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Quarantine cold treatment against C*eratitis capitata* (Wiedemann) (Diptera: Tephritidae) to export clementine mandarins to Japan

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As a consequence of the presence in Spain of the Mediterranean fruit fly *Ceratitis capitata* (Wied), the export of citrus to countries which are exempt of this pest is restricted by plant quarantine regulations. A series of trials using standard bioassay procedures followed by large-scale export tests, were conducted on Clementine mandarins. Fruits infested with eggs, young larvae and old larvae of this insect were stored for various periods at 2±0.5 ºC. The eggs were the stage most susceptible to cold, and no significant difference in cold tolerance was seen between young and old larvae. The effectiveness of 16 days storage at 2±0.5 ºC against the insect was demonstrated by 100% mortality when treating more than 30,000 *C. capitata* in Clementine mandarin under industrial conditions. No substantial modifications in the qualitative characteristics of the fruits were observed. No physiological alterations have been found and the rotes are the usual in any marketing process.

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Key words: Mediterranean fruit fly, *Citrus reticulata*, citrus, export Japan.

INTRODUCTION

Spain is the main exporter country of citrus fresh fruits in the world. However, as a consequence of the presence in Spain of the Mediterranean fruit fly *Ceratitis capitata* (Wied), the export of citrus to countries which are exempt of this pest is restricted by plant quarantine regulations. These barriers to free trade may be overcome by the application of suitable post-harvest disinfestations treatments.

Japan has authorized the entry of Spanish lemons which have received a quarantine cold treatment for 16 days at 2 ºC, (SANTABALLA y LABORDA, 1987) and oranges belonging to the groups Navel and Valencia-late (SANTABALLA *et al*.1995) and Salustiana, (SANTABALLA *et al*.,1997) treated for 17 days with temperatures below 2ºC which have started their quarantine cold treatment at 1.5ºC.

Here we describe the results of cold treatment studies performed to determine the storage temperature and period that will ensure 100% mortality of the most tolerant stages of *C. capitata* to cold when the insect is present in Clementine mandarins. For the execution of these tests, the Japanese Ministry of Agriculture, Forestry and Fisheries

has established a trial guideline requiring quarantine levels of disinfestations to be demonstrated through conducted series of trials. The first steep is demonstrated what is the most tolerant pest stage to the cold. The second steep is conduct a series of large-scale trials under simulated export conditions, to achieve cero survivors in three consecutive tests with > 10,000 forms of the most tolerant pest stage exposed to the treatment. The trials were conducted at the laboratories of the Department of Plant Protection of the Universidad Politécnica de Valencia (Spain).

MATERIALS AND METHODS

Test fruit

Fruits of mandarin (*Citrus reticulata*) of the variety Clementina, standardized size number 3 (54-64 mm of diameter, average weight per fruit: 65-70 g/fruit) with a homogeneous grade of maturity which has reached its normal colour, suitable for the survival of immature stages of test insects at the time of infestation. The fruits were picked in the same week of the trials. Fruits have received the following field treatments: mineral oil 1,5 % + Malathion 50 at 0,2%, in summer (5 months before harvesting). Fruits for test of development and survival of *C. capitata* on Clementine mandarins, when they were received in the laboratory, were dipped in a solution of Imazalil at 2.500 mg/kg a.i. and later allowed dip dry.

Fruits for the determination of the most cold tolerant stage of *C. capitata* went through the next post harvest packing process which was finished the day before to the use of the fruits.

The products used on each treatment of the process have been:

FOAM CURTAIN: 20% SOPP 10% (2% a.m)

WAXING: (water emulsion wax 18% s.s +TBZ 5.000,g/kg+ Imazalil 3.000mg/kg.

Test insects

Wild strains of *C. capitata* breed in laboratory. For this, fruits of sweet oranges (*Citrus sinensis* Osbeck) with attack symptoms of *C. capitata* from different citrus orchards of the province of Valencia were collected. Insects were reared on sand trays in order to obtain pupae of the wild strain. The flies were gradually adapted to the rearing laboratory conditions, with periodical renewals of pupae obtained by the above mentioned method.

For breading the insect, 3,500 pupae were introduced into a methacrylate cage (40x 25x 20cm) with its front part cover by a 0,5 mm mesh. Through this mesh the eggs are oviposited and recollected in a container with water placed on the base of the cage. Once produced the emergence, feeding and pairing of adults, eggs are daily picked by infiltration and sown in 37 x 12 cm containers with a depth of 1.5 cm. The trays are full with the following diet.

Wheat mill-feed	300gr
Sacarose	75gr
Brewers yeast	36gr
Nipagin- Na	2gr
Propil paraben	2gr
Benzoic- acid	2.4 gr
Destilated water	600cm^3

Each tray, with the diet sown with *C. capitata* eggs, is covered by another container with the same dimensions in order to avoid desiccation. Also, temperature and RH are kept, 25±0,5ºC, 70±10 %RH, with absence

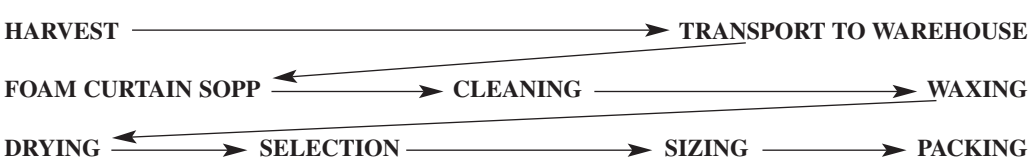

Figure 1. Inoculation of *Ceratitis capitata* eggs solution into fruits.

of light. After eggs have hatched out and larvae have reached their highest development, they leave the tray, pupating outside, inside the double filter paper. Helped by a fine brush, pupae are picked and the picked and they are placed in a clean cage to start a new cycle. Cages with adults are under the same temperature and relative humidity conditions as larvae, illuminated by 40 W fluorescent tubes, which are placed with a separation of 20 cm among them and 30 cm from the top of the cage, with a photoperiod of 12/12 hours light/darkness. Adults are fed by a mixture of sacarose and protein hydrolisate (ratio:4/1). Water is provided by permanent humid match-rope.

Fruits infestation

C. capitata does not readily oviposit on citrus with sufficient number of eggs to prepare satisfactory disinfestation trials. To obtain sufficient insects of the different life stages it was necessary to artificially infest the fruits. For so, eggs of *C. capitata* laid during the daytime (12±5 h old) are prepared by mixing them with a finely divided 0.25 % agar gel containing a 1% mixture of 10% aqueous solution of alkyldimethylbenzylamonium chloride, such a way that a suspension of 1000 eggs/cm^3 is formed.

Fruits were infested by a glass syringe (FRIEND, 1957) fitted to insert eggs in the separation area pulp-albedo. They were injected 0.2 cm^3 of solution (200 eggs) on each fruit (Figure 1). To prevent eggs medium leakage and fungi entry, a drop of polyvinylacetate glue was applied to the needle puncture.

Insect Development:

To determine the *C. capitata* development (evolution of the different life stages in the time) on the Clementine mandarins 140 fruits were inoculated with 200 eggs/ fruit. Fruits were stored in laboratory under conditions of 24± 1ºC and 65± 2% RH. From the second day after inoculation, five fruits were

Figure 2. Refrigerator with fruits introduced into net bags.

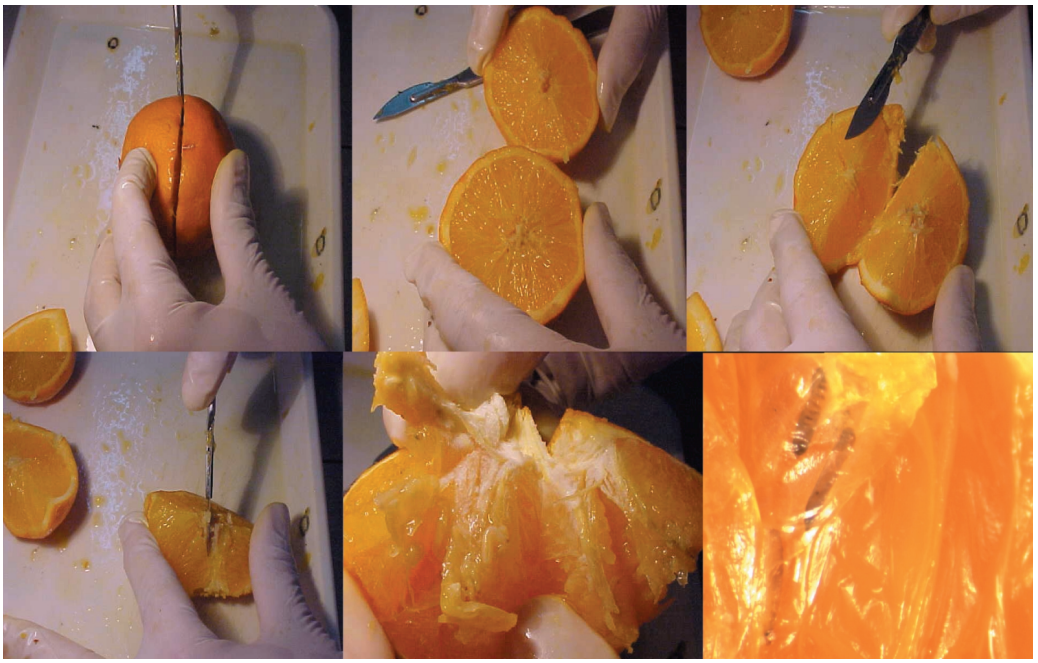

Figure 3. Cut of fruits for extraction of the larvae.

cut in a half every day, scoring the number of larvae found, their stage of development and number of pupae in the bottom of the container prepared for so. Three replications of each test have been made.

Determination of the Most Tolerant Stage to Cold Treatment

A total of 500 Clementine mandarin fruits were inoculated with 200 eggs/fruit (0.2 cm^3 of a solution of 1000 eggs/cm^3). 160 fruits were used for each stage of the insect: 20 hours eggs; young larvae (50% L1 and 50% L2): 6 days after inoculation; mature larvae (mainly L3): 14 days after inoculation. For each of these stages, 160 fruits (8 groups of 20) were introduced in a refrigerator with a precision thermostat at 2ºC±0.5ºC, leaving as control 20 fruits, under laboratory conditions (24±1 ºC and 65% RH), in one recipients with a double filter paper in the bottom to allow the insect pupation. Each group of 20 fruits was introduced into net bags to make their extraction after the treatment easier (Figure 2). Temperatures have been recorded by THERM LOGGER of Lang & Stelman A/S. with 3 channels for temperature sensors with a range of temperatures from -32ºC to 70ºC, 0.2 ºC precision and 0.1ºC resolution, and a range of relative humidity from 0 to 98% RH). The records of temperature are made every hour with a ±1 second/week precision. The following temperature measures have been made: In the pulp of 4 fruits placed in different positions and in 2 different points of the treatment chamber (inlet and outlet air temperatures of the cooler). All thermistor probes were calibrated in melting ice with a certified thermometer, before and after each trial to verify their accuracy. The treatment is considered started when the 50% of the sensor fruits reach 2ºC of temperature.

After 2, 4, 6, 8, 10, 12, 14, 16 days groups corresponding to each insect stage were

removed from the refrigerator. The fruits were cut after storage in laboratory until they reached 25ºC in order to observe the presence of larvae alive. Each of the next fruit groups were cut after 7 days in case of eggs, 4 days in case of young larvae and 2 days in case of mature larvae. Each fruit was cut in four pieces and they were crumbled in water. The obtained solution was filtered through a fine mesh, retiring large pulp pieces. The rest was poured into a 10% common salt solution in order to larvae over float. Microscopic examination determined that this method extracted all the larvae presents. Larvae and macerated fruit pulp were then examined under magnification and illumination, and the number of live individuals was recorded (Figures 3 and 4). Survivors are considered the larvae that after extraction make any movement. The effects of the cold treatment have been evaluated obtaining the corrected mortality, according to the ABBOTT's method (1925), from the number of larvae alive found in cut fruits.

Large-scale trials

These trials involved that the most tolerant stage of *C. capitata* is completely destroyed by cold treatment at 2±0,5ºC for 16 days under commercial conditions.

Fruits of *Citrus reticulata* of the variety Clementine, with the above-mentioned characteristics, were packed in Dutch wood boxes of 10 kg (44 cm height x 29 cm base) with 140/154 fruits/box.. A total of 1,050 fruits (7 boxes) were infested; 750 fruits (5 boxes), randomly placed with non infested boxes, in four pallets of 100 x 120 cm set up in solid block configuration with 9 boxes per level and 10 boxes of height, were treated in semi industrial cold chambers of small volume (15 m^3) and 300 fruits (2 boxes) randomly selected from the 7 boxes with infested fruits were left as control fruits (Figure 5). Load factor of the cold chamber was 77% in volume (351 kg of fruit/m^3).

Temperatures have been recorded with the same type of recorders used in the determination of the most tolerant stage.

Figure 4. Extraction method of larvae from fruit pulp.

The location of sensors was:

a) Pulp sensors: have been placed in 12 points located in 3 different plans: high, medium and low (Figure 5), in the center of the load, exit of cold air, opposite part of the exit of the cold air flow and one corner of the chamber.

b) Air sensors: Sensors have been placed in three points: cold air delivery, air return and in the center of the top of the chamber.

Three replications have been made.

Treatment is considered started when the 50% of the pulp sensors have reached the temperature of 2. ºC.

Figure 5. Storage of the fruits in the large-scale test and location of the temperature sensors at different plans.

Effects of cold treatment on the fruits quality:

Treated fruits have been stored in the same conditions and during the same period of time than the necessary to transport the fruits to final customers: 16 days at 2ºC. (Quarantine period of treatment) + 12 days at 5ºC (The rest of the time to transport the fruits to Japan) + 7 days at room temperature (marketing period). After this time, the quality of the fruits has been evaluated in accordance with the International Quality Standards (OCDE, 1971). For this purpose, 12 boxes have been selected from 4 different positions at 3 different heights of the cold chamber. Before the introduction of the fruits into the cold chamber and at the exit of the boxes from it, once the transport and marketing period has expired, each box has been weighted and it has been determined the qualitative characteristics of the fruits, pathologic and physiological alterations, appearance, flavor and taste. Three replications have been made.

RESULTS AND DISCUSION

Insect Development:

The details of development of *C. capitata* were established before every series of trials.

Results are exposed in Table 1 and average values are shown in Figure 6.

Table 1. **Development of *C. capitata* inside mandarin fruits of the variety Clementina stored at. 24± 1ºC and 65± 2% RH. . Number of different stages daily collected after inoculation L1 First larval instar; L2 Second larval instar; L3 Third larval instar; P: pupae.**

Stage	Days																				
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
First replication																					
L1	73	63	34	27	28	15	12	11	6												
L2			13	23	26	30	37	30	17	17	7										
L3								9	9	32	34	36	28	36	19	9	2	3	2		
P												7	13	15	26	29	35	36	31	34	37
Second replication																					
L1	47	50	24	17	29	20	3														
L2		8	24	18	27	24	32	20	5	3	2										
L3							0	30	36	29	26	18	24	12	17	8	6	3	2		
P												5	5	36	35	27	36	47	38	39	
Third replication																					
L1	50	47	44	20	21	12	6	5	2												
L2	50	0	0	17	20	15	23	36	35	14	20	17									
L3									15	24	22.5	18	24	28.5	12	1.5	6	3	3		
P												5	5	8	29	27	41	30	36	47	

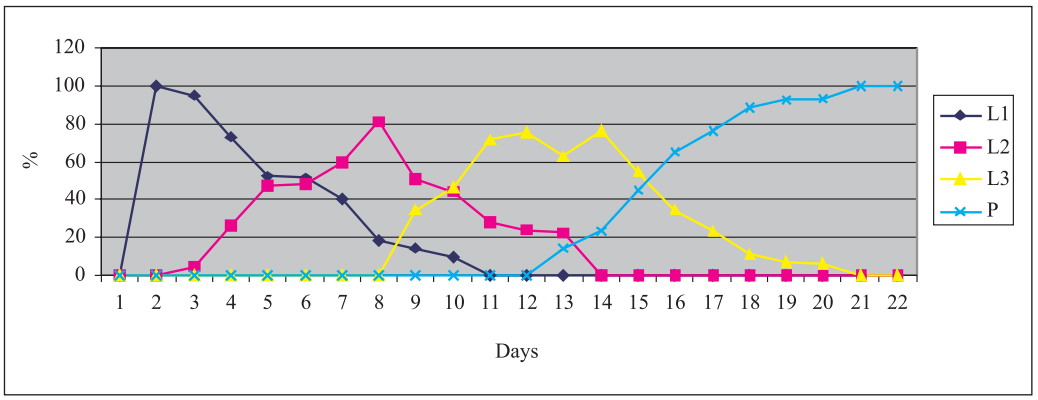

Figure 6. Evolution of *C. capitata* in Clementine mandarin at 22 ºC.

The duration of the life cycle (from inoculation to 100% pupae) is 22 days. The young larvae (50% of L_1 and 50%L_2) is reached between the 5th and the 7th day after inoculation and the maximum of old larvae (L_3) occurs after 12 days of inoculation, when the fruits are stored at 24± 1ºC and 65± 2% RH. The results were used to determine the date which the insect reach each stage.

Most cold tolerant stage

Data of the percentage dying at each exposure period in days are given in Tables 2 and 3.

The resume of the recorded temperatures registered in the pulp sensors is the following.

Replications	Temperatures in ºC			
	T. max	T. min	Average Temperature	Standard Deviation
1	2,6	1,4	2,0	0,1669
2	2,6	1,3	2,1	0,2438

The distribution of frequencies on each of the two replications was:

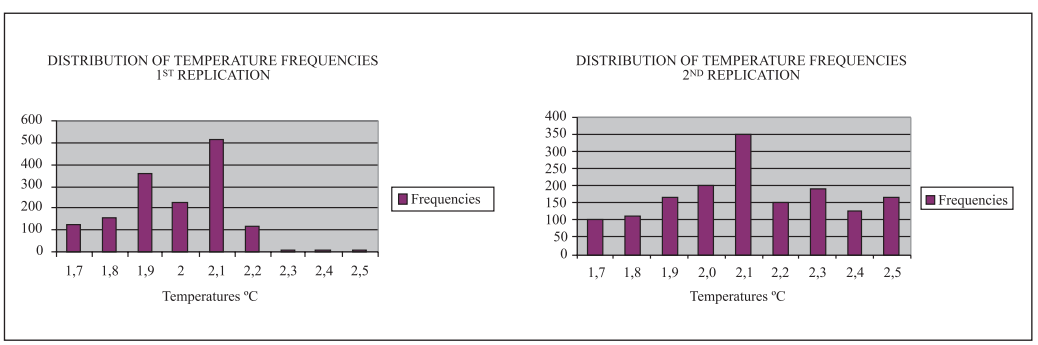

Table 2. **Mortality of each stage of *C. capitata* in Clementine mandarin fruits treated at 2ºC during different exposure periods. First replication:**

Insect stage	Exposure period (days)	Number of fruits	Number of eggs inoculated	Alive insects	% Corrected Mortality (Abbott's method)
Eggs	**Control**	**20**	**4,000**	**295**	**————**
	2	20	4,000	145	50.48
	4	20	4,000	32	89.15
	6	20	4,000	10	95.60
	8	20	4,000	2	99.32
	10	20	4,000	0	100
	12	20	4,000	0	100
	14	20	4,000	0	100
	16	20	4,000	0	100
Young larvae L1-L2	**Control**	**20**	**4,000**	**417**	**————**
	2	20	4,000	211	49.4
	4	20	4,000	86	79.38
	6	20	4,000	25	94.00
	8	20	4,000	12	97.12
	10	20	4,000	7	98.32
	12	20	4,000	4	99.04
	14	20	4,000	0	100
	16	20	4,000	0	100
Mature larvae L3	**Control**	**20**	**4,000**	**437**	**————**
	2	20	4,000	214	51.03
	4	20	4,000	90	79.40
	6	20	4,000	33	92.45
	8	20	4,000	9	97.94
	10	20	4,000	5	98.86
	12	20	4,000	3	99.31
	14	20	4,000	0	100
	16	20	4,000	0	100

Table 3. **Mortality of each stage of *C. capitata* in Clementine mandarin fruits treated at 2ºC during different exposure periods. Second replication:**

Insect stage	Exposure period (days)	Number of fruits	Number of eggs inoculated	Alive insects	% Corrected Mortality (Abbott's method)
Eggs	**Control**	**20**	**4,000**	**295**	**————**
	2	20	4,000	140	50.18
	4	20	4,000	25	31.10
	6	20	4,000	4	98.58
	8	20	4,000	1	99.64
	10	20	4,000	0	100
	12	20	4,000	0	100

Eggs	**Control**	**20**	**4,000**	**295**	————
	14	20	4,000	0	100
	16	20	4,000	0	100
Young larvae L1-L2	**Control**	**20**	**4,000**	**403**	————
	2	20	4,000	226	45.90
	4	20	4,000	89	77.91
	6	20	4,000	22	94.54
	8	20	4,000	11	97.27
	10	20	4,000	9	97.77
	12	20	4,000	2	99.50
	14	20	4,000	0	100
	16	20	4,000	0	100
Mature larvae L3	**Control**	**20**	**4,000**	**498**	————
	2	20	4,000	226	54.62
	4	20	4,000	109	78.11
	6	20	4,000	43	91.36
	8	20	4,000	15	96.99
	10	20	4,000	5	98.99
	12	20	4,000	2	99.60
	14	20	4,000	0	100
	16	20	4,000	0	100

Using the Probit response models (Probit Analysis) it has been checked that there are no significant differences between replications (P<0,05), so it has been considered not necessary to make the third replication, according to the protocol agreed with Japanese authorities. It has been carried out a Probit regression analysis, checking the suitability of the fit of the data to the lineal model of and test of parallelism for the groups. The calculations have been made with the statistic package SPSS for Windows. In the Figure 7 has been represented the corresponding regression lines.

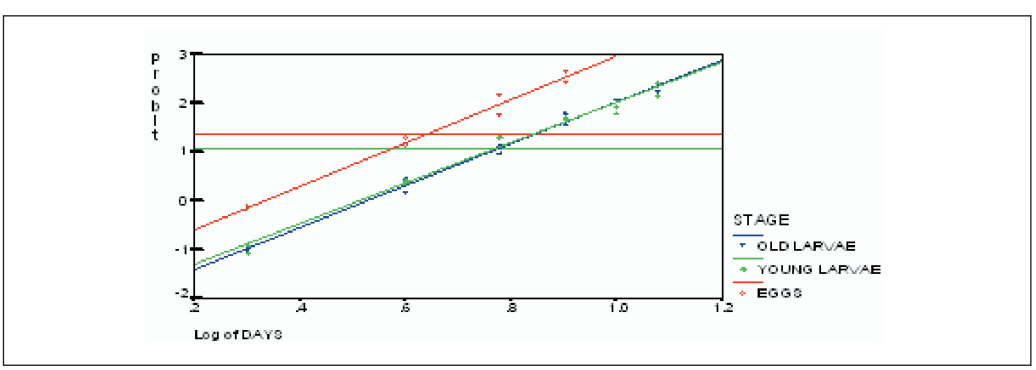

Figure 7. Probit transformed responses.

Survival of eggs and larvae on infested fruits decrease with the increasing of the time of storage at 2ºC. With treatments of 14 days the larval mortality has been total. With 10 days of treatment no larvae alive has been found in treated fruits with eggs. The data reasonable fit to the lineal model (P<0,05) and the hypothesis of parallelism of the lines can be accepted. The 20 hours eggs are significantly more sensible to cold than the larvae. No significant differences have been found between mature and young larvae (P<0,05).

The confidence intervals at 95% for the necessary days to reach the level of mortality probit 9 for each of the insect stages studied are:

Stage	**Days of CT**	**Confidence interval**
Eggs	7.33	6.89-7.84
Young larvae L1-L2	11.22	10.60-11.92
Mature larvae L3	11.70	11.06-12.43

This result is concordant with the results obtained by HILL *et al.* (1988) with Valencia oranges at 1.5±0.5 ºC and with the results obtained in Valencia-late and Navel oranges (SANTABALLA *et al.*,1995) and Salustiana oranges (SANTABALLA *et al.*,1997).

Large-scale trials

The resume of the recorded temperatures registered in the pulp sensors is the following.

Replications	Temperatures in ºC			
	T. max	T. min	Average Temperature	Standard Deviation
1	2.5	1.9	2.2	0.0823
2	2.5	1.9	2.0	0.1
3	2.5	2.0	2.2	0.737

The distribution of temperature frequencies on each of the three replications has been:

Results obtained are shown in table 4.

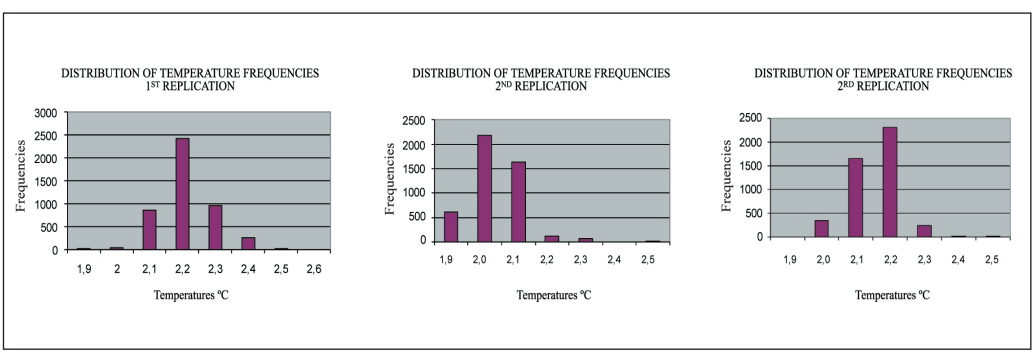

Table 4. **Results of the large scale-test. Mortality of old larvae of *C.capitata* on Clementine mandarin fruits.**

Replication	Control Fruits		Treated Fruits			
	Number of fruits	Alive insects	Number of fruits	Treated insects	Number of survivors	% Mortality
1	294	4,527	735	11,317	0	100
2	286	4,118	715	10,295	0	100
3	302	4,167	752	10,376	0	100
Total	882	12,812	2,202	31,988	0	100

In the control fruits the number of pupae recovered from 3 replicates exceeded 12,800 in 882 fruits, whereas no pupae were obtained from 12,812 treated fruits

The cold treatment at 2 ± 0,5ºC during 16 days has shown its total effectiveness against more than 30,000 insects (31,988) treated.

Qualitative characteristics of the fruit:

Table 5. **Variation of the qualitative characteristics of the fruits. Average values after three replications.**

	Box weight	% Juice	º Brix	Acidity (NaOH)	Ripeness Index
Before treatment	10.00	53.1	10.4	7.51	11.77
After treatment	9.68	50.4	11.3	7.23	12.45
% Variation	-3.2	-5.08	+8.6	-3.73	+5.77

*There is no appreciation of variation in the flavor, taste or appearance of the fruits.

Table 6. **Pathologic and physiological alterations**

Evaluated fruits	Rotten fruits	% Rotten fruits	Physiological alterations Pitting, Adustosis, Membrosis peteca, Red blotch
5.292	7	0.13	0

CONCLUSIONS

The development of *C. capitata* in Clementine mandarin fruits is similar to the development in oranges and faster than in lemons.

The larval stages are more resistant to cold than the eggs, without significant differences between young larvae (50% L1+50% L2) and mature larvae (L3).

After 14 days of cold treatment the larval mortality is total.

Cold treatment at 2ºC for 16 days in industrial conditions has shown a total effectiveness against the most cold tolerant stage of *C. capitata* and it can be taken with a total guarantee as a quarantine treatment against this pest.

Refrigerated treatments, followed by transport and marketing period of the fruit, have not provoked substantial modifications in the qualitative characteristics of the fruits. No physiological alterations have been found and the rotes, caused all by *Penicillium* sp., are the usual in any marketing process.

RESUMEN

SANTABALLA, E., R. LABORDA, M. CERDÁ. 2009. Tratamiento frigorífico de cuarentena contra C*eratitis capitata* (Wiedemann) (Diptera: Tephritidae) para la exportación de clementinas a Japón. *Bol. San. Veg. Plagas*, **35**: 501-512.

Como consecuencia de la presencia en España de la mosca mediterránea de la fruta, *Ceratitis capitata* (Wied), la exportación de cítricos, a países que están libres de esta plaga, está restringida por regulaciones de cuarentena.

En el presente trabajo, se han llevado a cabo una serie de ensayos en mandarinas clementinas usando los procedimientos de bioensayos estandardizados, seguidos por pruebas a gran escala.

Los frutos infestados por huevos o primeros estadíos larvarios (L_1, L_2) y el 3er estadío larvario (L_3) de este insecto, fueron almacenadas durante diversos periodos a 2±0.5 ºC. Como resultado se demostró que los huevos son el estadío más susceptible al frio, mientras que no se encontraron diferencias significativas entre los distintos estadíos larvarios.

La efectividad del tratamiento de frío sobre *Ceratitis capitata* aplicado durante 16 días a 2±0.5 ºC, ha quedado demostrada al obtenerse un 100% de mortalidad en más de 30.000 larvas tratadas bajo condiciones industriales.

No se observaron modificaciones sustanciales en las características cualitativas de la fruta. Tampoco se encontraron alteraciones fisiológicas y las rutinas son las normales de cualquier proceso de marketing.

Palabras clave: Mosca mediterránea de la fruta, *Citrus reticulata*, cítricos, exportación Japon.

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