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A PROTOCOL FOR STORAGE AND LONG-DISTANCE SHIPMENT OF MEDITERRANEAN FRUIT FLY (DIPTERA: TEPHRITIDAE) EGGS. II. ASSESSMENT OF THE OPTIMAL TEMPERATURE AND SUBSTRATE FOR MALE-ONLY PRODUCTION

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ABSTRACT

The present study has been conducted to assess the effect and interaction of various storage substrates and conditions on eggs of the Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann). Tests were carried out with the genetic sexing strain VIENNA 8/D53, a strain that carries a *temperature sensitive lethal* (*tsl*) mutation that allows the selective killing of female zygotes. This study identifies strategies to enhance the storage and transport conditions through assessment of effect on egg, pupal and adult survival in order to facilitate the establishment of satellite mass rearing facilities for the production of male medflies. Eggs were immersed in two different substrates and stored at different temperatures and for different time periods. Findings from this study suggest that egg storage periods, and to some extent, the storage substrates have significant effects on pupal and adult survival. For 72-h storage periods, the eggs preserved in agar solution at 10°C produced the most pupae. There was an inverse relationship between the concentration of dissolved oxygen in the substrate during storage and the quality and survival of the stored/transported eggs. Apparently low levels of dissolved oxygen reduce metabolic rates, allowing the storage period to be prolonged.

Key Words: SIT, *Ceratitis capitata*, Mediterranean fruit fly, egg storage, genetic sexing, egg shipment

RESUMEN

El presente estudio fue conducido para evaluar el efecto e interacción de varios substratos y condiciones de almacenamiento en huevos de la mosca mediterránea de la fruta, *Ceratitis capitata* (Wiedemann). Las pruebas se realizaron con la cepa en la cual es posible separar los sexos genéticamente VIENNA 8/D53, la cual contiene una mutación letal sensible a la temperatura que permite la eliminación selectiva de los zigotos femeninos. Este estudio identifica estrategias para mejorar las condiciones de almacenamiento y transporte por medio de la evaluación de su efecto en la supervivencia de huevos, pupas y adultos, esto para facilitar el establecimiento de laboratorios satélites de cría masiva para la producción de machos de la mosca mediterránea de la fruta. Los huevos fueron sumergidos en dos substratos diferentes y almacenados a diferente temperatura a diferentes periodos tiempos. Los resultados de este estudio sugieren que el periodo y hasta cierto punto el substrato de almacenamiento tienen un efecto significativo en la supervivencia de las pupas y los adultos. Para un periodo de almacenamiento de 72-h los huevos almacenados en solución de agar a 10°C producen un número mayor de pupas. Hubo una relación inversa entre la concentración de oxigeno disuelto en el substrato durante el almacenamiento y la calidad y supervivencia de los huevos almacenados/transportados. Aparentemente los niveles bajos de oxigeno disuelto reducen el metabolismo y permiten que el periodo de almacenamiento pueda ser prolongado.

Previous studies (Cáceres et al. 2007) have identified various protocols that can be used in order to store and transport eggs of the Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann) from a central facility to satellite facilities where the eggs would be used to produce only sterile males. These protocols considered 3 variables, the age of the eggs, the temperature of storage in water, and the length of the storage period.

Currently, all programs applying the sterile insect technique (SIT) against the medfly utilize genetic sexing strains based on a *temperature sensitive lethal* mutation and a male linked translocation (Cáceres et al. 2004; Dyck et al. 2005). These strains produce only males following elimination of females as a result of a high temperature treatment during embryo development (Fisher 1998). It is therefore important to assess the effect of different storage conditions on the high temperature treatment that would be given to the eggs following transport to a satellite mass rearing facility.

For the present study, eggs of the Mediterranean fruit fly genetic sexing strain VIENNA 8/D53 (Franz 2005) were routinely collected and

stored in either water or a dilute agar solution at different temperatures and for different time periods. The purpose of using the agar solution was to provide a medium with a higher density than distilled water in order to prevent egg sedimentation, and thus avoid damage. Following storage, the eggs were incubated according to the standard protocol for elimination of females (Fisher 1998; Fisher & Cáceres 2000; Cáceres 2002). Following the temperature treatment, the embryos were placed on standard larval diet and various quality control parameters were assessed during the larval, pupal, and adult stages. In addition, the dissolved oxygen was measured during storage to determine any possible relation between the dissolved oxygen concentration and the quality and survival of the stored eggs.

The results are discussed in the framework of improving the efficiency of programs integrating the SIT by providing a strategy for long distance egg shipment to supply satellite medfly mass-rearing facilities with fertile eggs for male-only pupal production.

Materials and Methods

Strain

The genetic sexing strain VIENNA 8/D53 was selected for all experiments. This strain carries a male-linked translocation, a chromosome inversion, and the females are homozygous for two selectable markers, *white pupae* (*wp*) and *temperature sensitive lethal* (*tsl*) (Robinson et al. 1999; Franz 2005). Females are eliminated by exposing the eggs to 34°C for 12 to 24 h during the last half of embryo development (Franz et al. 1996; Cáceres et al. 2004).

Egg Treatment

Eggs from strain VIENNA 8/D53 that were 24 h old were immersed in different storage substrates, and subjected to different storage temperatures and time periods. The storage conditions consisted of 2 substrates, distilled water or agar (0.1%) solution, 2 temperatures 10 or 25°C, and 3 storage periods, 0, 24, and 72 h. The egg collections were performed following standard guidelines and protocols for medfly genetic sexing strains (Cáceres 2002). Following collection, the eggs were incubated inside plastic bottles containing distilled water (v/v ratio of 1:20, respectively), into which compressed air was provided via an aquarium stone, for 24 h at 24°C. For this study, nine 8-mL aliquots of eggs were removed. One served as control and was incubated for an additional 12 h at 34°C to eliminate the female zygotes, and then maintained for an additional 12 h at 24°C. The other 8 aliquots were stored under different test conditions, which consisted of the combination of the 2 temperatures, the 3 storage periods and the 2 substrates. Each aliquot was stored in 70 mL of substrate.

Following storage, the eggs were transferred back into the plastic bottles to complete the 48-h incubation period as follows, 12 h at 34°C to eliminate the female embryos and then for another 12 h at 24°C.

Larval Rearing

Following the incubation period, the treated and the control eggs were washed with distilled water to remove traces of the storage substrate, and transferred into trays containing 5 kg of larval diet (Tanaka et al. 1969). An aliquot of 8 mL of eggs was put into each tray. The 3rd instars were collected in a tray containing sawdust, which served as the pupation medium. Larvae were collected for 3 consecutive days and the volume measured.

Quality Control

Quality control tests were performed following the FAO/IAEA/USDA "Manual for Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies" (2003).

Egg Hatch

Samples of 1000 eggs were collected for each treatment and the control group, before transfer to the larval diet. Five days later, the number of unhatched eggs was counted and the percentage egg hatch calculated. During the 5-day incubation period, the egg samples were placed under the same conditions as the larval trays.

Egg to Pupal Survival

Egg to pupal survival was calculated based on the estimated original number of eggs (8 mL × 25000 egg/mL) transferred to the larval diet and the number of pupae produced for each treatment. The number of pupae recovered for every treatment was estimated volumetrically.

Egg to Adult Survival

Egg to adult survival was calculated based on the estimated number of eggs transferred to the larval diet for each treatment, the number of pupae recovered, as estimated for the egg to pupal survival, and the percentage of adult emergence from 5 mL of 8-d-old pupae. The total number of adults was determined 5 d after the first fly emerged.

Dissolved Oxygen

Dissolved oxygen and temperature were measured with a portable dissolved oxygen meter

microprocessor-based instrument, consisting of a cell-containing electrolyte enclosed by a selective membrane and two metallic electrodes (PAKTON® 35640-series). The electric current produced by the consumption of oxygen by the cathode is proportional to the partial pressure of the oxygen in the sample. The dissolved oxygen and temperatures were measured for all treatments, before and after the storage periods.

Data Analysis

Numerical data were analyzed by Analysis of Variance (ANOVA). The effect of the storage times, substrates, and temperatures on the egg hatch and egg to adult survival was analyzed by 3-way ANOVA and the means compared by the Tukey multiple range test. Interaction between the variables, storage temperature and time was determined by means of the generalized linear model of variance to perform univariate ANOVA. Results were analyzed with the statistical software MINITAB® for windows.

RESULTS

Egg Hatch

The length of the storage period had a significant effect on egg hatch (F = 11.31, P = 0.002). Eggs stored for 72 h (as well as control eggs) displayed higher hatch than those stored for 24 h (Table 1). Moreover, within a given storage period, egg hatch was higher for eggs stored at 10°C. Overall egg hatch did not differ between storage in water and in an agar solution, although storage in water tended to result in slightly higher egg hatch than storage in agar. The highest egg hatch was recorded for the eggs stored in water and in the agar solution at 10°C for 72 h (59.7 ± 7.2% and 59.4 ± 9.9%, respectively).

Egg to Pupal Survival

Significant variations were observed when assessing the egg to pupal survival among the different treatments (Table 1). In contrast to egg hatch, 0 and 24 h storage periods produced higher values for egg to pupal survival (F = 12.39, P = 0.029) than 72 h storage. For eggs stored for 72 h, the highest egg to pupal survival was recorded for eggs stored at 10°C. The lowest egg to pupal survival values were observed among the eggs stored for 72 h at 25°C, regardless of the storage substrate. Nevertheless, statistically significant differences were only found between those eggs stored in agar solution at 25°C for 24 h (27.4 ± 3.0%), and at 10°C for 24 h (26.4 ± 2.2%), when compared with those stored at 25°C for 72 h either in agar solution (19.9 ± 1.4%), or water (8.4 ± 3.4%) (F = 6.72, P < 0.5). Eggs stored for 24 h produced higher numbers of pupae than those stored for 72 h, regardless of the storage substrates or temperatures. Overall, the storage of medfly eggs in agar solution at 25°C for 24 h appeared to be the most suitable condition for pupal production. However, when considering 72 h storage periods, the eggs preserved in agar solution at 10°C produced the most pupae (22.9 ± 5.0%), but was not significantly different from the treatment in agar solution at 25°C for 24 h that produced the highest number of pupae (27.4 ± 3.0%) or the control (27.1 ± 4.4%).

Egg to Adult Emergence

There was a significant correlation in terms of the storage conditions between egg to pupal survival and egg to adult survival (R^2 = 0.984, P =

TABLE 1. EGG HATCH, EGG TO PUPAL SURVIVAL, AND EGG TO ADULT SURVIVAL (MEAN % ± SE) FROM 24-H-OLD EGGS OF GENETIC SEXING STRAIN VIENNA 8/D53 HELD UNDER DIFFERENT STORAGE CONDITIONS. CONTROL EGGS WERE INCUBATED IN WATER FOR 12 H AT 34°C TO ELIMINATE THE FEMALE ZYGOTES, AND THEN MAINTAINED FOR AN ADDITIONAL 12 H AT 24°C. MEANS WITHIN COLUMNS FOLLOWED BY THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT, P < 0.05.

Treatments			Parameters		
Storage substrate	Time (h)	Temperature °C	Egg hatch	Egg to pupae efficiency	Egg to adult efficiency
Agar	24	25	45.6 ± 6.2 b	27.4 ± 3.0 a	21.9 ± 3.3 a
Water	24	25	47.5 ± 6.3 b	25.7 ± 3.1 ab	21.3 ± 2.2 a
Agar	24	10	48.3 ± 7.8 b	26.4 ± 2.2 a	21.0 ± 2.6 ab
Water	24	10	50.3 ± 13.1 ab	25.3 ± 4.1 ab	19.8 ± 2.8 ab
Agar	72	10	59.4 ± 9.9 a	22.9 ± 5.0 ab	18.4 ± 3.8 ab
Water	72	10	59.7 ± 7.2 a	21.3 ± 4.8 ab	17.3 ± 4.2 ab
Agar	72	25	54.7 ± 11.7 ab	19.9 ± 1.4 b	16.0 ± 1.2 b
Water	72	25	56.4 ± 1.2 ab	8.4 ± 3.4 c	6.5 ± 2.8 c
Control	0	—	55.3 ± 7.1 ab	27.1 ± 4.4 a	21.5 ± 3.4 a

0.05). However, since this was contrary to the effect seen with egg hatch, this indicates that storage directly affects larval and pupal survival. As for the egg to pupal survival, egg to adult survival was greater for those eggs stored for 24 h than those stored for 72 h (Table 1), although the survival was only significantly lower for the eggs stored in water at 25°C or 72 h (6.5 ± 2.8%). For eggs stored for 72 h in agar solution at 10°C, the egg to adult survival was 18.4 ± 3.8%, which was not statistically different from the control (21.5 ± 3.4%).

Dissolved Oxygen

Concentrations of dissolved oxygen were 5 times higher in water than in the agar solution prior to and following storage (Fig. 1). A relationship between the concentration of oxygen available for the duration of the different treatments and the storage substrates, temperatures and periods was observed. The water substrate, as well as low temperatures (10°C), were associated with higher oxygen concentrations. Moreover, at any given temperature higher concentrations of oxygen occurred during the shorter storage periods. The water substrate at 10°C for 24 h displayed the highest concentrations of dissolved oxygen available at the end of the storage period (Fig. 1). Nevertheless, in spite of these findings, there was no direct effect of the concentration of dissolved oxygen on adult fly emergence. There was no control for this test as bubbling and aerated was continuous and there was no variation in the concentration of dissolved oxygen at the beginning and the end of storage period.

Discussion

The optimal storage conditions for 72 h storage are at 10°C in an agar solution. In general increasing storage reduces egg hatch, pupal production and adult emergence. Storage periods of 24 h appeared to have no damaging effects on adult emergence and pupal production, although there was a direct effect on egg hatch. Storage periods of 72 h on the contrary, appeared to have no effect on egg hatch, whereas there was a negative effect on the pupal and adult production, but only when stored at 25°C. Overall, storage in agar solution appeared to be better than storage in water, although the effect was only significant for 72 h storage at 25°C. The detrimental effect of storage in water at 25°C for 72 h on the rate of adult emergence and pupal production observed in this study are in line with data reported by Cáceres et al. (2007) and with Arakaki et al. (1984), who showed that with melon fly *Bactrocera cucurbitae* Coquillett at temperatures of 5 or 10°C, eggs preserved in water maintained high hatch rates, even after 7 d of storage.

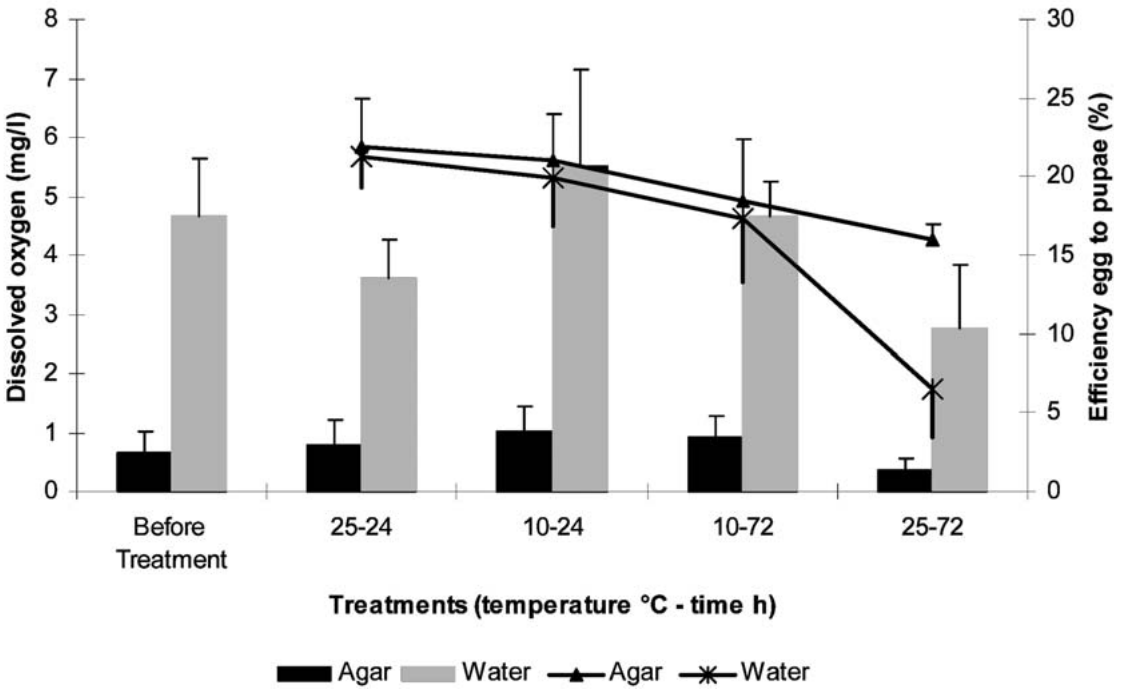

Fig. 1. Dissolved oxygen concentration (columns) and egg to pupal efficiency (lines) before and after storage of VIENNA 8/D53 eggs under different temperature conditions and storage periods.

The results gathered from this study on the number of pupae produced and the rate of adult emergence suggest that storage in agar at 25°C for 24 h constitutes the most suitable condition for eggs of the VIENNA 8/D53 genetic sexing strain. This is acceptable for situations where the satellite mass rearing facility is within a 24-h shipment period from the central facility mass-producing the eggs. However, for storage up to 72 h, storage in agar solution at 10°C provides the best conditions in terms of pupal production and adult emergence. Overall, the effect of the storage times was not significant for egg hatch, but for the larval development stage this effect was reflected directly on the significantly diminished number of pupae collected particularly on the treatments where eggs were stored up to 72 h. The concentration of dissolved oxygen available in the storage substrate affected the emergence of adult flies. As initially suggested, we can assume that the agar solution provides better physical conditions for egg storage, as it prevents egg sedimentation. In addition, the low levels of dissolved oxygen may reduce metabolic rates, allowing the storage period to be prolonged. Experiments with housefly *Musca domestica* (L.) have demonstrated that the gaseous composition of the atmosphere during incubation has an effect on the survival of the embryos and oxygen-enhanced environments during cold storage appear to have a detrimental effect on survival (Bucher et al. 1947). However, recent experiments have demonstrated that although exposure of housefly embryos to hypoxic conditions was not detrimental, it did not increase chilling tolerance (Leopold 2000). In the present study it was established that egg storage in agar solution is better than in water, but the specific characteristics that enhance this storage effectiveness, were not determined.

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