Controlled *in vivo* infestation of mandarin fruit with *Ceratitis capitata* for development of quarantine treatments

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Abstract

Movement of citrus fruit from *Ceratitis capitata*-infested areas requires mandatory quarantine treatments. Development of such treatments requires the use of infested fruit. The *in vivo* approach is the most realistic way to obtain these fruit. However, it requires previous studies to determine the optimal fruit:fly ratio to minimize the number of decayed fruit and to maximize the number of flies per fruit obtained. In this study, the optimal fruit:fly ratio for the *in vivo* infestation of mandarin fruit was investigated. The effect of different fruit:fly ratios from 1:5 to 1:50 for an exposure time of up to 3 days on the number of both decayed fruit and puparia per fruit was determined. Provided that an adequate fungicide treatment is applied before the infestation to avoid fruit decay, the use of a 1:10 fruit:fly ratio for 48 h is enough to obtain almost 20 healthy puparia per fruit. These results allow the use of the *in vivo* approach to develop quarantine treatments against *C. capitata* in mandarins.

Additional key words: citrus, Mediterranean fruit fly, postharvest.

Resumen

Infestación *in vivo* controlada de mandarinas con *Ceratitis capitata* para el desarrollo de tratamientos cuarentenarios

La fruta producida en áreas infestadas por *Ceratitis capitata* está sujeta a tratamientos cuarentenarios cuyo desarrollo requiere la utilización de fruta infestada. La manera más realista de obtener esta fruta es mediante técnicas de infestación *in vivo*. Sin embargo, previamente hay que realizar estudios para determinar la proporción óptima frutos:mosca a utilizar, de manera que se minimicen las pérdidas de fruta por pudrición, y se maximice el número de moscas obtenido por fruto infestado. En este estudio se ha investigado esta proporción para mandarinas. Se ha determinado el efecto de distintas proporciones frutos:mosca, entre 1:5 y 1:50, durante un periodo de hasta 3 días, tanto sobre el número de frutos podridos como sobre el de puparios obtenidos por fruto. Siempre que se aplique un tratamiento fungicida fuerte previamente a la infestación para evitar pudriciones, el uso de una proporción frutos:mosca de 1:10 durante 48 h es suficiente para obtener casi 20 puparios sanos por fruto. Estos resultados permiten utilizar la infestación *in vivo* en el desarrollo de tratamientos cuarentenarios contra *C. capitata* en mandarinas.

Palabras clave adicionales: cítricos, mosca mediterránea de la fruta, post-recolección.

Introduction

Among a multitude of insect pests of quarantine importance, fruit flies of the family Tephritidae are probably the most important group worldwide. The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), has become the single most important pest species in the family because of its worldwide distribution. In Mediterranean countries, it is particularly damaging on citrus and peaches. *Ceratitis capitata* is thought to have originated in the Paleotropical region from where it spread to the Mediterranean basin and parts of Central and South America and Australia (EPPO, 2007). However there are some areas in Asia, Oceania and America which remain free of *C. capitata* (EPPO, 2007). When host fruit such as citrus from *C. capitata*-infested areas are shipped to these pest-free areas where the fly could become established, fruit must be subject to quarantine treatment ensuring that no viable insects are present at destination. The most widely used postharvest disinfestation treatment for citrus against this fruit fly involves exposure of the fruit to near-freezing temperatures. In the case of the USA, the U.S. Department of Agriculture established a minimum exposure during overseas transit of 14-18 days at 1.1-2.2°C (USDA, 2002). Extensive research is currently focused on the development of alternative or complementary quarantine treatments, especially for cold sensitive commodities such as citrus. As a consequence alternative or additional treatments are under development for Spanish citrus exports (Alonso *et al*., 2005a, 2007; Palou *et al*., 2007, 2008).

Although a quarantine treatment ideally would be devised using feral insects infesting the fruit naturally, this approach is rarely considered because of the difficulty in obtaining the sufficient number of insects of the correct stage in good condition (Hallman, 2004). Therefore, fruit fly larvae for development of quarantine treatments have been usually obtained following one of the following procedures (Hallman and Loharanu, 2002): (a) *in vitro* with or without a rearing medium (e.g. Balock *et al*., 1963; Benschoter, 1987; Sharp and Chew, 1987; Mansour and Franz, 1996), (b) reared on one medium followed by insertion into fresh fruit (e.g. Kamburov, 1972; Alonso *et al*., 2002a,b, 2005b; Palou *et al*., 2007, 2008), and (c) *in vivo* reared in artificially

infested fruit (e.g. Seo *et al*., 1973; Windeguth and Gould, 1990; Hallman and Worley, 1999; Hallman and Martínez, 2001; Agnello *et al*., 2002). The latter approach is *a priori* the most realistic case, but requires studies to determine the optimal fruit to fly ratio (fruit:fly ratio henceforth) during infestation and the duration of the exposure to minimize the number of fruit where the life cycle of *C. capitata* will not be completed. An important aspect of this study is to counter fruit decay which can significantly affect fruit fly survival. It is important to have sufficiently high number of flies per fruit to allow robust statistical analyses. Hallman and Worley (1999) and Hallman and Martínez (2001) in their studies with grapefruit (*Citrus paradisi* Macf.) and the Mexican fruit fly, *Anastrepha ludens* Loew., used a fruit:fly ratio ranging from 1:50 to 1:100 for a period of 24 to 48 h. However these authors did not provide any data to estimate the number of decayed fruit, or the mean number of flies obtained per fruit.

Spanish citrus exports to *C. capitata*-free countries consist mostly of mandarins, especially clementines (76×10^3 Mg during the 2006-07 season; Font de Mora, 2007), such as 'Clemenules' or 'Marisol' cultivars. Recent market access and postharvest studies have focused on these citrus cultivars (Alonso *et al*., 2002b, 2005b, 2007; Palou *et al*., 2007, 2008) and did not use the *in vivo* approach. Before such an approach could be used it is necessary to define an efficient infestation procedure. Therefore the objective of this research was to establish a convenient fruit:fly ratio and appropriate exposure times for the development of quarantine treatments in mandarins.

Material and Methods

Ceratitis capitata

Insects used in this assay originated from a laboratory colony established in 2001 at the Institut Valencià d'Investigacions Agràries (IVIA). This colony has been periodically supplemented with the introduction of wild flies from naturally-occurring infested fruit during summer and fall. Adult *C. capitata* were reared in a controlled environment cabinet at $25 \pm 1°C$ and $75 \pm 5\%$ relative humidity under illumination by fluo-

rescent tubes (Sylvania F-18W/Grolux; 16 h day^{-1}; 2,500 lux). Unless otherwise stated, the same environmental conditions were applied to the all assays reported in this study. Flies were kept in perspex cages (40×40×30 cm) with a density of 2,000 flies per cage. These cages had two round holes (8 cm in diameter) on the upper side covered by a mesh and the front covered by gauze used by females for oviposition. Water and a diet consisting of a mixture of enzymatic autolyzed brewer's yeast and sugar (1:4, w:w) were supplied to the flies. Eggs were laid through the gauze and fell into a dish containing water from which they were collected daily by filtering (Jacas and Viñuela, 1994). Immature stages were reared on an artificial diet containing 400 g of wheat bran, 112 g of sugar, 58 g of brewer's yeast, 4.5 g of methyl paraben, 4.5 g of propyl paraben, 4 g of benzoic acid, and 900 mL of water, using a density of 4 eggs g^{-1} diet (Alonso *et al*., 2002a). Mature puparia were used in all assays.

Infestation

Infestation took place in plastic cages (2×20×30 cm) with a lid consisting of a gauze hold in place by a frame. A glass vial (5 cm high and 1 cm in diameter) containing 0 (control) to 500 puparia of *C. capitata* was fixed with tape to one of the corners of the cage. Water was supplied in a 50 mL glass Erlenmeyer with a wick. The same diet as described for the rearing was spread on top of the lid for adults to feed *ad libitum*. Five petri dishes each containing 10 puparia were assembled to assess adult emergence and sex ratio. Upon fly emergence, cages were checked daily until newly laid eggs were observed outside the gauze. Two days later, both cages and fruit were prepared for the infestation. Fruit

from orchards in the Valencia area were harvested at commercial maturity and transferred to the IVIA postharvest facilities where they were sorted, randomized, washed with tap water and dipped in a fungicide solution for 1.5 min (as described in Table 1). After the fruit had air dried, groups of 10 fruits were introduced in the cages which had been previously cooled at 12°C for 1 h to prevent flies from flying away during manipulation. After the introduction of the fruit at 25°C, the cages were left undisturbed for 24 to 72 h depending on the assay. After this time, fruit were removed from the cage and individually placed in 1-L cloth covered plastic boxes where both decayed fruit and the number of puparia were scored after 20 days. From these data, the percentage of decayed fruit per cage and the number of puparia per fruit were calculated.

Three separate experiments were conducted, each one using the mandarin cultivar that was commercially available at that time (Table 1). The first experiment used clementine mandarins (*Citrus reticulata* Blanco) cv. Marisol previously treated with the fungicide imazalil sulphate at a concentration of 0.5% (Fecundal-S 7.5, Janssen Pharmaceutica N.V., Beerse, Belgium) and were exposed to either 0, 5, 10, 20, 30 or 50 flies per fruit for one day. Three cages per fruit:fly ratio were used. The second experiment used hybrid mandarins cv. Nova [*C. reticulata* × (*C. paradisi* × *C. reticulata*)] treated with a mixture of the fungicides imazalil sulphate at 2.5% (Fecundal-S 7.5), guazatine acetate at 0.8% (Textar 20G, Tecnidex S.A., Paterna, Valencia, Spain) and thiabendazole at 1.5% (Tebezeta-45, Fomesa Fruitech S.L., Valencia, Spain) and exposed to either 0, 5, 10, 20 or 30 flies per fruit for either one, two or three days. One cage per each combination dose/ exposure was used. The third experiment used clementine mandarins cv. Clemenules which received a fungi-

Table 1. Assays performed: postharvest fungicide dip treatments applied before artificial infestation, mean emergence (percentage ± SE) and sex ratio (percentage females ± SE) of the flies used, and physical characteristics of infested fruit. Data followed by the same letter were not significantly different according to Duncan's multiple comparison procedure ($P \leq 0.05$)

Assay	Fruit cultivar tested	Fungicide treatment (% a.i.)	Emergence (%) (n = 5)	Sex ratio (% females) (n = 5)	Rind width (mm) (n = 10)	Rind color index[1] (n = 35)
1	'Marisol'	Imazalil sulphate (0.5%)	94.0 ± 6.0	52.6 ± 5.6	2.19 ± 0.07b	7.5 ± 1.65
2	'Nova'	Imazalil sulphate (2.5%) + guazatine acetate (0.8%) + thiabendazole (1.5%)	88.0 ± 8.0	51.8 ± 3.5	2.78 ± 0.21a	22.0 ± 1.50
3	'Clemenules'	Imazalil sulphate (2.5%) + guazatine acetate (0.8%) + thiabendazole (1.5%)	96.0 ± 2.4	50.0 ± 3.2	2.01 ± 0.13b	16.56 ± 0.50

[1] CI = 1000a/L*b (Hunter parameters).

cide treatment as in the previous assay and exposed to either 0, 5, 10, 20, 30 or 50 flies per fruit for two days. Three cages per fruit:fly ratio were used. Each cage was considered as a single replicate for decayed fruit analysis whereas each fruit constituted a replicate for the analysis of the number of puparia per fruit. Prior to infestation, rind color of 35 fruit was measured as Hunter parameters (L, a, b) using a colorimeter (Minolta, Model CR-300). For each fruit, three measurements along the equatorial area were performed. The specific color index (CI) for citrus was calculated as CI = 1000a/L*b (Jiménez-Cuesta *et al*., 1981). The rind width was measured using a digital Vernier caliper, where 10 fruits were equatorially divided in two halves and one measurement was conducted on each half.

Statistical analyses

Data were subjected to either one- or two-way analyses of variance (ANOVA) and, where appropriate, means were separated by Duncan's multiple comparison procedure ($P \leq 0.05$). If necessary, data were arcsine-transformed before analyses to prevent the violation of the assumptions underlying ANOVA (Sokal and Rohlf, 2000). Analyses were performed using the Statgraphics Plus 4.1 software package (Manugistics Group Inc., Rockville, MD, USA).

Results

Both rind width and color of the fruit used in these assays depended on the variety used (Table 1). Rind width ranged from 2.0 to 2.8 and 'Nova' mandarins were significantly the thickest ones. Rind color depended on the variety used and ranged from 7.5 to 22.0 for 'Marisol' and 'Nova' mandarins, respectively.

The adult emergence and sex ratio of the flies used to infest those fruit are shown in Table 1. Emergence ranged from 88.0 to 94.0% and around 50% of these flies were females. These values fit within the values usually obtained under laboratory conditions. Based on the percentages of adult emergence obtained, the actual fruit: fly ratios achieved in our assays were slightly lower than desired (from 4.4 to 48 flies per fruit instead of 5 to 50 flies per fruit, respectively).

The percentage of decayed fruit was very high in the first experiment (Fig. 1) and this level of decay was dependent on the fruit:fly ratio used for infestation ($F = 15.66$; $df = 5, 12$; $P < 0.0001$). There was no decay
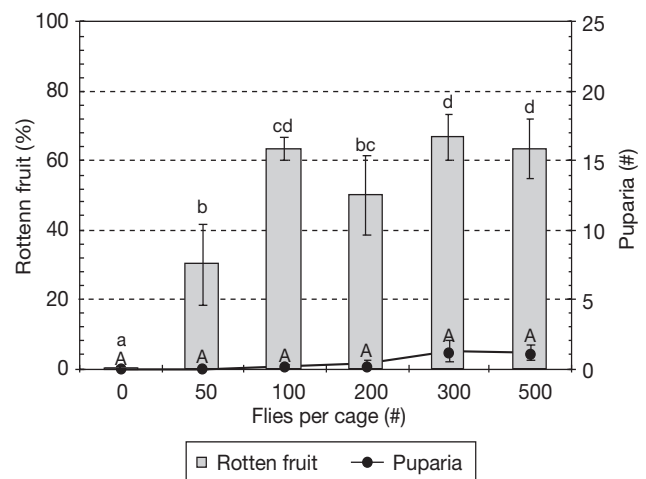
Figure 1. Percentage of decayed fruit and number of puparia obtained per fruit (mean ± SE) after infestation of 'Marisol' clementine mandarins using different fly densities (0-500 flies per cage) during 1 day. Means based on three cages containing 10 fruits for each fly density tested. Bars or points exhibiting the same letter were not significantly different according to Duncan's multiple comparison procedure ($P \leq 0.05$).

in the control fruit but it significantly increased from 30.0% to 63.3% for 1:5 and 1:50 ratios, respectively. Such a high percentage of decay drastically reduced the number of fruit were *C. capitata* larvae could complete their life cycle. As a consequence, the mean number of puparia obtained per fruit was very low (0.55 individuals) irrespective of the fruit:fly infestation ratio used ($F = 1.16$; $df = 3, 5$; $P = 0.4108$).

Because of the high percentage of decayed fruit obtained in the first assay, fruit used in subsequent assays were subjected to a more intense fungicide regime (Table 1). This new treatment dramatically reduced the percentage of decayed fruit (Fig. 2 and 3) and as a consequence, the number of larvae that could complete their development increased (Fig. 4). This depended on both the fruit:fly ratio used ($F = 6.92$; $df = 4, 135$; $P < 0.0001$) and the length of exposure ($F = 6.16$; $df = 2, 135$; $P < 0.0001$) but there was no interaction between these two factors ($F = 1.77$; $df = 8, 135$; $P = 0.0880$). The number of puparia per fruit was the lowest for the 1:5 ratio (1.43 puparia per fruit) and the highest for the 1:30 ratio (39.7 puparia per fruit). Ratios of 1:10 and 1:20 were not significantly different from each other and yielded a mean of 16.35 puparia per fruit. A one day exposure resulted in a mean of 5.00 puparia per fruit and this value was significantly lower than that obtained when exposure lasted either two or three days (mean of 18.30 puparia per fruit). Therefore two
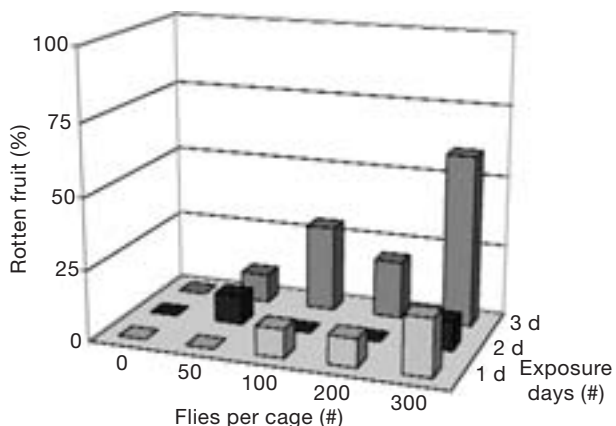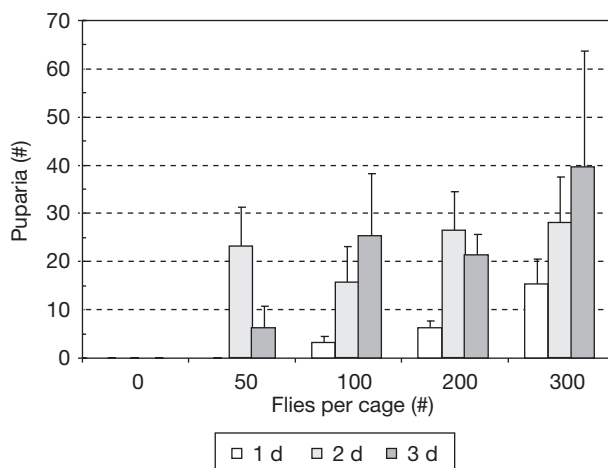
Figure 2. Percentage of decayed fruit obtained after infestation of 'Nova' mandarins using different fly densities (0 to 300 flies per cage) during either 1, 2 or 3 days. Values based on one cage containing 10 fruits for each combination of fly density and exposure time tested.

days was the only exposure time selected for the third experiment.

The percentage of decayed fruit in the third experiment did not depend on the fruit:fly ratio ($F = 1.60$; $df = 5, 17$; $P = 0.2336$) and ranged from 3.3 to 10.0% (Fig. 3). However this ratio significantly affected the number of puparia obtained per fruit ($F = 4.59$; $df = 5, 174$; $P = 0.0006$, Fig. 3), which was significantly lower for the 1:5 ratio (9.70 puparia per fruit) than for the rest of ratios tested (mean of 18.38 puparia per fruit).
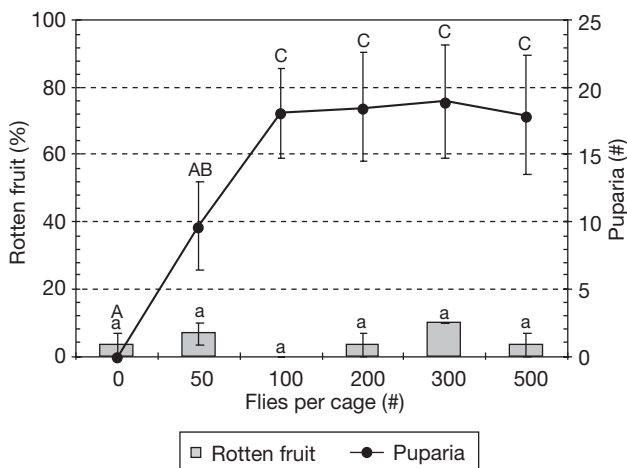
Figure 3. Percentage of decayed fruit and number of puparia obtained per fruit (mean ± SE) after infestation of 'Clemenules' clementine mandarins using different fly densities (0-500 flies per cage) during 2 days. Means based on three cages containing 10 fruits for each fly density tested. Bars or points exhibiting the same letter were not significantly different according to Duncan's multiple comparison procedure ($P \leq 0.05$).

Figure 4. Number of puparia obtained per fruit (mean ± SE) after infestation of 'Nova' mandarins using different fly densities (0 to 300 flies per cage) during either 1, 2 or 3 days. Values based on one cage containing 10 fruits for each combination of fly density and exposure time tested.

Discussion

Three different mandarin cultivars were used in these infestation experiments. Therefore differential sensitivity to decay and differential resistance to medfly infestation could hamper the validity of the results obtained. As observed in the first experiment with 'Marisol' mandarins, the percentage of decayed fruit depended on the fruit:fly ratio used and was nil for uninfested fruit. Because our aim was to develop a protocol that could be applied to infested fruit, a worst case scenario was chosen whereby a postharvest fungicide treatment strong enough to prevent fungal decay in the punctures made by adult females during oviposition. Consequently any cultivar difference in relation to decay became irrelevant.

A differential resistance to medfly infestation could appear from the occurrence of either antixenotic or antibiotic mechanisms (Wiseman, 1999) differentially occurring in the mandarin cultivars tested. Antixenosis describes a situation in which the insect is either repelled from or not attracted to its normal host plant. All cultivars were at commercial maturity when tested (Table 1). Although both rind color and width (which can affect oviposition and therefore infestation) were not the same between cultivars, and presumably other chemical characteristics of fruit, these fruit would be accepted in these no-choice tests because the only condition of acceptance is fruit maturity. Antibiosis describes a situation in which the insect's normal relationship with

a host plant causes physiological or developmental detriment to the insect. Previous work with these and other cultivars has been via infesting fruit by inserting larvae that had been grown in an artificial medium into the fruit (Alonso *et al.,* 2002a,b, 2005b; Palou *et al.,* 2007). The observations and results from these studies have shown no antibiotic phenomenon.

The results of these experiments clearly showed the importance of the fungicide treatment to prevent fruit decay. The use of a postharvest solution containing 5% imazalil sulphate was not suitable to keep the infested 'Marisol' fruit healthy enough to allow the larvae of *C. capitata* to complete their development (Fig. 1). Only the use of higher doses of this fungicide in combination with guazatine acetate and thiabendazole (second and third experiments) kept infested fruit in good enough condition to allow *C. capitata* larvae to pop out from the fruit and safely pupate. These postharvest fungicide application doses are higher than those authorized for practical use (MAPA, 2007) and therefore can not be applied for commercial purposes. The use of this fungicide mixture resulted in a percentage of decayed fruit higher than 50% only when the highest fruit:fly ratio (1:30) was applied during three days to 'Nova' mandarins (Fig. 2). For any other ratio, this percentage infrequently reached 25%, and was almost nil for ratios below 1:20 when fruit was exposed to flies for up to two days. Under these conditions, the number of pupae obtained per fruit increased with both decreasing the fruit:fly ratio and increasing exposure time (Fig. 4). *C. capitata* flies exploit fruit wounds (including pre-existing oviposition punctures) as oviposition sites (Papaj *et al*., 1989). Therefore females tend to lay more eggs into already wounded fruit than into unpunctured fruit. The occurrence of this synergistic phenomenon could explain why the number of puparia obtained per fruit did not increase linearly with either the fruit:fly ratio or exposure time (Fig. 4). When a period of two days was used as exposure time, the number of puparia obtained per 'Clemenules' fruit did not change for fruit:fly ratios between 1:10 and 1:50 (Fig. 4). Any of these ratios allowed the collection of almost 20 puparia per fruit. Previous assays using *in vivo* infestation techniques for citrus (Hallman and Worley, 1999; Hallman and Martínez, 2001) did not provide data on the number of decayed fruit, or the mean number of flies per fruit obtained. Hence, it is not possible to copare those results to those obtained in this work. Working with mangoes (*Mangifera indica* L), Torres-Rivero and Hallman (2007) obtained about 45 *C. capi-*

tata larvae per fruit when using fruit:fly ratios of 1:210 to 1:275. These ratios are 4 to 20-fold those reported here and resulted in a 2-fold increase in the number of pupae.

Previous studies for the infestation of mandarins consist of inserting 10 third instar larvae reared on artificial diet into fresh fruit (Alonso *et al*., 2002b, 2005b, 2007; Palou *et al*., 2007, 2008). These studies yielded less puparia per fruit than those that could be obtained in 'Clemenules' mandarins using the *in vivo* infestation fruit:fly ratio of 1:10 or higher for two days. These results will therefore improve the current infestation practices involving *C. capitata* and mandarins. Provided that an adequate fungicide treatment is applied before the infestation to satisfactorily avoid fruit decay, the use of a 1:10 fruit:fly ratio for two days guarantees the collection of almost 20 healthy puparia per fruit. This *in vivo* approach can now be used to develop quarantine treatments against *C. capitata* in mandarins.

Acknowledgments

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