *Catolaccus grandis* and *Bracon* sp. These parasitoids have been reared using Parafilm® "M" as a host containment cell for third instar boll weevil larvae (Cate 1987). Waxfilm (Pat. Pend.) is also used as an alternative film for rearing parasitoids (Aquino et al. 1993). Due to the high cost for Parafilm® "M" importation, Aquino et al. (1996, 1997) found that Waxfilm may be a plausible alternative film to use in Brazil, especially in the Northeastern region where farmers cannot afford expensive pest control measures.

Precise assessment of behavioral aspects in a mass rearing program of any beneficial insect is a valuable tool for continued improvement of their manipulation in the laboratory. Under natural conditions, careful examination of holes in cotton squares, and

square contents (cocoons, dead bodies, etc.) may indicate boll weevil mortality factors (Sturm and Sterling 1986; Ramalho and Gonzaga 1991; Ramalho et al. 1993), such as predator and parasitoid attacks. After inundative releases of *C. grandis* against the cotton boll weevil in the Lower Rio Grande Valley, Texas, Morales-Ramos et al. (1995), estimated parasitism by observing the densities of emergence holes from cotton (*G. hirsutum* L.) floral buds (squares) and bolls by the parasitoid or host.

Because Waxfilm (Pat. Pend.) has been used recently for rearing *C. grandis* in the laboratory (Aquino et al. 1996) and has a potential for future use in Brazil, it is important to study some behavioral aspects of *C. grandis* when reared in this film. The objective of this study was to determine the adult post eclosion emergence time of *C. grandis* from different films, and based on the shape, size, and number of escape holes, propose a key for identification of the emerged insects.

MATERIALS AND METHODS

In this study, two films were used: Parafilm® "M" and Waxfilm (Pat. Pend.). Each film contained 70 cells with immature stages of *Catolaccus grandis* on parasitized larval boll weevils (*Anthonomus grandis* Boheman). The parasitism was performed according to the procedures suggested by Cate (1987). In order to observe more closely the emergence behavior of the parasitoids, a CCD-IRIS color video camera, Sony®, model No. SSC-C374 (AC 24 V 50/60 Hz 4.9W) was used, plugged into a 20" Panasonic TV, model No. CT-2083Y (120 V 60 Hz) and recorded by a JVC video cassette recorder, model No. HR-J610U (AC 120 V~60Hz 19W). Two components were measured: first, the time required to emerge from the cells, and second, the shape and size of emergence holes made by the parasitoids.

Emergence Time Technique: A Completely Randomized Design (CRD) was used to analyze the data. Male and female were compared within the two films (Parafilm® "M" and Waxfilm (Pat. Pend.) and male to male, and female to female, between the two films. The level of significance used was 0.05, using Fisher's LSD. The percentage of parasitoids emerging from the films was measured. The health of the parasitoids was also evaluated after they emerged from the holes, by recording their walking, flying, and mating behavior using a video camera.

External Diagnostic of Emergence Holes: After placing the 3rd instar boll weevil larvae into the cells, films were placed in a cage measuring 45.8 cm x 25.5 cm x 48.5 cm, and the

cage placed within a growth chamber (Percival, model # 1-35 LVL, 115 volts, 60 Hz) with 50-70 gravid *Catolaccus grandis* females (5 days and older), at 26°C, 80% relative humidity (Morales-Ramos and Cate 1992), for 24h parasitism. After parasitism, the sheets were removed and placed into plastic petri dishes (100 x 15mm) for emergence.

RESULTS AND DISCUSSION

Post eclosion emergence time from the rearing films differs between male and female. *Catolaccus grandis* made only a single round emergence hole in all Waxfilm (Pat. Pend.) cells while in Parafilm® "M" the parasitoids made a number of holes (varying from one to eight) of asymmetric shapes. The duration of time from eclosion to emergence from Parafilm® "M" cells varies from 10 minutes (one hole) to two days (7 holes). The time for emergence from Waxfilm (Pat. Pend.) varies from 1 to 4 hours. The shape of the emergence hole using Waxfilm (Pat. Pend.) is a single round hole measuring 0.8-1.4 mm, very similar to those found in cotton squares under natural emergence behavior conditions of *C. grandis* (Ramalho et al. 1993). The smaller holes are generally made by males, while the larger holes are made by females. Interestingly, out of 100 Waxfilm (Pat. Pend.) cells, 65 of the parasitoids (males and females) emerge from Waxfilm (Pat. Pend.) in a specific location: the corner of the cell. Not surprisingly, this location is the weakest point in the film. My discussion with Drs. Eduardo Missawa and Hongbing Lu (OSU, Mechanical and Aerospace Engineering) suggests that this is due to three mechanisms: 1) Substantial strength reduction due to the Waxfilm (Pat. Pend.) edge damages such as voids and micro-cracks developed during the formation of the indentation (by pressing the film into the mold); 2) Concentration of residual stress caused by abrupt cross section changes of the structure; and 3) Local wall-thickness reduction of the cell in that area. Parafilm® "M", however, displayed different types of holes of different sizes (Fig. 1). By looking at the surface of the Parafilm® "M" cell we can see that the parasitoid struggles

hard to escape (emerge). That is not the case with Waxfilm (Pat. Pend.), in which the parasitoid make a single hole to escape.

Assessement of Morphological Differences of Emergence Holes: After parasitism, it was observed that in both films males start emerging earlier than females. In Parafilm® "M", when temperature was 26°C, males emerge 13 days after parasitism, and females 15 days after parasitism. This result was similar to those found by Morales-Ramos & Cate (1993), at 27°C. However, both males and females emerge six times faster from Waxfilm (Pat. Pend.) than from Parafilm® "M" (Fig. 2 and 3). When parasitoids pupation is finished, *Catolaccus grandis* chew emergence hole in cells approximately the same size as its body. Males make a single round escape hole (0.8-1.0-1.4 mm). Females also make a single round escape hole (1.1-1.4 mm). Waxfilm (Pat. Pend.) prevents the host from escaping in three stages of the process. First, it prevents the larvae from escaping before parasitism. The thickness of the film (two-three times thicker than Parafilm® "M") makes break through more difficult; second, the Waxfilm (Pat. Pend.) prevents breaking through and escaping because of its suitability for chewing, which holds the host until it is stung by the parasitoid. When the third instar boll weevils are removed from the diet to the film, they still may have some feeding activity, attempting to chew the films. Because Waxfilm (Pat. Pend.) is more chewable than Parafilm® "M", this may entice the boll weevil larvae to stay longer inside the cell, increasing the probability of being parasitized. Boll weevil larvae were observed to have a circular behavior inside the Waxfilm (Pat. Pend.) cell. This is its natural behavior in the cotton square (Ramalho et al. 1993), when the larvae tries to enlarge a place in the square (Sturm and Sterling 1986) for its pupation and eclosion.

Chewing and circling behavior are less frequent in Parafilm® "M". Third, Waxfilm (Pat. Pend.) prevents adult boll weevils from escaping from the cells when parasitism is not completed. Waxfilm (Pat. Pend.) traps the boll weevil adult in the region between the thorax and the abdomen, and it eventually dies. Considering that the mass rearing of parasitoids will eventually involve shipping the rearing cells to the cotton fields, this technique gives more assurance that no viable boll weevil adults will be shipped along with parasitoids to cotton fields. It was observed that in Waxfilm (Pat. Pend.), in the absence of parasitism, 80% of unparasitized boll weevils died inside the film. The treatment using 20% of carnauba wax resulted in 97% of unparasitized boll weevils dying without escaping from the cells (Fig 4). Boll weevils that make a hole in the surface of the film or get stuck on their way out (Fig. 5). The 3% of boll weevils that do escape, make a single round hole (3 mm) [Fig. 6] or a irregular one (4 x 2 mm) [Fig. 7], generally on the corner of the cell. After emergence, still in the petri dish, boll weevils, apparently attempting to feed, may punctuate the Waxfilm (Pat. Pend.) cell with a single hole (Fig. 8) or several holes (Fig. 9). The boll weevil may also puncture the cell it just emerged from as well as cells where *C. grandis* emerged (Fig. 10 and 11).

Using this proposed key (Table 1), it is possible to identify precisely the exact number of cells that yield parasitoids, the sex ratio of the parasitoids, cells that did not emerge, as well as the exact number of boll weevils emerged when parasitism was not completed. If cells are found that show boll weevil emergence, some measures can be taken in order to increase parasitism and more closely monitor cell construction. Good rearing practices aim to decrease or prevent undesirable insects in the rearing cages. Some

of these action may include: checking the age of boll weevil larvae before parasitism (3rd instar is desirable), time of exposure for parasitism and larvae/parasitoid/time ratio.

A small circular hole ca. 0.8 mm in diameter for male (Fig. 12) and 1.3 mm for female (Fig. 13), are indications that *C. grandis* parasitoids have successfully killed the boll weevil larvae and have emerged from the Waxfilm (Pat. Pend.) cell.

It is important to keep track of the sex ratio of the parasitoids in mass production of *C. grandis* or of any other parasitoid. Sex ratios obtained during mass production of *C. grandis* on Waxfilm (Pat. Pend.) can be easily assessed by the proposed key (Table 1). In a cage with a 1,000 emerged parasitoids, for instance, it is not practical to count the insects one by one to check the sex ratio. We also noticed that even sampling the parasitoids in the cage by using the insect aspirator, parasitoids get hurt, and that is not a good practice. However, by following the proposed key, observing the diagnostic features of the types of emergence holes on Waxfilm (Pat. Pend.), one can easily know the sex ratio of the culture.

Even though there is no information in the literature about the time of emergence of *C. grandis* on cotton squares, the single hole this parasitoid makes naturally (Ramalho et al. 1993) may indicate that it does not have trouble in getting out of the cotton square. This quick escape of parasitoids from the Waxfilm (Pat. Pend.) - one single hole like in nature - gives us a certainty that the parasitoids are not hurt when struggling to escape from the cells. No apparent sexual behavior differences were shown by parasitoids reared in Parafilm® "M" and Waxfilm (Pat. Pend.), and sex ratios obtained from both films were close to 1:1. Males were sexually active immediatelly following emergence. When a male

finds a female, it starts constantly moving its wings very fast for 1-12 seconds and swaying left and right. This stops immediately before mounting on the thorax of the female. Then, it stays quiet until the appropriate moment, when it bends under the female abdomen for the 'sex act', which lasts 3-10 seconds. In summary, adults of both sexes exhibited normal physical characteristics as well as normal locomotion (walking, flying and jumping) and sexual behavior (copulation and laying eggs).

We noticed that approximately 35% of all the cells containing larvae did not yield parasitoids because they either were not parasitized and/or dried or were attacked by the black fungus *Aspergillus niger*, also known as black mold. We observed that the fungus *A. niger* attacks primarily the boll weevil larvae in Parafilm® "M" cells. When that happens, it causes the death of the host larvae inside the cell, which makes it non-viable for the success of the parasitism. There were some cases in which the parasitism succeeded, however, the *C. grandis* pupae was affected by the fungus and, consequently, there was no yield of parasitoids. Also, we noticed that when *C. grandis* adults could not emerge from Parafilm® "M" cells, they became weak, died, and eventually became contaminated by the fungus. We did not find *A. niger* in Waxfilm (Pat. Pend.). This may be due to the fact that beeswax has some anti-fungal properties like propolis (Pepeljnjak and Jalsenjak 1984; Grzybowski and Szewczyk 1987; Johnson et al. 1994).

During this study, the presence of the mold mite *Tyrophagus putrescentiae* (family Acaridae) was also observed in the growth chamber. *T. putrescentiae* was found feeding on drops of honey as well as on the dead boll weevils and/or parasitoids (larvae, pupae, adult), which agrees with the feeding behavior described by Harwood and James (1979).

Additionally, the temperature and RH where *C. grandis* colony was kept is the ideal range for the mite *T. putrescentiae* (Boczek 1991). During manipulations of rearing sheets and petri dishes contaminated with *T. putrescentiae* the senior author had a moderate patchy or coalescing dermatitis, probably caused by the presence of mites in the colony, which, according to Harwood and James (1979), may cause this skin allergy.

An understanding of the emergence behavior of *Catolaccus grandis* from host containment cells in laboratory make it possible to manage this and other parasitoids under the same artificial conditions. Additional studies are necessary, however, in order to determine the physiological stress on the parasitoids after several hours of intense activity trying to emerge from the films. Further studies can be focused on the fecundity, vigor, and longevity of the parasitoids after emerging from different films.

ACKNOWLEDGMENTS

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TABLE 1

KEY FOR IDENTIFYING *CATOLACCUS GRANDIS* EMERGENCE
FROM WAXFILM (PAT. PEND.)

1. Hole present	2
Hole absent	no parasitism (host mortality: larvae, pupae or adult) [Fig. 14]
2. Hole circular	3
Hole non-circular/irregular (~ 2 x 4 mm)	boll weevil emergence [Fig. 7]
3. Hole diameter > 0.5 mm	4
Hole diameter (one or more holes) measuring 0.1-0.2 mm	boll weevil feeding [Figs. 8 and 9]
4. Hole diameter < 2mm	5
Hole diameter ~ 3 mm	boll weevil emergence [Fig. 6]
5. Hole ~ 0.8 mm	*Catolaccus grandis* male [Fig. 12]
Hole ~ 1.3 mm	*Catolaccus grandis* female [Fig. 13]

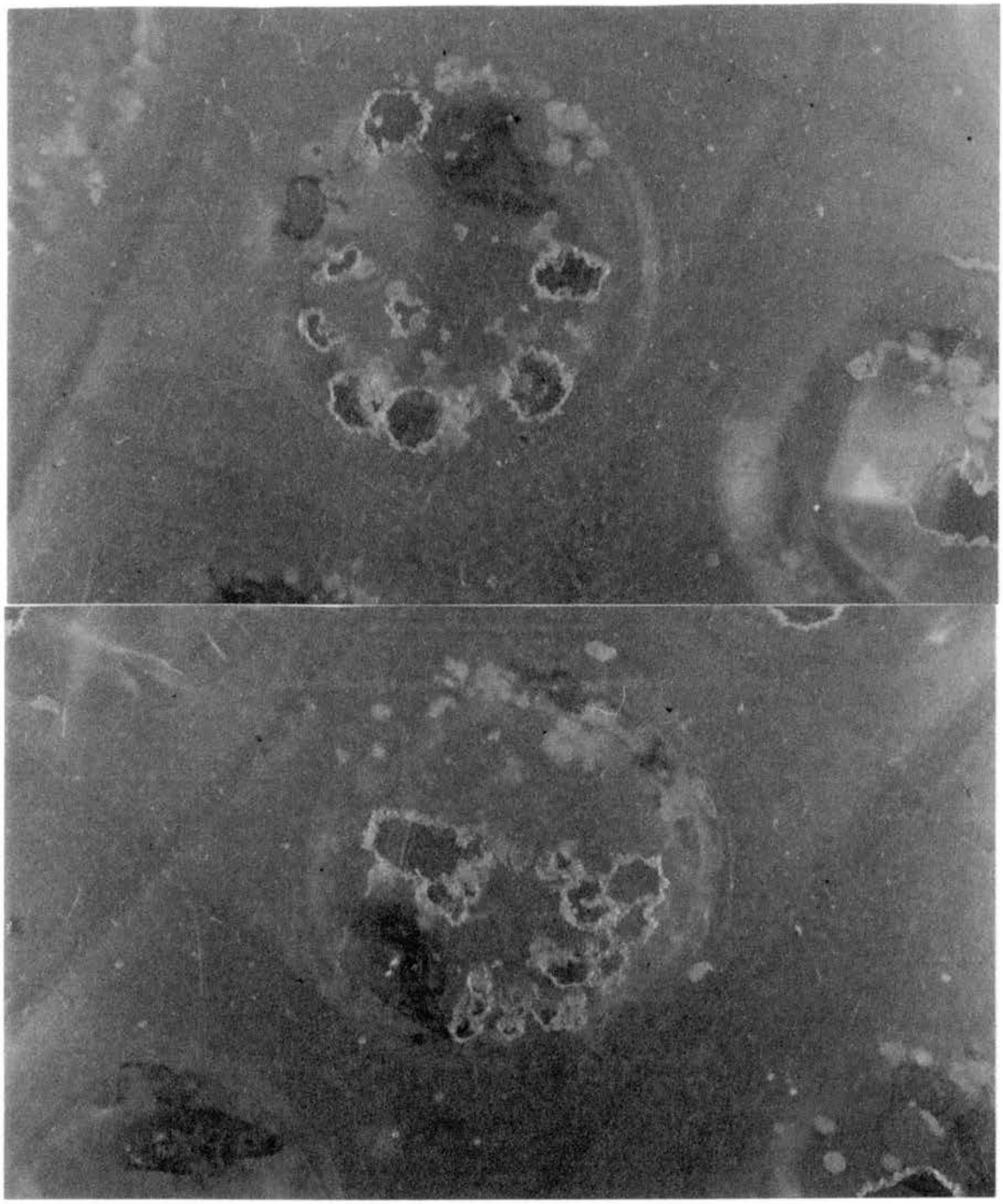

Fig. 1 - Parafilm® "M" showing multiple emergence holes of *Catolaccus grandis*.

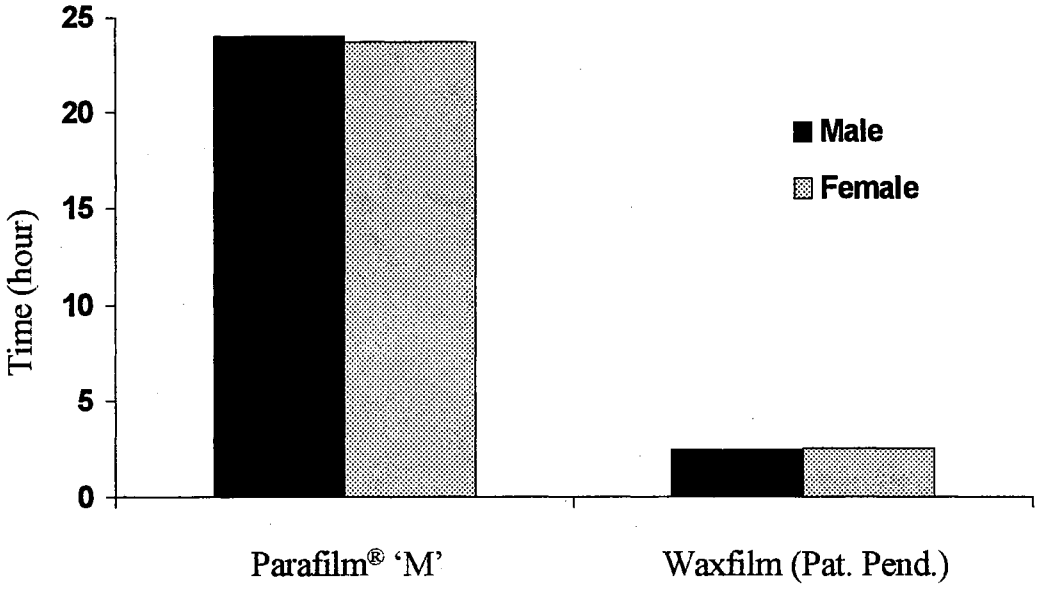

Fig. 2 - Average time of emergence of *Catolaccus grandis*.

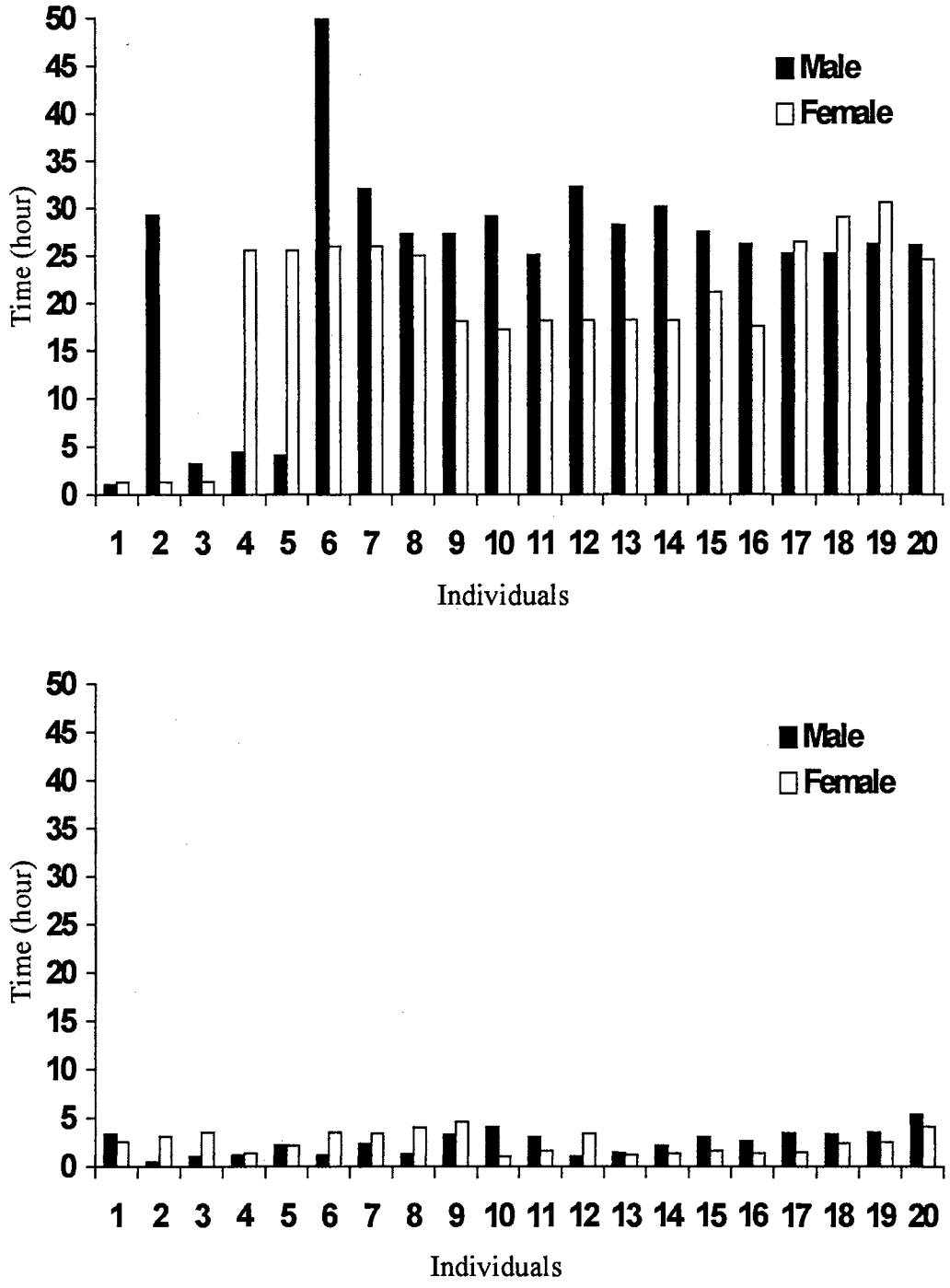

Fig. 3 - Time of emergence of *Catolaccus grandis* from Parafilm® "M" and Waxfilm (Pat. Pend.).

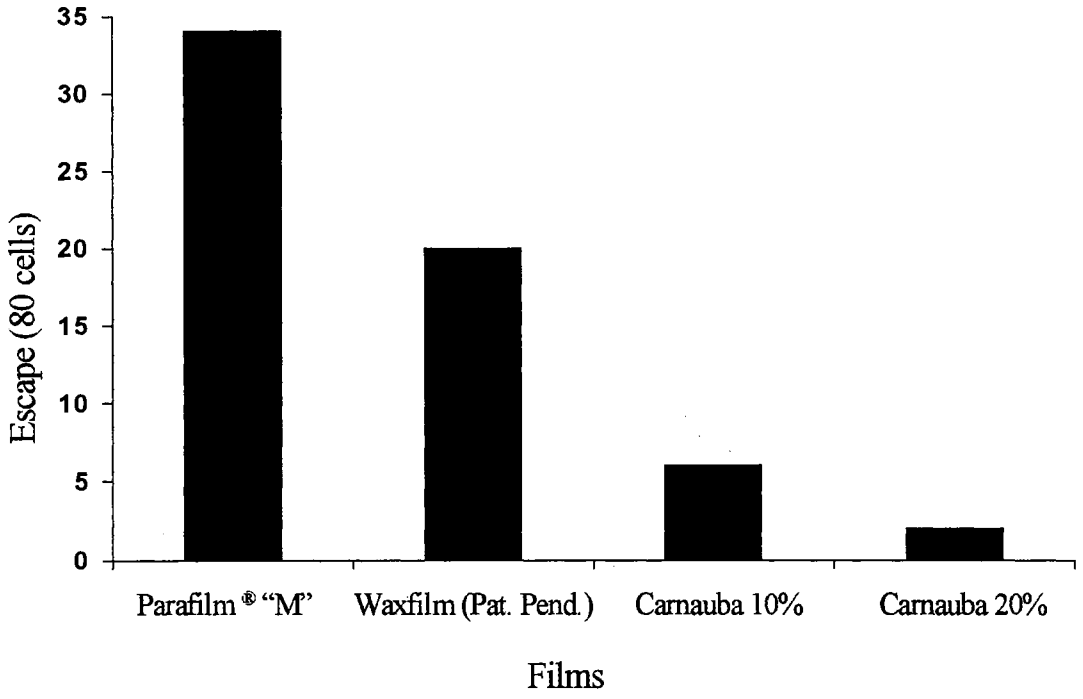

Fig. 4 - Adult boll weevils escaping from films when parasitism has not ocurred.

Fig. 5 - Boll weevil stuck in the Waxfilm (Pat. Pend.) on its way out.

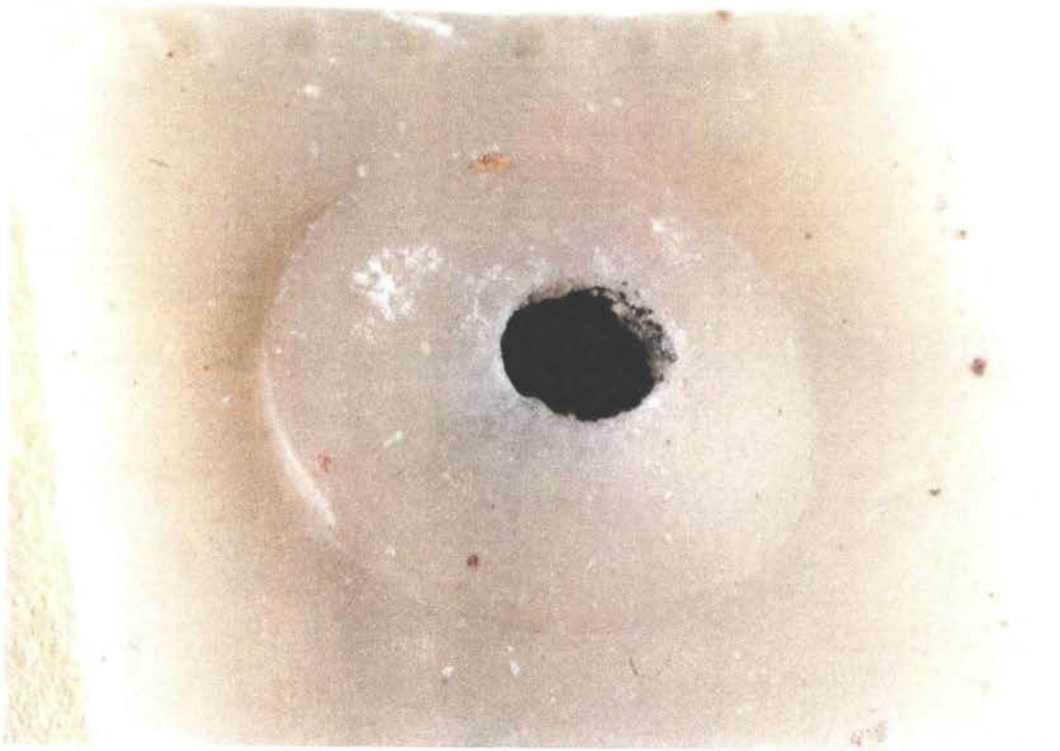

Fig. 6 - Waxfilm (Pat. Pend.) cell with boll weevil round emergence hole.

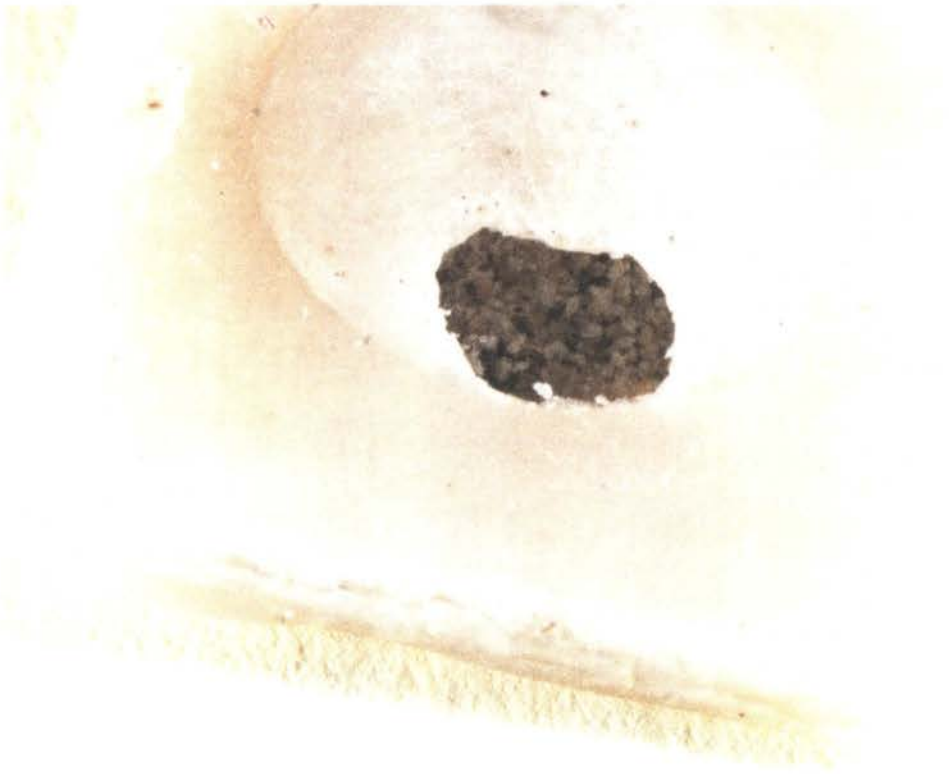

Fig. 7 - Waxfilm (Pat. Pend.) cell with boll weevil irregular emergence hole.

Fig. 8 - Waxfilm (Pat. Pend.) cell with boll weevil feeding hole.

Fig. 9 - Waxfilm (Pat. Pend.) cell with several boll weevil feeding holes .

Fig. 10 - Waxfilm (Pat. Pend.) cell with both feeding and emergence holes of the boll weevil.

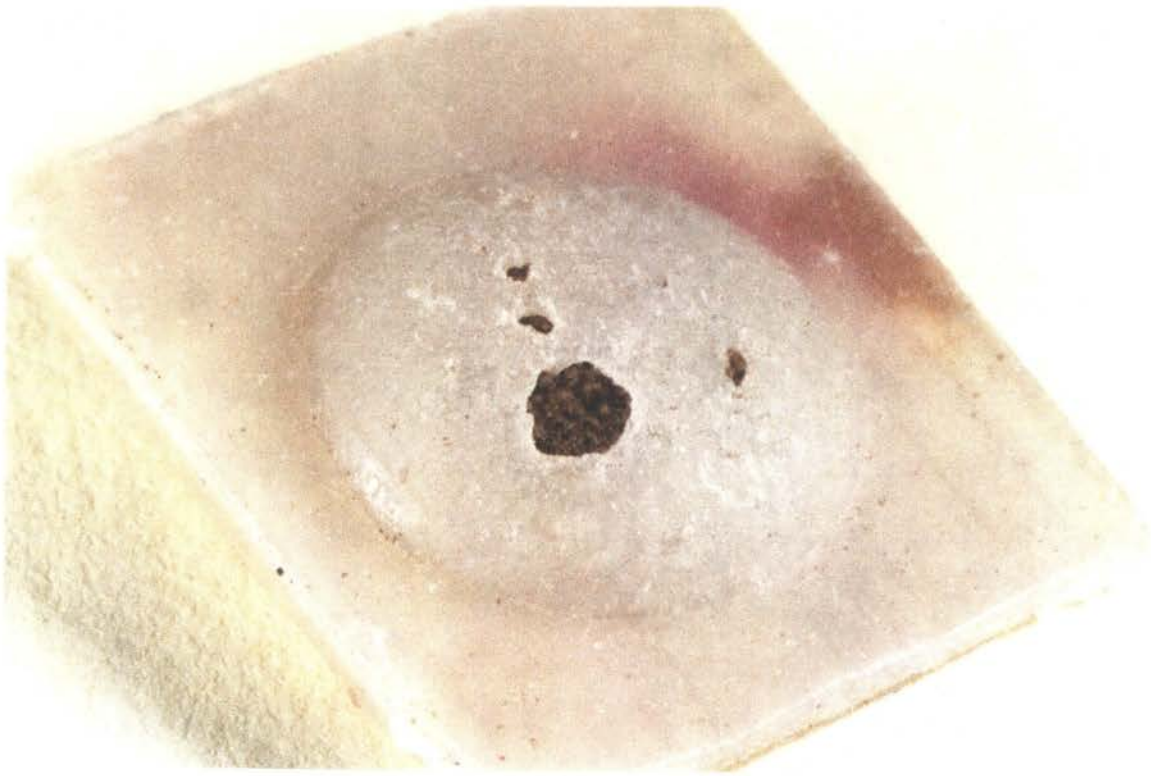

Fig. 11 - Waxfilm (Pat. Pend.) cell with both boll weevil feeding holes and *C. grandis* emergence hole.

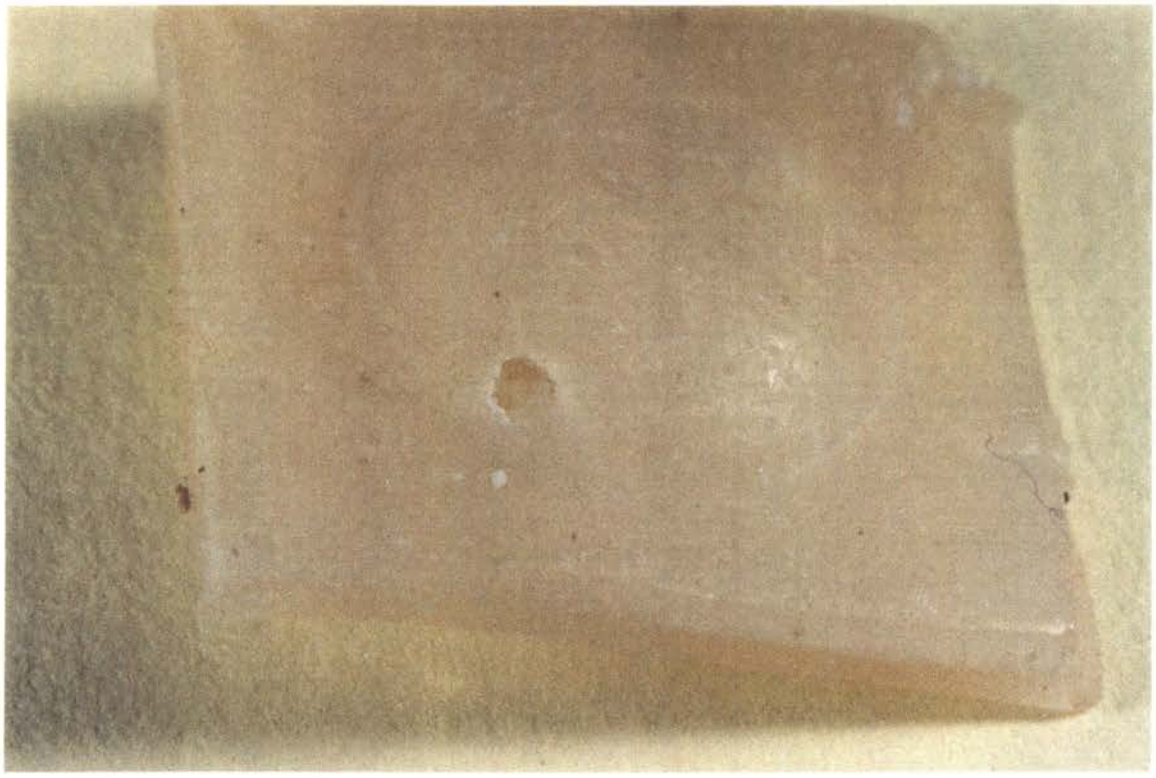

Fig. 12 - Waxfilm (Pat. Pend.) cell with male *C. grandis* emergence hole.

Fig. 13 - Waxfilm (Pat. Pend.) cell with female *C. grandis* emergence hole.

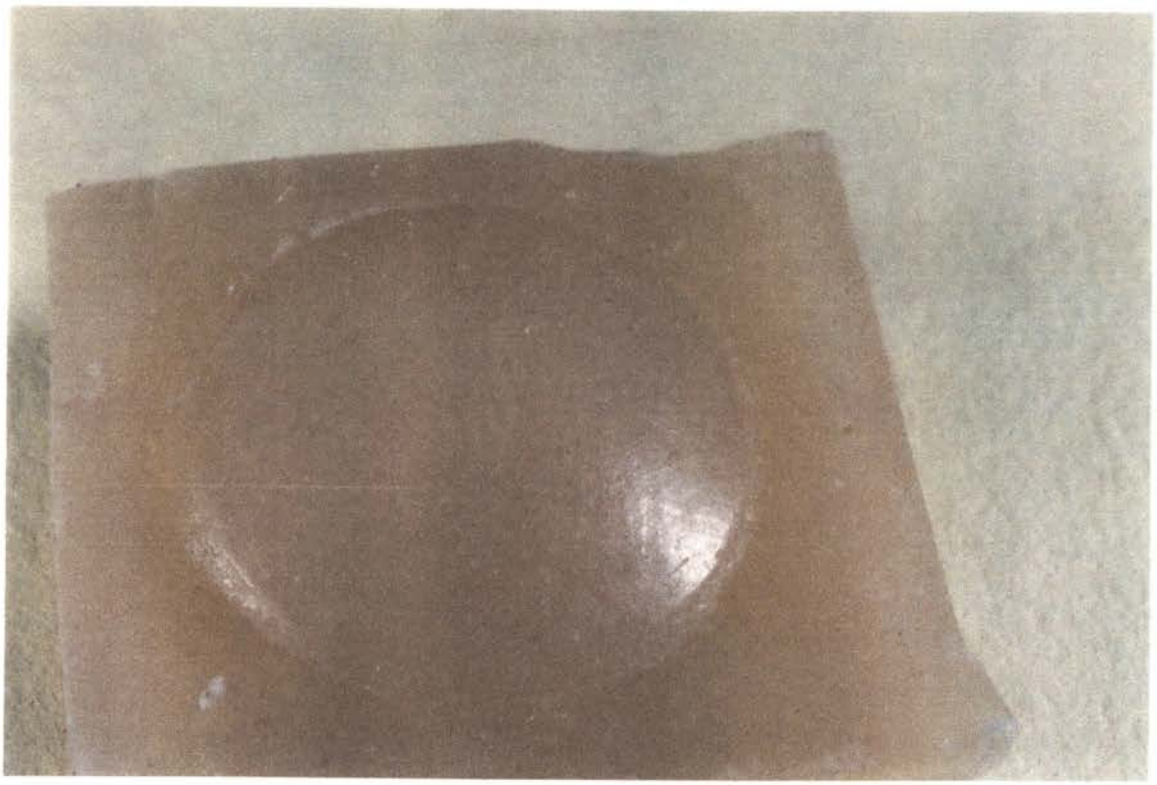

Fig. 14 - Waxfilm (Pat. Pend.) cell without emergence hole.

CHAPTER V

MANAGING HUMIDITY AND DRINKING WATER IN ACRYLIC CAGES FOR REARING PARASITOIDS

SCIENTIFIC NOTES

Catolaccus grandis Burks (Hymenoptera: Pteromalidae) has been one of the most common parasitoids reared in the laboratory for biological control purposes. The standard technique is the one proposed by Cate (1987). A necessary task during the laboratory rearing of *C. grandis* is to maintain the proper high humidity levels, and provide drinking water. Basically, there are two ways to generate humidity and drinking water inside the cage: 1) by leaving a petri dish with water; or 2) spraying with a mister. The problem by leaving a petri dish with water is the significant drowning of parasitoids and consequent reduction of colony size. Spraying water using a manual spray, which is better than the petri dish, still represents a problem - spraying enough to provide drinking water makes for a messy cage and soaked parasitoids. It becomes a difficult task to remove the dead parasitoids with a paper tissue without hurting the healthy ones. It has been observed in our laboratory that by leaving a petri dish (100 x 15 mm) containing water on the top of the cage after spraying inside with a mister, the humidity in the cage tends to concentrate under the petri dish - inside the cage. The difference of temperature condenses water on the inside top of the cage. Eight hours after placing the petri dish on the top of the cage, a round pool of dew can be seen under the petri dish. After 24 hours, a very nicely shaped pool of water will be available for the parasitoids to drink from (Fig. 1). We have noticed that when the parasitoids are drinking from this 'pool' they do not get trapped. This may be because they have a large dry area to land on and can approach the pool in a controlled manner. In our observations, we have not seen any parasitoid death when this

pool is available. Also, because water condensation could be placed in whatever spot desired on the top of the cage, cleaning tasks become easier. The petri dish needs to be permanently on the top of the cage in order to provide a constant dew pool. To better distribute the drinking water for the parasitoids, up to four petri dishes of water on the top of the cage (45.8 cm x 25.5 cm x 45.8 cm) can be used. When the rearing cage is covered by cotton cloth on at least one side, the humidity of the cage will be the same as in the growth chamber and, therefore, spraying can be avoided.

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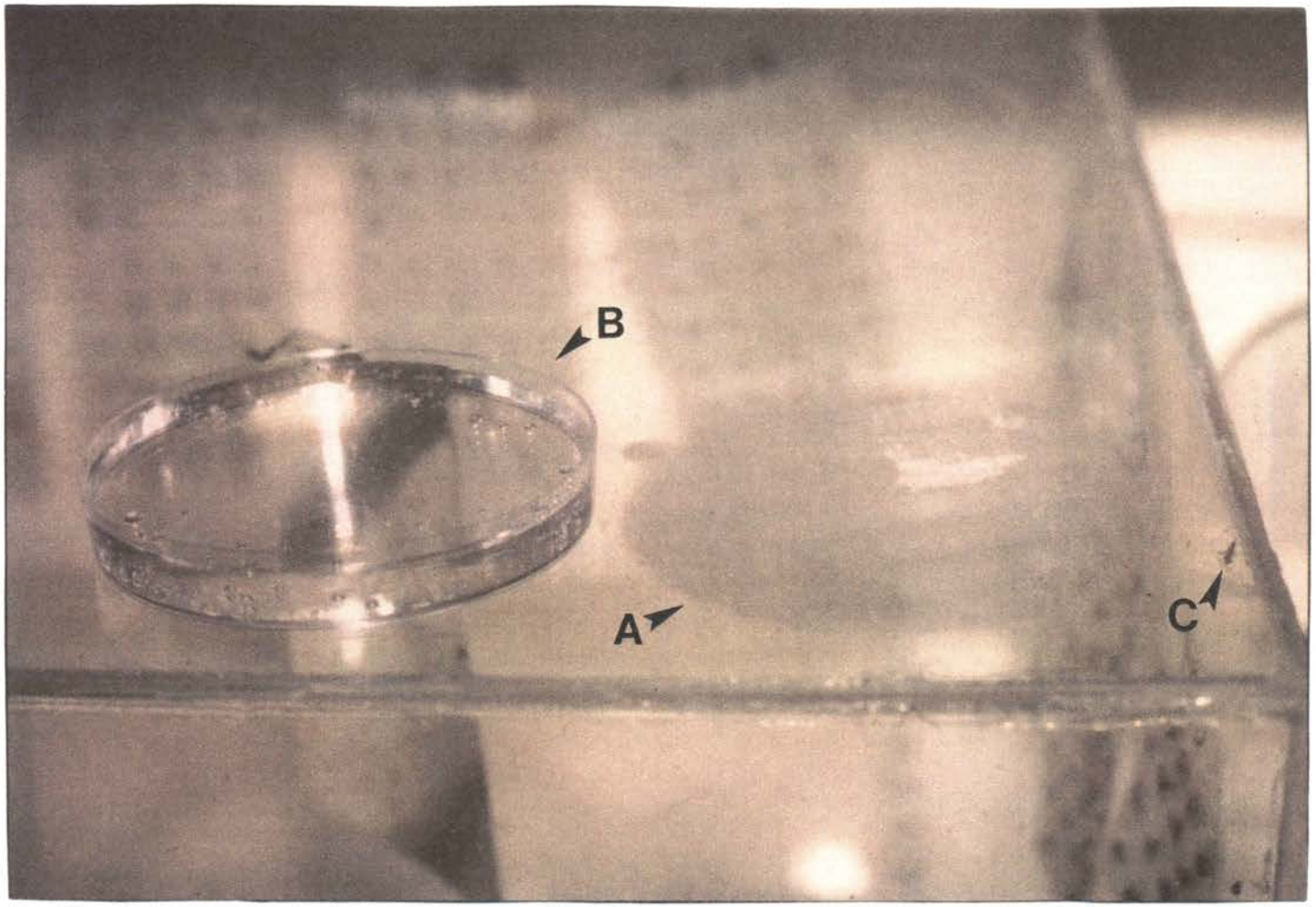

Fig. 1 - Condensed water (petri-dish shape) on the inside of an acrylic rearing cage (A), created by the pictured petri dish containing tap water left on the outside the cage for 24 h (B). *Catolaccus grandis* near pool (C).

VITA

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Doctor of Philosophy

Thesis: BEESWAX BASED FILMS AS ALTERNATIVE SUBSTRATES FOR REARING PARASITOIDS OF THE COTTON BOLL WEEVIL, *ANTHONOMUS GRANDIS* BOHEMAN (COLEOPTERA: CURCULIONIDAE)

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