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USAGE OF GREEN COCONUT WATER AND DIFFERENT TISSUE CULTURE MEDIA FOR IN VITRO HONEY BEE SEMEN STORAGE (*Apis mellifera;* HYMENOPTERA: APOIDEA)

Rosana Almeida and Ademilson Espencer Egea Soares

SUMMARY

*Honey bee semen was stored in green coconut (*Cocos nucifera *L.) water plus dihydrostreptomycin and in commercial tissue culture media at different temperatures. Glass capillary microtubes of 0.1cm diameter and centrifuge microtubes 0.2ml capacity were used for semen storage. Sperm motility was assessed after 1, 2, 5, 10, 15, 30, 50, 80 and 120 days. Queens were instrumentally inseminated with diluted semen and their laying behavior evaluated. Storage in coconut water shows living sperm until 80 days. However, the queen's laying was normal and resulted in viable worker brood only when semen stored up to 15 days in coconut water medium was used for insemination. Coconut water seems to be an ideal natural diluent for short periods* in vitro *storage of honey bee semen and can be an appropriate method for genetic improvement programmes for honey bees.*

RESUMEN

*Se almacenó semen de abejas en agua de coco (*Cocos nucifera *L.) verde con dihidroestreptomicina y en medios de cultivo comerciales, a diferentes temperaturas, utilizando microtubos capilares de 0,1cm de diámetro y microtubos de centrífuga con 0,2ml de capacidad. La motilidad de los espermatozoides fue evaluada luego de 1, 2, 5, 10, 15, 30, 50, 80 y 120 días. Reinas fueron inseminadas instrumentalmente con semen diluido y se evaluó su comportamiento reproductivo. El almacenamiento en agua de coco resulta en espermatozoides móviles hasta 80 días. Sin embargo, las reinas ovaron normalmente y resultaron obreras normales solamente cuando se utilizó para la inseminación semen almacenado en agua de coco hasta por 15 días. El agua de coco parece ser un diluente natural ideal para el almacenamiento* in vitro *de semen de abejas por períodos cortos y puede ser un método apropiado para programas de mejoramiento genético de abejas.*

Introduction

The instrumental insemination in *Apis mellifera* queens is one of the most important techniques for genetic studies, enabling mating with drones from specific lineages for the establishment of improvement programs or the maintenance of genetic markers.

With the advances of the instrumental insemination technique many researchers (Novak *et al*., 1960; Poole and Taber, 1969; 1970; Camargo, 1975; Verma, 1978; Moritz, 1983; Harbo, 1986; Collins, 2000) have attempted to develop efficient methods for the in vitro storage of semen using different kinds of diluents with physical and chemical properties that allow them to be used as an effective culture media for spermatozoa survival.

Among culture media tested, the coconut water (*Cocos nucifera* L.) presents a rich nutrient composition. Some of the nutrients have been found in the queen's spermatheca fluid (Verma, 1973) and seminal plasma (Novak *et al*., 1960). The aim of this study was to adapt some honey bee semen storage conditions in a green coconut water natural medium and different commercial storage media used for in vitro tissue culture with a similar composition as the coconut water.

Materials and Methods

Storage media

Media prepared from green coconut water. The storage medium was prepared with green coconut water to which dihydrostreptomycin (1mg/ml) was added. The medium pH was corrected with NaOH to 7.0 (pH of the semen; culture medium I) or 9.7 (pH of the spermathecal fluid; culture medium II). Coconut water packed in UHT system (pH 7.0; culture medium III) was also tested. The solutions were sterile-filtered by means of a 0.2µm/47mm membrane and separated in centrifuge microtubes before freezing.

Commercial tissue-culture media. The media tested were: Medium 199 (Sigma M-4530; culture medium IV), Minimum Essencial Medium Eagle (MEM; Sigma M-4780; culture medium V) and Cell Freezing Medium Glycerol (Sigma C-6039; culture medium VI). These media were chosen as they have a similar composition to the coconut water. The products were

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RESUMO

*O sêmen de abelhas foi estocado em água de coco verde (*Cocos nucifera *L.) com dihidroestreptomicina e em alguns meios de cultura comerciais, a diferentes temperaturas, utilizando microtubos capilares de 0,1cm de diâmetro e microtubos para centrífuga com capacidade para 0,2ml. A motilidade dos espermatozóides foi avaliada após 1, 2, 5, 10, 15, 30, 50, 80 e 120 dias. As rainhas foram inseminadas instrumentalmente com sêmen diluído e tiveram o seu comportamento de postura avaliado. A estocagem em água de coco apresentou espermatozóides com motilidade até 80 dias. Todavia, a postura das rainhas foi normal e resultou em operárias somente quando foi utilizado para a inseminação instrumental sêmen estocado em água de coco até 15 dias. A água de coco parece ser um diluente natural ideal para a estocagem* in vitro *de sêmen de abelhas, por curtos períodos e pode ser um método apropriado para programas de melhoramento genético de abelhas.*

stored according to the producers' instructions. All the glassware and equipment used was sterilized in an oven at 150ºC for 40 minutes.

Semen collection and storage

Drones were collected at 12-15 days of age. The ejaculation was provoked by manual pressure on the drone's abdominal region and the semen was collected in glass capillary tubes from the everted endophallus of the drone. The central region of the glass capillary (50µl) was pulled under heat and then cut, smoothed and sharpened at one end; the other end was adapted to a metal piston for the capillary handling (AEE Soares and JJ Santos, non-published data; Figure 1). In order to test the efficiency of each semen conservation medium, the media were individually mixed with a pool of semen (≅ 5µl) in 0.5ml centrifuge microtubes, and homogenized with a micropipette. Four samples from each condition were stored in 50µl glass capillary tubes (medium/semen mixture 3:1) and in 200µl centrifuge microtubes (medium/semen mixture 10:1), refrigerated (8-10ºC for coconut water media I, II and III; 2-5ºC for commercial culture media IV and V) or frozen (-20ºC for coconut water media I, II and III; -20º C and -70º C for commercial culture medium VI). Non-diluted semen samples were stored in glass capillary tubes at each of the above temperatures for control groups of queens inseminated with pure semen.

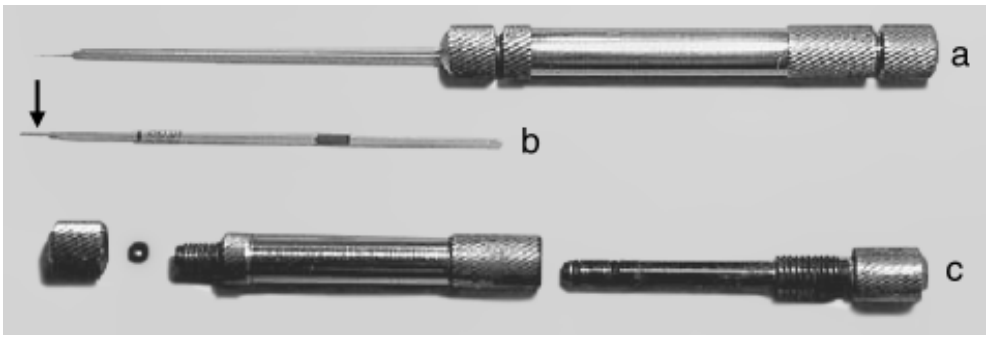

Figure 1. a: Metal piston developed by AEE Soares and JJ Santos (unpublished) used for semen collection from *Apis mellifera* drones' and for instrumental insemination of queens; b: capillary with modified end; c: disassembled metal piston.

After 1, 2, 5, 10, 15, 30, 50, 80 and 120 days the stored samples were observed under an optical microscope and the spermatozoa activity was estimated from the percentage of motile spermatozoa observed soon after mounting on a glass slide. The semen quality was evaluated following the laying behavior of the instrumentally inseminated queens.

Preparation of the hives and instrumental insemination of the queens

Several orphan nucleus colonies were prepared under similar conditions. A queen exclusion web was placed at each colony entrance. The queens were produced by the larvae transferring method (Doolittle, 1899). After emergence from the cell, each queen had its wings clipped, its thorax marked and was placed in a hive.

Two queens were inseminated with the stored semen from each different storage time and each different media. When the motility index was null, the samples were not tested. After the instrumental insemination, the queens were put back into their colonies.

Some steps of the honey bee instrumental insemination are shown in Figure 2.

Oviposition control and brood evaluation

The hives were checked daily until the presence of eggs was noticed, indicating the beginning of the queen's laying. A representative group of cells was chosen, and following the bees' ontogenesis it was possible to verify whether the emerging brood consisted of workers (from fertilized eggs) or drones (unfertilized eggs), in order to confirm if fertilization had occurred.

Statistical analysis

The Kruskal-Wallis One Way Test of Variance on Ranks was applied. Dunnett's Method for multiple comparison was used to indicate which groups differ from each other.

Results and Discussion

The activity of spermatozoa stored under different conditions (media, pH and temperature) is summarized in Table I. The statistical analysis shows differences between the median values (H=22.247; df=7; P=0,002).

In the refrigerated samples (media I, II and III) stored in glass capillary tubes at 8-10ºC, the sperm kept alive (1-10%) for until 80 days in medium I and up to 30 days in media II and III. The natural coconut water media (I and II) appear more efficient than the coconut water packed in UHT (medium III) but there is not a statistically significant difference (H=1,258; df=2; P=0,533).

The motility of spermatozoa stored in commercial media IV and V at 2-5ºC and in medium VI at -70ºC was observed only in the initial 5 days.

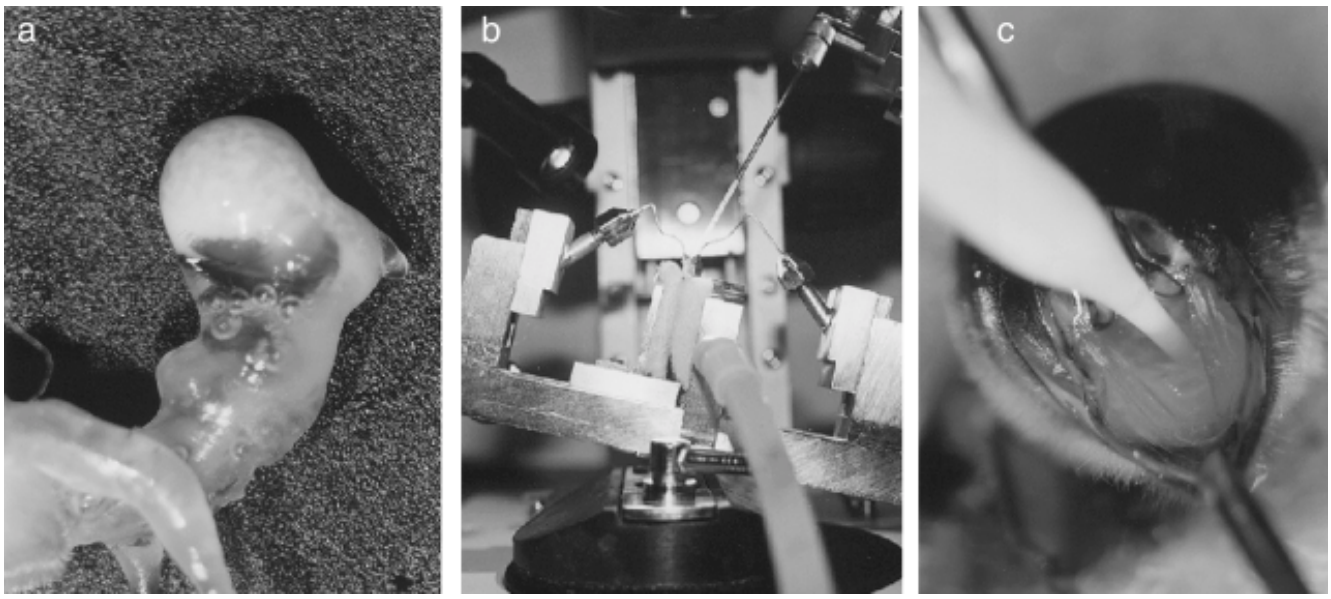

Figure2. Some steps of the honey bee instrumental insemination technique. a: Everted endophallus of the drone; b: position of the queen for the instrumental insemination; c: correct position of the glass capillary tube inside the queen.

Although the stored semen maintained their motility in green coconut water culture media as time passed, it notably decreased after 15 days of storage. In studies with different diluents, including coconut water (Verma, 1978; Locke and Peng, 1993), it was also observed that sperm tends to gradually lose motility after the first weeks of culture.

After the best semen storage conditions had been established, the second part of the experiment ensued: the viability of eggs in queens instrumentally inseminated with old diluted semen samples was studied. Table II summarizes the results of screening emerging brood, indicating whether worker or drone brood was observed. Six queens died during the procedure or in the course of the experiment.

The performance of all instrumentally inseminated surviving queens was followed for three months. The commercial storage media (IV, V, VI) did not give good results; spermatozoa motility was observed but fertilization did not occur. Studies on sperm storage have shown that no egg fertilization occurs in *A. mellifera* queens inseminated with Ringer-maintained semen at 1.7 and 32ºC (Taber and Blum, 1960) and that a significant percentage of sterile eggs is found when semen diluted in saline plus 10% dimethyl sulfoxide (DMSO) is used (Harbo, 1986).

Nevertheless, in the present work tests with instrumental insemination showed that when queens were insemi-

TABLE I
PERCENTAGE OF MOBILE SPERMATOZOA AFTER DILUTION WITH CULTURE MEDIA AND STORAGE IN GLASS CAPILLARY TUBES AT SEVERAL TEMPERATURES

	I a		II a		III a		IV b		V b		VI b		control groups 1b	2b	3b	4b
Days	8-10ºC	-20ºC	8-10ºC	-20ºC	8-10ºC	-20ºC	2-5ºC	-20ºC	2-5ºC	-20ºC	-20ºC	-70ºC	2-5ºC	8-10ºC	-20ºC	-70ºC
1	4	0	4	0	4	0	3	0	3	0	0	1	3	4	0	0
2	4		4		4		3		3			1	2	4		
5	4		4		2		1		1			1	0	0		
10	4		4		2		0		0			0				
15	4		3		2											
30	1		1		1											
50	1		0		0											
120	0															

Motility Index
0 = 0%
1 ≅ 01 – 10%
2 ≅ 11 – 50%
3 ≅ 51 – 75%
4 ≅ 76 – 100%

Medium I = green coconut water plus dihydrostreptomycin (pH 7.0)
Medium II = green coconut water plus dihydrostreptomycin (pH 9.7)
Medium III = green coconut water (packed in UHT system) plus dihydrostreptomycin (pH 7.0)
Medium IV = Medium 199 (Sigma M-4530)
Medium V = Minimum Essencial Medium Eagle (MEM; Sigma M-4780)
Medium VI = Cell Freezing Medium Glycerol (pfs; Sigma C-6039)
KRUSKAL-WALLIS (H=22.247; df=7; P=0.002)

* Dunnett´s Method: The treatment groups with the same letter are not significantly different.

nated immediately after mixing fresh semen with commercial culture media, normal oviposition by the queen took place. Although these media are not efficient for the storage of honey bee semen, they can possibly be used as a diluent for increasing the volume of semen for instrumental insemination. Only dihydrostreptomycin was added to the green coconut water media. According to Poole and Taber (1970) the addition of antibiotic seems to be of great importance in order to assure the survival of the spermatozoa over long periods.

The evaluation of the queen's laying behavior confirms that medium III, made with green coconut water packed in UHT system, showed to be less efficient than the media I and II, which were prepared with coconut water extracted from the fresh fruit.

When semen stored for up to 15 days in green coconut water (media I and II) were used, the eggs fertilization occurred and both the queen's laying and the worker brood viability were highly satisfactory. However, when semen stored for 30 days in coconut water was used for the instrumental insemination of queens, only drone brood occurred, suggesting that the low concentration (1–10%) of the sperm alive was not enough for egg fertilization.

The concentration of Na^+ and K^+ present in the honey bee queen spermatheca is one of the important factors for the reversible suppression of sperm motility and longevity (Verma, 1973). Arginine and lysine contents of honey bees spermatozoa and seminal plasma prolong the period of sperm motility (Novak *et al.*, 1960), and coconut water contains these nutrients. Based on this data, the coconut water medium preparation included only pH correction and antibiotic addition. Our interest is to develop an effective and more natural storage medium for the *A. mellifera* semen, having in view its cost/benefit. Although the commercial media tested have a chemical composition similar to that of coconut water, they were not efficient for semen preservation.

After observation of the laying behavior, the females' spermathecae were dissected. All queens that produced only drone brood presented empty spermathecae, indicating that they did not migrate into this receptacle. On the other hand, queens that produced worker brood showed living sperm into the spermatheca.

The sperm viability was null for negative temperatures and plastic centrifuge microtubes. Plastic centrifuge microtube utilization is not indicated for semen storage, because semen maintained within plastic recipients, in general, became non-viable independently of temperature conditions. The semen diluted with culture medium VI was an exception, but presented a very low percentage of motility (< 2%) up to at most 5 days of storage in centrifuge microtubes (at -20 and -70°C). The storage in glass capillary tubes at -70°C showed better results than at -20°C, although at most 10% of motile spermatozoa were observed after 24 hours of storage.

TABLE II
EVALUATION OF STORAGED SPERM VIABILITY IN THE EGG FERTILIZATION OF THE INSTRUMENTALLY INSEMINATED QUEENS

Medium	Period of Storage (days)	Motility Index [1]	Emerging Brood (%)
I	1	4	82.0**
	"	"	88.0**
	15	4	97.0**
	"	"	96.7**
	30	1	18.2*
	"	"	–
	50	1	28.5*
	"	"	23.0*
	80	1	4.6*
	"	"	–
II	1	4	44.6**
	"	"	50.0**
	15	3	95.7**
	"	"	–
	30	1	9.1*
	"	"	9.7*
III	1	4	72.5**
	"	"	49.0**
	15	2	3.8*
	"	"	–
	30	1	28.0*
	"	"	24.5*
IV	1	3	7.8*
	"	"	24.5*
V	1	3	32.0*
	"	"	–
VI	1	1	–
	"	"	5.8*
Control group 2-5°C	1	3	72.0**
Control group 8-10°C	1	4	86.0**

** honey bee workers (from fertilized eggs)
* honey bee drones (unfertilized eggs)
– dead queens
[1] Motility Index: 0 = 0% 1 ≅ 01 – 10% 2 ≅ 11 – 50% 3 ≅ 51 – 75% 4 ≅ 76 – 100%

The results reveal that the spermatozoa viability as well as their motility in green coconut water plus dihydrostreptomycin is not affected in a significant manner up to two weeks of storage. After this period, viability strongly decreases. However, the coconut water seems to be an ideal natural culture medium for sperm preservation, at least for short periods. This technique may facilitate work that needs controlled crossings and cannot be realized because of the absence of drones and queens simultaneously available, with the possibility of loss of biological material after a few days. Besides, the coconut water usage for semen preservation allows its transportation, which can facilitate this biological material exchange.

The piston used for the capillary handling was extremely efficient for semen collection directly from the drone's genital apparatus as well as for the instrumental insemination, making it easy to insert the capillary into the medial oviduct of the queen, thus assuring the artificial crossings success.

This study gives encouraging evidence to continue the diluent exploration for semen storage, and contributes to the establishment and maintainance of a bank of sperm and genetic improvement programmes for *A. mellifera* bees.

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