Scientific Note

Ezequiel C. Quenta*, Carlos C. Huaynate, Marleny O. Ruidias, Tania T. Arambulo and Grimaldo Febres

New quarantine cold treatment for medfly *Ceratitis capitata* (Diptera: Tephritidae) in pomegranates

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Abstract: The medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most important agricultural pests worldwide due to its impact on harvest and market restrictions for host fruits in several countries. Pomegranates (*Punica granatum* [L.]; Lythraceae) are sensitive to chilling injuries caused by existing quarantine cold treatments. This study aimed to establish a new cold-treatment schedule at 5 °C for fresh pomegranates by determining the required exposure time to mitigate *C. capitata* in the pomegranate 'Wonderful' variety. The third-instar larva was deemed the most cold-tolerant instar and was used for this study. The combination of 5.2 °C for 36 days achieved 100 % mortality.

Keywords: physical control; phytosanitary measure; fruit flies; cold treatment

Resumen: La "moscamed" *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) es una de las plagas agrícolas más importantes a nivel mundial debido a su impacto en la cosecha y restricciones de mercado de frutos hospedantes en varios países. Las granadas (*Punica granatum* [L.]; Lythraceae) son sensibles a las lesiones por frío causadas por los tratamientos cuarentenarios en frio existentes. Este estudio tuvo como objetivo establecer un nuevo programa de tratamiento en frío a 5 °C para granadas frescas mediante la determinación del tiempo de exposición necesario para mitigar *C. capitata* en la variedad de granada "Wonderful". La larva del tercer estadio fue considerada la más tolerante al frío y fue usada en los experimentos. La combinación de 5.2 °C durante 36 días logró un 100 % de mortalidad.

Palabras clave: control físico; medida fitosanitaria; moscas de la fruta; tratamiento de frio

The international trade of fresh fruits has increased in recent decades because of the growing global demand for such products. Consequently, scientific initiatives to support new strategies and measures to mitigate quarantine pests have intensified. To facilitate trade of pomegranates (*Punica granatum* L.; Lythraceae) to countries regulating the medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), quarantine treatments are required. The United States Department of Agriculture (USDA), through its Agricultural Commodity Import Requirements database (USDA 2023), has established cold treatment T 107-a schedules (1.11 °C for 14 days; 1.67 °C for 16 days; and 2.22 °C for 18 days) against the medfly for several fruit species, including pomegranates, and for all countries.

Pomegranates have limited shelf life, which makes cold storage necessary. However, physiological disorders, such as husk scald, splitting, and chilling injury, are common challenges that reduce the marketability of the fruit (Sunil et al. 2015). Early harvested pomegranates were particularly susceptible to chilling injury, and it was found that their optimum storage temperatures were between 5 °C and 7.5 °C (Kashash et al. 2016). Taghipour et al. (2020) also found that keeping pomegranates below 5 °C for longer than one month resulted in chilling injury. The sensitivity of pomegranates to low storage temperatures triggers serious economic concerns, as cold storage is an important quarantine treatment requirement for exporting fruit to fly-free zones internationally (Kashash et al. 2016). The aim of this study was to demonstrate the efficacy of a new cold-treatment schedule based on 5 ± 0.5 °C to mitigate *C. capitata* in fresh pomegranates of the 'Wonderful' variety.

***Corresponding author: Ezequiel C. Quenta**, Servicio Nacional de Sanidad Agraria (SENASA), La Molina 1915, 15024, Lima, Peru, E-mail: equenta@senasa.gob.pe. https://orcid.org/0009-0009-1258-7682
Carlos C. Huaynate, Servicio Nacional de Sanidad Agraria (SENASA), La Molina 1915, 15024, Lima, Peru, E-mail: cahuaynate@senasa.gob.pe. https://orcid.org/0009-0006-0470-9721
Marleny O. Ruidias and Tania T. Arambulo, Asociación de Productores de Granada del Peru, Guillermo Perata 215, Santiago de Surco 15038, Lima, Peru, E-mail: marleenglish2021@gmail.com (M.O. Ruidias), soraya0400868@hotmail.com (T.T. Arambulo)
Grimaldo Febres, Universidad Nacional Agraria La Molina, Av. Universitaria s/n, La Molina, Lima, Peru, E-mail: gjfebres@lamolina.edu.pe. https://orcid.org/0000-0003-4557-1539

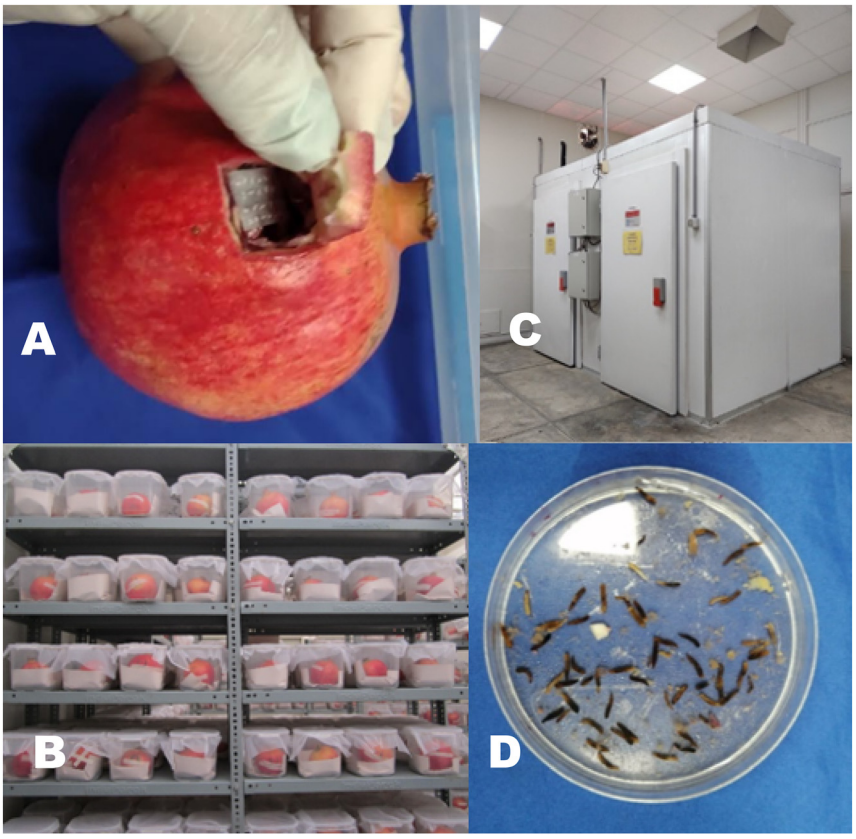

Figure 1: Procedure for experimental tests of cold treatment in *Punica granatum*: (A) pomegranate fruit showing the "window" method for medfly *Ceratitis capitata* egg inoculation; (B) infested fruit storage containers for development of the immature stage; (C) two experimental cold chambers; and (D) medfly third instar larvae found dead after cold treatment.

This research was developed at the Phytosanitary Treatment Research Laboratory of the Peruvian Servicio Nacional de Sanidad Agraria del Perú (SENASA), La Molina (Lima, Peru), under controlled conditions of temperature (25–27 °C) and relative humidity (50–60 %).

A wild colony of *C. capitata* was obtained from peach, *Prunus persica* (L.) Batsch (Rosaceae), and the apple variety 'Ana Israel', *Malus domestica* (Suckow) Borkh. (Rosaceae) fruits. The adult flies were conditioned in 40 × 30 × 30 cm cages lined with a tulle mesh on one side that served as a substrate for laying their eggs. The cages contained food (sugar + enzymatic protein) and water. The collected eggs were placed in artificial larval diets, whose composition followed the International Atomic Energy Agency (FAO/IAEA/USDA 2019) and López et al. (2006). The collected larvae were kept in dark rooms for 24 h and put in a pupal maturation room for 10 days. Subsequently, they were returned to the adult colony room for emergence and to repeat the cycle. The colony developed under 25.1 °C and 57.4 % relative humidity and were no more than five generations removed from the wild when used for cold-treatment experiments.

Artificial infestation was accomplished by taking a whole fruit and cutting a "window" or a square flap that could be opened and closed (Figure 1A). Medfly eggs were collected and lined up in groups of 100 on a piece of filter paper (1.5 × 1.0 cm). Fruits were inoculated by putting a piece of filter paper into the "window" or under the flap of fruit skin, which was then closed and sealed with paraffin and masking tape. Each infested fruit was placed in individual plastic containers with thick absorbent paper at the bottom to facilitate pupation and then covered with a tulle mesh (Figure 1B).

When applying the cold treatments, it was important that the treatments were applied to the most cold-tolerant stage of *C. capitata* within infested fruit. Consequently, we initially monitored the development and mortality of eggs and the larval instars in inoculated fruits. Three sets of 70 fruits each were established as replicates. Starting 1 day after inoculation and continuing for 14 consecutive days, we recorded the number of live eggs and instars.

The tolerance test was conducted following methodology provided by De Lima et al. (2007), Gazit and Kaspi (2017), and guidelines of the International Sanitary and Phytosanitary Norm (ISPM) # 28 (FAO 2016). Eggs and larval instars (first, second, and third) were assessed to determine the most cold-tolerant instar/stage. A probit model was developed for each replicate to determine the most cold-tolerance instar/stage. Then, a statistical evaluation of slopes and intercepts was done to test if they were statistically equal at 5 % significance level.

A small-scale cold treatment test was conducted at 5 °C. Cold treatment was applied 8 d after egg inoculation in order to ensure a high proportion of 3rd instar larvae (the most cold-tolerant stage) yet avoid the "jumping" behavior that marks the end of larval development. Fruits under cold treatment were evaluated (cut open to determine larval mortality) after 13 periods (12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, and 36 days). The longest time period, 36 days, was considered to simulate an in-transit cold treatment from Peru to Asia. A total of 1,140 infested pomegranate fruits were used as follows: 40 infested fruits per each of the 13 time periods (13 × 40 = 520), and 50 infested fruits for the control group (520 + 50 = 570). This number of fruits was used for each of the two replicates (570 × 2 = 1,140). The replicates were conducted in different chambers (Figure 1C) and at different times. Fruits from the control group were stored at 26.3 °C and 51 % relative humidity, on average, during the trial period. A probit model was made with 95 % fiducial limits of mortality days.

A large-scale disinfestation test was made using an average temperature of 5.2 °C for an exposure time of 36 days. It was conducted using a total of 960 fresh pomegranate fruits that were artificially infested. This experiment was designed to use more than 30,000 test insects. For each replicate, 320 fruits were used (240 for treatment and 80 for the control). The three replicates were conducted separately and at different times.

Fruits were placed in 33.5 × 29.5 × 12 cm cardboard boxes (same as those used for export). Infested fruits were placed in 36 boxes. Sensors to register the treatment temperature were placed into 10 non-infested pomegranates (one sensor per fruit). Temperatures were recorded every 60 min to verify the target temperatures during treatment. Ninety-one boxes containing the same variety of pomegranates also were placed inside the treatment chamber for each of the three replicates to simulate real conditions.

Treated fruits were evaluated (cut open to determine larval mortality) 24 h after finishing the treatment, and the control group was evaluated 24 h after starting the treatment. Fruits from the control group were stored under similar conditions as the small-scale test.

The following formula from Couey and Chew (1986) was used to calculate the upper confidence limit (C), where pu is the survival rate, and n is the number of test insects:

$$C = 1 - (1 - pu)^n$$

A test of fruit quality was conducted to evaluate the damages caused by cold in pomegranate fruits stored in equivalent conditions as in the quarantine treatment. Three boxes containing eight export quality size pomegranate fruits each were treated together during the confirmatory large-scale treatment test at 5.2 °C on average for 36 days. Twenty-four fruits were evaluated and analyzed at the beginning of the treatment and 36 days after finishing. The following was determined: Brix degrees (measured using an ATAGO® CO., LTD Hybrid PAL-BX/ACID 1 Brix 0–60 % and Acid 0.1–4.0 %, Shiba-Koen, Minato-ku Tokyo, Japan), soluble solids (%), acidity, maturity index (measured with ATAGO® Brix-Acidity Meter), and initial and final weights. The appearance, aroma, and flavor were evaluated through sensory (hedonic) tests, while physiological and pathological changes were recorded through observation. The weight data were analyzed with paired t test, and Brix degree, acidity, and maturity index were analyzed with the non-parametric Mann–Whitney test.

The development study resulted in eggs being found only on days 1 and 2, the first-instar larvae on days 2, 3, and 4, the second-instar larvae on days 4, 5, and 6, the third-instar larvae on days 6–13 (the majority on day 8), pupae on days 9–14, and pupae only from day 14 onward. In the tolerance test, the third-instar larva was found to be the most cold-tolerant instar as demonstrated by probit analysis in Table 1 and illustrated in Figure 2. Data were found to be statistically equal, then the two replicates were pooled and the probit model was applied. After that, an analysis of fiducial limits (LD) of the lethal times was done. As a consequence of this finding, the third instar larvae was used for the small and large-scale cold treatment experiments.

Results of the small-scale test at 5 °C demonstrated full mortality (100 %) of the 3rd instar larvae of *C. capitata* after 28 days (Table 2). These results are framed within a simulated 36-d in-transit cold treatment.

In the large-scale test, with a temperature of 5.2 °C, full mortality (100 %) was achieved using the same simulated exposure time (36 days). The number of dead medfly third-instar larvae reached 45,762 individuals (14,955; 15,405 and 15,402 for replicates 1, 2 and 3, respectively). Follet and

Table 1: Probit model at 95 % fiducial limits of mortality days or lethal time (LT) to 95 %, 99 %, 99.99 % and 99.99683 % mortality on immature stages of *Ceratitis capitata* in the pomegranate variety 'Wonderful' stored at 5 °C on average. It can be noticed that the mature larvae (third instar larvae) take longer to die when compared to egg and young larvae (first and second instar larvae).

Stage	LT (upper, lower limit)			
	LT95	LT99	LT99.99	LT99.99682
Egg	7.85 (6.88, 8.80)	9.33 (8.39, 10.43)	12.35 (11.16, 14.05)	12.96 (11.62, 14.81)
Young larvae	7.94 (6.80, 9.03)	9.15 (8.08, 11.32)	11.62 (10.39, 13.41)	12.11 (10.83, 14.05)
Mature larvae	9.95 (9.57, 10.36)	11.59 (11.14, 12.09)	14.93 (14.27, 15.69)	15.60 (14.89, 16.42)

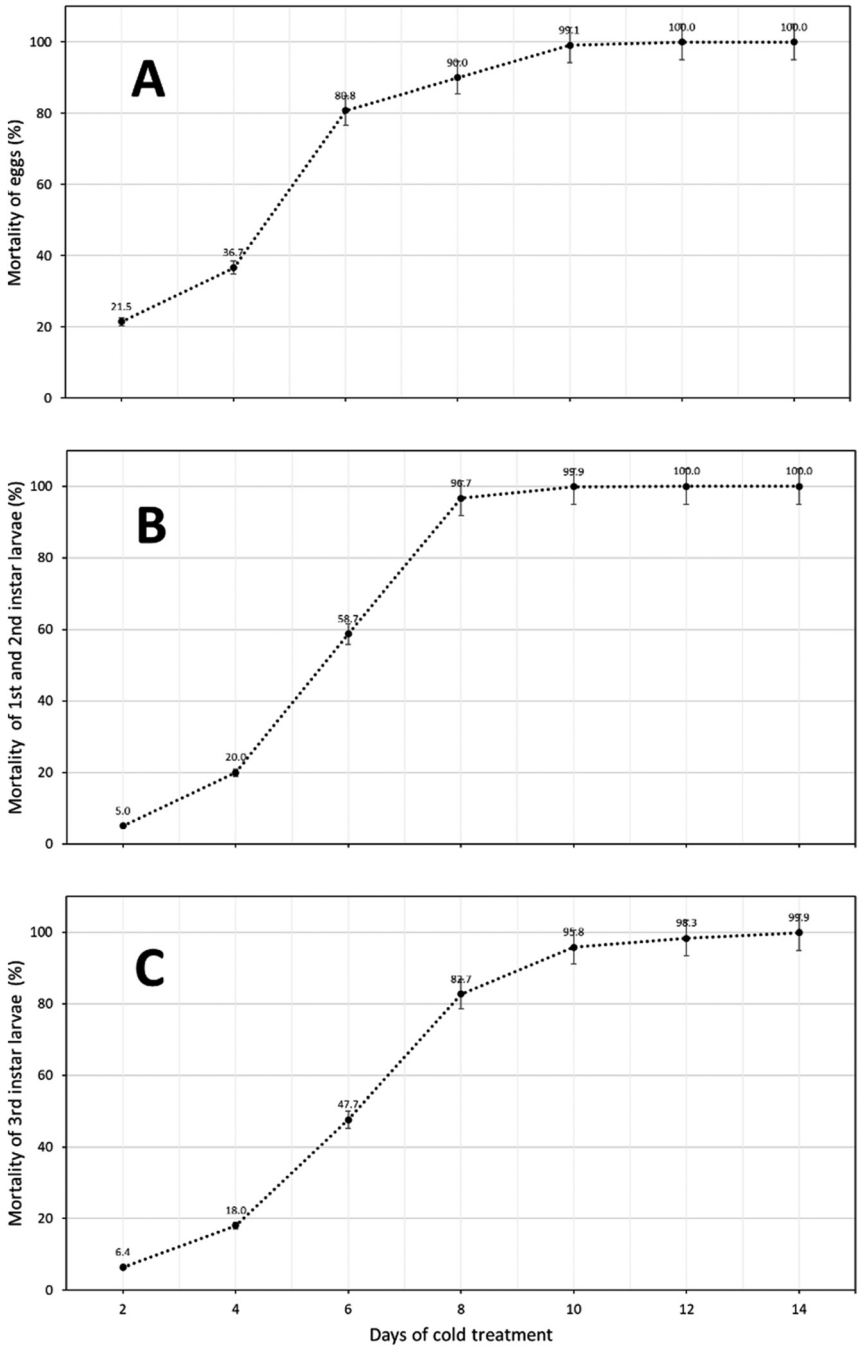

Figure 2: Mortality of medfly *Ceratitis capitata* immature stages in pomegranate fruits during the tolerance test subjected to cold treatment (5 °C). (A) Eggs; (B) first and second instar larvae; and (C) third instar larvae. It can be noticed that the third instar larvae take the longest time to reach complete mortality compared to eggs and other larval instars.

Table 2: Probit model estimates and 95 % fiducial limits of mortality days or lethal time (LT) to 95 %, 99 %, 99.99 % and 99.99683 % mortality for mature larvae (third instar larvae) of medfly *Ceratitis capitata*. Small-scale test in the pomegranate fruit, varity 'Wonderful' stored at 5 °C on average.

LT	Lower	Estimate	Upper
95	9.52	11.58	13.13
99	15.34	16.62	17.70
99.99	25.53	26.91	28.71
99.99682	27.37	28.98	31.14

Neven (2006) pointed out that countries, such as Japan, Australia, and New Zealand, accept quarantine treatment efficacy at 99.99 % (at the 95 % confidence level), which is obtained by treating 29,956 insects with no survivors. Likewise, Schortemeyer et al. (2011) expressed that to achieve a probit of 8.719 requires 29,956 test insects and 99.99 % mortality. After applying the formula from Couey and Chew (1986) to our data, the upper confidence limit equals 98.9 % (C = 0.9897084162; considering n = 45,762 test insects, and pu = 0.000065461172 survival rate based on a probit of 8.719;

Table 3: Large-scale disinfestation test results. Mortality of the most cold tolerant stage of *Ceratitis capitata*, in the pomegranate variety Wonderful stored at 5.2 °C on average.

Replication	Control fruit		Treated fruit			
	Nº of fruits	Total survivors	Nº of fruits	Estimated Nº of treated insects	Total survivors	Mortality %
1	80	4,985	240	14,955	0	100
2	80	5,135	240	15,405	0	100
3	80	5,134	240	15,402	0	100
Total	**240**	**15,254**	**720**	**45,762**	0	**100**
Upper confidence limit (*C*)						0.989708416
C (%)						98.90

Upper confidence limit calculated according to Couey and Chew (1986).

Table 3). The percentage of survivorship was 0.0001 (99.99 % mortality) beginning on day 26; therefore, it can be assured that there will not be survivors after the 5.2 °C cold treatment with 99 % confidence.

In addition, the results for the qualitative parameters, after 36 days, were optimal. Pomegranates were 'good' for appearance, 'nice' for aroma, and no fruits were affected in terms of physiological and pathological changes. Differences were found for the weight variable (p-value = 0.000 < 0.05), meaning that there was a decrement. With respect to the other variables, the statistical results were all significant, meaning that there was an increment for Brix degree (p = 0.002) and maturity index ($p \leq 0.001$), and a decrement for acidity ($p \leq 0.001$).

To conclude, full mortality of *C. capitata* 3rd instar larvae was accomplished by applying a cold treatment with 5.2 °C for 36 days. Full mortality was accomplished under the framework of a 36-day time period to simulate an in-transit cold treatment from Peru to Asia. These results comply with international standards and a probit of 8.7.

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Data availability: The raw data for the cold treatment of pomegranates have been included at the following link. https://www.senasa.gob.pe/senasa/cold-treatment-test/.

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