Scientia Horticulturae 246 (2019) 437–447

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Insect repellent and chemical agronomic treatments to reduce seed number in 'Afourer' mandarin. Effect on yield and fruit diameter

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ARTICLE INFO

Keywords:
Insect repellent
Seed number
Afourer
Fruit diameter
Citrus fruits
Copper sulfate
Gibberellic acid
Seed reduction
Net-covered trees

ABSTRACT

Obtaining citrus fruits without seeds is a recurrent objective for farmers as it is one of the most valued characteristics, especially in mandarins. 'Afourer' tangor is a highly valuable well-established mandarin, and a high percentage of seeded fruits are produced under cross-pollination conditions. Several agronomic techniques have been suggested to control presence of seeds, such as covering with nets and copper sulfate ($CuSO_4$) and gibberellic acid (GA_3) treatments. Natural bee repellents are also proposed to reduce the number of seeds per fruit. In this study, we aimed to compare the effect of several agronomic treatments to reduce the seed number in 'Afourer' mandarin. To this end, we assessed the effect of chemical and bee repellent treatments on the seed number per fruit and the side effect on yield and fruit diameter. Under these experimental conditions the two bee repellents, one based on zinc and one based on *Capsicum annuum*, were not useful for reducing the seed number per fruit in 'Afourer' mandarin. The copper sulfate + GA_3 treatment reduced the seed number per fruit by only 35%, and this efficiency was clearly not enough to reduce the seed number for commercial purposes. The most effective method to reduce the seed number per fruit was covering with nets, but this technique led to markedly reduced yields. Yield data was highly variable. Fruit diameter correlated positively with the seed number, but it was a weak relationship as the seed number explained only 15% of fruit size variability (R^2 = 0.15).

1. Introduction

With a world production of 156 million tons (MT) in 2015, citrus is one of the most important crops worldwide (FAO, 2017). Eighty-four percent of this amount (131 MT) corresponded to fresh fruit (FAO, 2017). Internal and external fruit qualities are greatly demanded by the fresh market, and lack of seeds is one of the most highly valued characteristics, especially in mandarins (Roldán and Navarro, 2001; Gambetta et al., 2013). Consequently, obtaining citrus fruits without seeds has become a recurrent objective for farmers as it can improve price and sale expectations (Vardi et al., 2008).

The most cultivated mandarin varieties are parthenocarpic and self-incompatible and, therefore, they produce fruits without seeds in the absence of cross-pollination (Soost, 1965). However, when mandarin varieties are grown close to compatible ones, like other mandarins or tangor hybrids, they produce several seeds per fruit (Cronje et al., 2014). The use of new hybrid varieties with a better potential price or to prolong the offer period has been extended in the last decades (Bono et al., 2000). Many of these new varieties are pollen-compatible with traditional ones and are, therefore, directly involved in the presence of seeds.

'Afourer' tangor presents organoleptic and ripening characteristics that are well appreciated by the fresh market (Nadori, 2004). This variety, also known as 'Nadorcott', originated in Afourer (Morocco) and is probably a hybrid of cv. 'Murcott' ('Murcott' is a tangor hybrid of tangerine and orange, *Citrus reticulata* Blanco x *C. sinensis* (L.) Osbeck) and an unknown pollinator parent (Nadori, 1998, 2004). 'Afourer' is reported to be self-incompatible and produces seedless fruit if grown in isolation (Bono et al., 2000; Chao, 2005). However, under cross-pollination conditions, a high percentage of seeded fruits are produced (Agustí et al., 2005; Chao, 2005). The 'Afourer' flowering period widely overlaps many other pollen-compatible varieties, such as cv. 'Nova' (the

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https://doi.org/10.1016/j.scienta.2018.11.025
Received 19 June 2018; Received in revised form 8 November 2018; Accepted 13 November 2018

tangelo hybrid of *C. clementina* hort. ex Tanaka x [*C. paradisi* Macf. x *C. tangerina* hort. ex Tanaka.]) and cv. 'Clemenules' (a bud mutation of *C. clementina* hort. ex Tanaka).

New seedless varieties can be obtained through conventional breeding, triploid hybrids, induced mutations by gamma rays and emerging biotechnological approaches (Spiegel-Roy, 1990; Li et al., 2002; Olivares-Fuster et al., 2002; Jacquemond et al., 2003; Zhang et al., 2017). However, having no seeds does not ensure fruits are well accepted by the fresh market and new varieties can fail. Market preferences are unpredictable and do not depend on a single trait. In addition, new varieties' agronomic behavior may not be what is expected (Lladró, 2013; AVA-ASAJA, 2015). Consequently, a different approach is, having a successful variety, use an agronomical technique to avoid the presence of seeds.

Citrus pollen is heavy and sticky, which favors adherence to insect bodies and hinders wind pollination (Pons et al., 1995). Thus citrus pollination is generally entomophilous and bees (*Apis mellifera* L.) are the main pollinator (Pons et al., 1996). According to Gravina et al. (2016), covering nets is one of the most effective practices used to reduce seeds. The main disadvantages are its high cost and reduced yield per tree (Gravina et al., 2016). Mesejo et al. (2006) suggested that 25 mg/l of $CuSO_4 \cdot 5H_2O$ applied at full bloom to entire 'Afourer' trees significantly reduced the average number of seeds per fruit by 55–81%. The role of gibberellic acid (GA) in parthenocarpy and fruit set has been well studied (Pharis and King, 1985; Jacobsen and Olszewski, 1993; Fos et al., 2001). Exogenous GA_3 treatments have been shown to improve fruit set in numerous citrus cultivars (García-Martínez and García-Papí, 1979), but not in 'Satsuma' mandarin (Coggins et al., 1968) which had a high level of endogenous GA at anthesis (Talon et al., 1992). The exogenous application of GA_3 (10 mg/l) to 'Clemenules" flowers around anthesis impaired fertilization by either enhancing ovule abortion or reducing pollen tube growth (Mesejo et al., 2008). Gambetta et al. (2013) attempted different GA and $CuSO_4$ treatments to reduce the seed number in 'Afourer'. The most efficient treatment was proved to be three applications of GA_3 (50 mg/l) + $CuSO_4$ (25 mg/l), with a reduction from 3.7 to 2.3 (38%) in the number of seeds per fruit (Gambetta et al., 2013).

Historically, insect repellents have been developed for personal bite protection against disease-transmitting arthropods, such as mosquitoes and ticks (Katz et al., 2008). N,*N*-diethyl-3-methylbenzamide (DEET) is the most widely used and most efficacious insect repellent for personal protection (Katz et al., 2008; Dickens and Bohbot, 2013). Although DEET can be used occasionally for agronomic purposes, it is not economically and environmentally viable.

Bee repellents have been specifically studied for a long time, and also for personal protection but, in this case, more emphasis has been placed on agronomical use (Shaw and Bourne, 1944; Acevedo, 2017). Most efforts have focused on repellent additives to reduce pesticide hazards to honey bees (Atkins et al., 1975; Mayer et al., 2001; Sahebzadeh et al., 2009). Phenols, ketones and many other chemical compounds have been tested to provide honeybees in pesticide-treated fields with functional protection (Mishra and Sihag, 2009, 2010). Less information is available on natural bee repellents. Some studies, mostly conducted on the lab scale, have demonstrated natural compounds to repel bees. Pheromonal compounds, essential oils and pyrethrins have been tested (Free et al., 1985; Rieth and Levin, 1988; Larson et al., 2014). Recent studies indicate that pheromones can be toxic to bees (Larson, 2017) and that essential oil effects have a limited duration given their high volatility (Moore and Debboun, 2007). Massive bee repellent use to prevent bees in fields must ensure that they are not toxic. Regulation (EC) No. 1107/2009 provides clear criteria to approve active substances, which cannot have acute or chronic effects on colony survival and development (OJ, 2009). New natural bee repellents are being proposed and used by farmers, but are they effective?

As far as we are aware, no comparative study that has involved chemical and insect repellent agronomic treatments to reduce the seed number in mandarins has been published. This causes doubts in citrus growers when they have to choose that which could provide the best results. In this study, we aimed to compare the effect of several agronomic treatments to reduce the seed number in 'Afourer' mandarin. To achieve this main objective, we aimed to assess: (i) the effect of chemical and bee repellent treatments on the seed number per fruit; (ii) the possible effect of treatments on yield per tree; (iii) the influence on style length and fruit diameter; (iv) the possible influence on pest presence.

2. Materials and methods

2.1. Experimental site

The experiment was conducted in the municipal district of Montserrat in the province of Valencia, Spain (39° 21′ 35″ N 0° 32′ 44″ W; Alt: 150 m) in the spring of 2016. The plot had a total surface area of 250 m^2 and it was close (less than 100 m) to other pollen-compatible mandarin plots (cv. 'Clemenules' and cv. 'Nova') which favored cross-pollination. The plot soil was homogeneous throughout the site. Soil was calcareous sandy clay loam with a pH of 8.06 and 5.2% of limestone. The general site climate was Mediterranean oceanic, with a long-term average annual rainfall of 450 mm and an average annual air temperature of 19 °C (w.s. 39° 22′ N 0° 27′ W 54 m.a.s.l.). In 2016 the annual rainfall was 517 mm and it rained 22 mm in winter (2015 Dec 21–2016 Mar 21 and 48 mm in spring (2016 Mar 21–2016 June 21).

The experiment was conducted on 9-year-old trees of cv. 'Afourer', grafted onto *Citrus macrophylla* Wester rootstocks in a commercial orchard 6 x 4 m apart with drip irrigation. All treated trees in the experiment were the same age and with the same irrigation. Management was carried out under standard cultural conditions with no other treatments, except for the experimental treatments, during the flowering period.

2.2. Experimental design

In this experiment, five treatments were carried out; (i) a negative control treatment: trees covered with an anti-insect net (C-); (ii) a treatment with copper sulfate and gibberellic acid (CuGA); (iii and iv) two treatments with insect repellent products (ZnRep and CapRep); (v) a positive control treatment, treated only with water (C+). The experiment was run with a randomized complete block design (RCBD) with eleven replicates per treatment, distributed into three blocks (B1 with three replicates; B2 with four replicates; and B3 with four replicates) (Fig. 1). Treatments were randomly distributed within blocks. Each replicate was two treated trees and an edge of at least ten untreated trees to minimize any possible interactions between treatments (Fig. 1).

2.3. Treatments

The $CuSO_4$ + GA_3 treatment was water with 25 mg/l of $CuSO_4 \cdot 5H_2O$ + 50 mg/l GA_3 and 0.1% v/v of buffer Tampotec (K_2O solution to correct pH). Two formulated commercial insect repellents were used in this research. The first repellent treatment was a water solution 0.2% v/v of an under evaluation zinc-based product (2% w/w soluble zinc + 2% w/w complexed Zn) and 0.1% v/v of buffer. The second repellent treatment was a water solution 0.2% v/v of a natural repellent based on the essence of a spicy variety of *Capsicum annuum* L. and 0.1% v/v of buffer. The positive control treatment was water with 0.1% v/v of Tampotec buffer. Finally, trees were covered with nets for the negative control treatment to avoid pollinators.

Treatments were applied using a 15 l knapsack sprayer, to which a conical nozzle (PULMIC) was added to ensure a constant pressure of 2.5 bar, and 2.5–3 liters per tree were used. Treatments were applied 3 times throughout the flowering period on these data (year/mm/dd): 2016/04/01 (25% of opened flowers); 2016/04/11 (60%); 2016/04/18

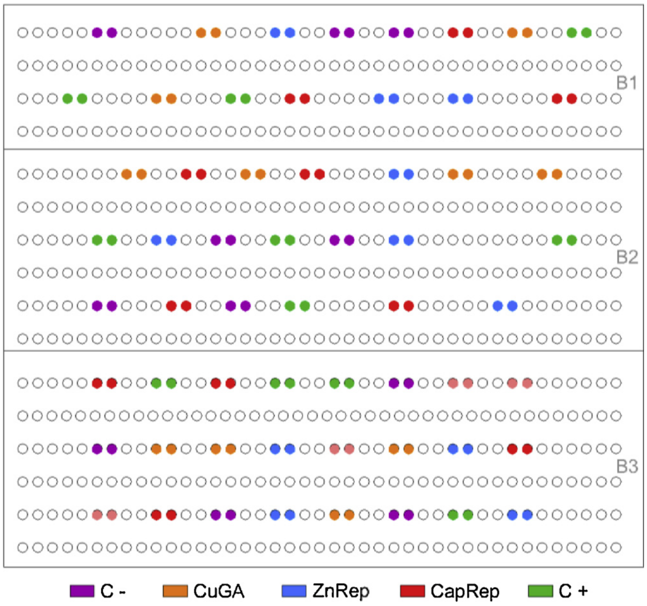

Fig. 1. Experimental design. Field distribution of treatments. Treatments are noted as C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control. Blocks are noted as B1 block 1; B2 block 2; B3 block 3.

(90%).

2.4. Measurements

When fruits had totally developed (March 2017), treated trees were harvested and yield per tree was recorded in kilograms. The number of fruits per tree was also noted. The seed number per fruit and fruit diameter was measured in 10 fruits per tree randomly selected. Fruit diameter was measured with a vernier caliper. The seed number and fruit diameter was correlationally noted for each fruit.

Style length and presence of aphids was measured 3 times, once after each application (T1: 2016/04/08, T2: 2016/04/15 and T3: 2016/04/25). Style length was measured in 10 flowers per tree. To avoid pollen contamination, negative control treatment styles were not measured as trees were covered with nets. The presence of aphids was examined in 10 young buds per tree. In this case, negative control treatment observations could be made without removing the nets.

2.5. Statistical analysis

The average, standard error, skew, kurtosis, frequency distribution and density curve of the numerical variables were assessed per treatment. ANOVAs were used to compare the mean values between treatments and blocks. Shapiro-Wilk tests were calculated to check the normality requirements. In some cases due to lack of normality, non-parametric methods were selected to compare the means among treatments and blocks by a Kruskal-Wallis rank sum test. When significant differences were found, Levene's test and eta-squared statistics were calculated to assess the homogeneity of variances and the effect size in the ANOVA, respectively. Tukey's honestly significant difference (HSD) was selected as the *post hoc* test. Violin plot were used which combines a density plot and a boxplot. The density plot computes and draws kernel density estimate, which is a smoothed version of the histogram. In the boxplot the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles), data beyond the end of the whiskers are called "outlying" points and are plotted individually.

The frequencies of aphids were analyzed by a chi-squared test. Linear models were selected to analyze the effect of numerical variables on yield, while Pearson correlations were calculated to assess the relationships between the independent variables. All the statistical analyses were done using R (R Core Team, 2017) with some extra packages: car (Fox and Weisberg, 2011); plotrix (Lemon, 2006); ggpubr (Kassambara, 2017); agricolae (Mendiburu, 2017); vcd (Meyer et al., 2006); writexl (Ooms, 2017); ggplot2 (Wickham, 2009); readxl (Wickham and Bryan, 2017); plyr (Wickham, 2011); tidyr (Wickham and Henry, 2017) and knitr (Xie, 2017). Database: Mendeley (https://doi.org/10.17632/7nm7mgddpv.1).

3. Results

3.1. Effect of treatments on the seed number per fruit

Including all the treatments, 110 trees were treated and 768 fruits were cut to count the seed number. Kruskal-Wallis test for the effect of treatments on the seed number showed significant differences among treatments (KW value = 101.43, p = 0.00). The average seed number per fruit was significantly lower in the fruits picked from the trees treated with $CuSO_4$ + GA_3 (0.64 ± 0.11) compared with the positive control treatment (0.99 ± 0.11), while no significant reduction was observed in the average seed number recorded from both repellent treatments versus the control fruits (Table 1; Fig. 2). The fruits from the net-covered trees (C-) had no seeds. The Violin plot showed that the CuGA treatment modified data distribution, while both repellent treatments had the same data distribution as the control (Fig. 3; Fig. S1).

Treatments are noted as: C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control; N, number of repetitions; KW, Kruskal-Wallis test by ranks, different letters mean significant differences for alpha = 0.05; sd, standard deviation; se, standard error; Shapiro, p-value for the Shapiro-Wilk normality test (p-value below 0.05 indicates a non normal distribution); Kruskal-Wallis values: Df = 763; KW-value = 101.43; p-value = 0.000.

Number of seeds per fruit showed significant differences among blocks (Fig. S2; Fig. 4). Block-2 had less seeds, probably due to its inner position (Fig. 1) that was more protected from pollinators. The treatments-blocks interaction (Fig. 4) showed that the differences among treatments in block-2 were attenuated. The CuGA-treated fruits had fewer seeds in blocks 1 and 3, while the repellent treatments showed more erratic behavior.

The Chi-square test showed significant differences in the percentages of the seeded and seedless fruits among treatments (chisq p = 5e-22). These differences were symmetrical to those found when the average seed number per fruit was analyzed (Fig. 5; Table S1). Both repellents and the positive control treatments had a similar percentage (approx. 50%) of seeded and seedless fruits, while the CuGA treatment lowered the percentage of fruits with seeds. The seedless percentages were also analyzed by blocks (Fig. S3). Once again, the differences among treatments were attenuated in block 2, and the repellent treatments showed erratic behavior (Fig. S3). This erratic behavior of repellents could be due to the fact that their effect is probably more exposed to other variable factors like wind, etc., while the CuGA treatment probably acted on stigmas being a more unchanging effect.

Table 1
The average seed number per fruit achieved in each treatment with the Kruskal-Wallis rank sum test values.

Treatment	N	Mean	KW	sd	se	skew	kurtosis	Shapiro
C-	118	0.00	c	0.00	0.00	NaN	NaN	–
CuGA	189	0.64	b	1.58	0.11	3.73	15.70	1.27E-23
ZnRep	168	1.11	a	1.48	0.11	1.36	1.45	3.88E-15
CapRep	144	1.15	a	1.68	0.14	2.20	6.55	1.75E-15
C+	149	0.99	a	1.30	0.11	1.43	1.75	3.03E-14

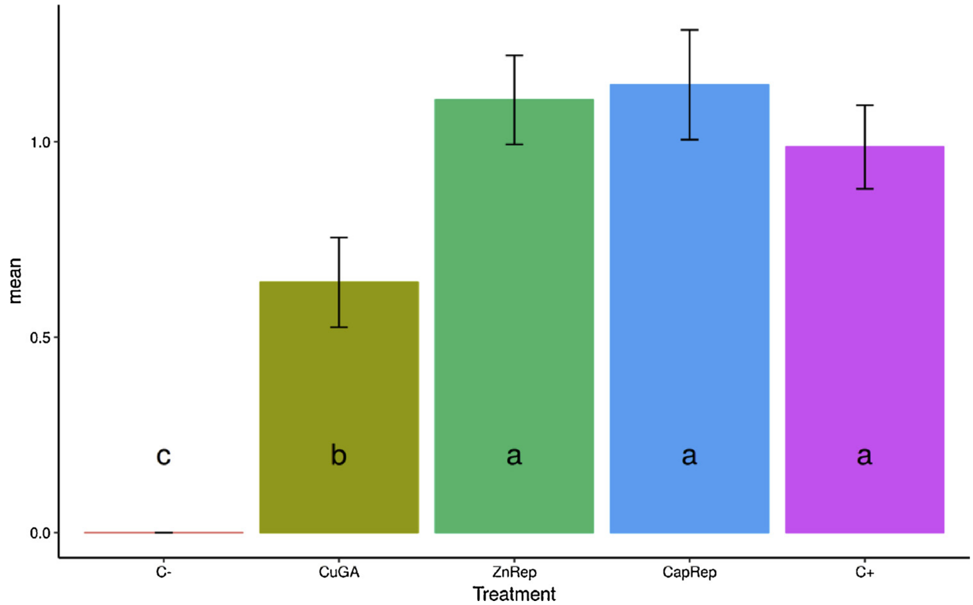

Fig. 2. Barplot for the effect of treatments on the seed number per fruit. Different letters mean significant differences in the Kruskal-Wallis test by ranks. Error lines indicate standard errors. Treatments are noted as C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control.

Finally, treatments were compared by eliminating seedless fruits. The ANOVA for the effect of treatments on the seed number per fruit having eliminated the seedless fruits showed no significant differences among treatments (Fig. S4). This result indicates that the CuGA treatment mostly reduced the number of fruits with seeds, but not the seed number in the seeded fruits.

3.2. *Influence of treatments on yield per tree*

The fruits of all the trees were collected and the total yield per tree was recorded. The total yield data did not fit a normal distribution (Fig. S5) and a high level of variability was found among individuals (Saphiro-W test in Table 2). The Levene's test p value of 0.038 confirmed that the standard deviations were not homogenous among treatments. So although the averages among treatments were very different, the Kruskal-Wallis test for the effect of treatments on yield showed only a weak significant difference between the negative control and the CuGA treatment (Fig. 6; p-value = 0.04, KW value = 10.07). This result probably indicates a limited ability to assess the effect of treatments on yield because of a strong individual effect. The Violin plot for the effect of treatment on yield clearly showed a change in data distribution for the net-covered trees (Fig. 7). The average yield per tree for the negative control treatment (trees covered with nets) was the lowest (2.15 Kg), but no significant difference was found with the positive control treatment (C+ = 4.94 Kg) (Table 2 and Fig. 6).

Treatments are noted as, C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control; N, number of repetitions; KW, the Kruskal-Wallis test by ranks, different letters mean significant differences for alpha = 0.05; sd, standard deviation; se, standard error; p-value for the Shapiro-Wilk normality test, p-value below 0.05 indicates a non normal distribution; Kruskal-Wallis values: Df = 105; KW-value = 10.07; p-value = 0.04.

3.3. *Effect of treatments on fruit diameter*

The diameter of 768 fruits was recorded where, in this case, the fruit diameter data perfectly fitted a normal distribution (Fig. S6 and Saphiro

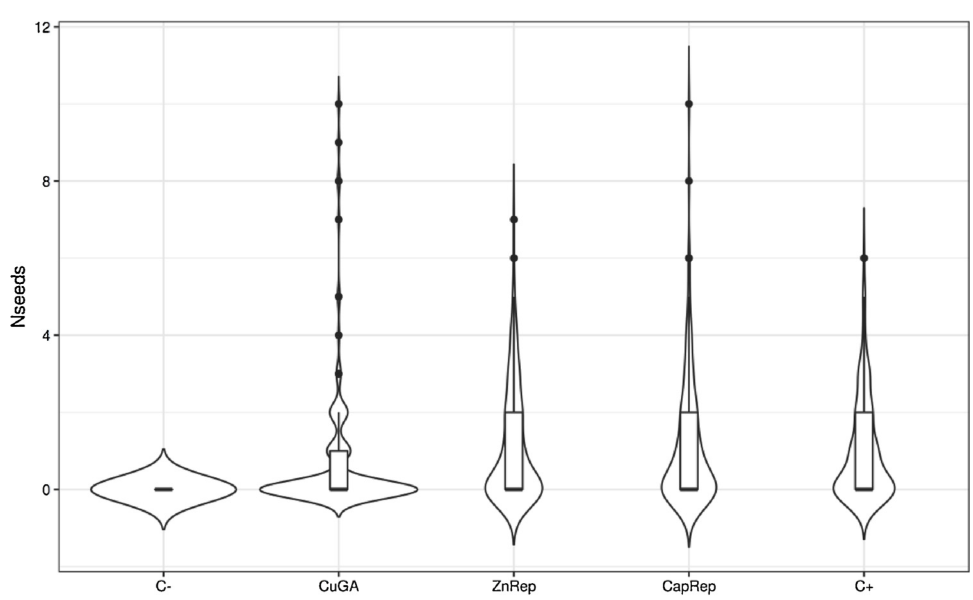

Fig. 3. Violin plot of the seed number per fruit for each treatment. Treatments are noted as C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control. Violin plot combines a density plot (kernel density estimate) and a boxplot (with hinges and outlying points).

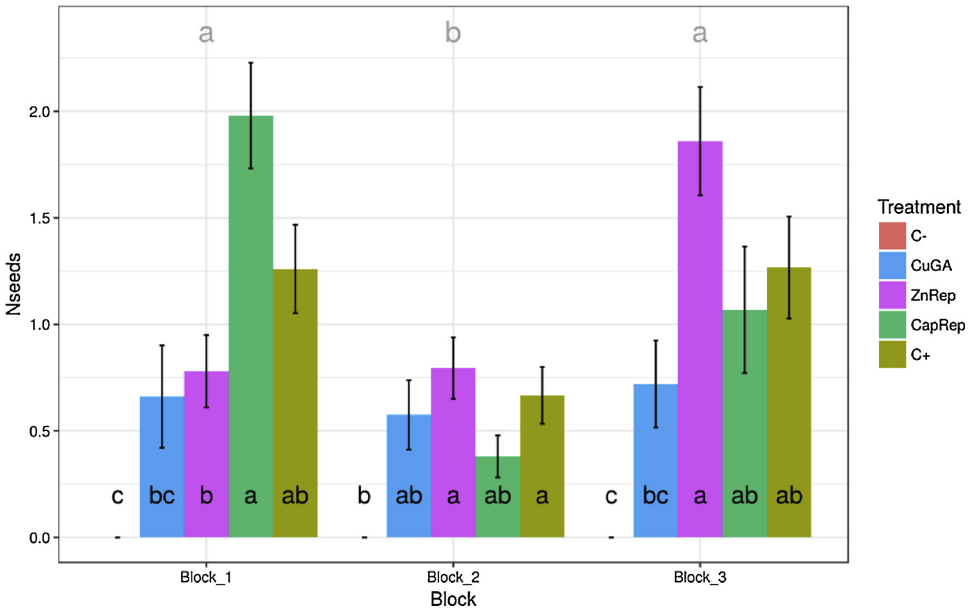

Fig. 4. Barplot for the effect of treatments on the seed number per fruit. Different gray letters mean significant differences among blocks, while different black letters mean significant differences among treatments inside the blocks in the Kruskal-Wallis test by ranks. Treatments are noted as C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control.

-W in Table 3). Therefore, the linear models and ANOVA were used to compare the mean values among treatments. The ANOVA for the effect of treatments on fruit diameter showed significant differences among treatments (F-value = 3.18, p = 0.01). The lowest average diameter was obtained in the fruits from the net-covered trees (56.56 ± 0.69 mm), while the highest average value went to the fruits from the positive control treatment (59.02 ± 0.60 mm), with significant differences (Table 3 and Fig. 8). The fruits from the trees treated with copper sulfate + GA_3 had an intermediate average diameter (57.96 ± 0.52 mm) between the two controls (Table 3). The zinc-based repellent fruit diameter (58.58 mm) came closer to the positive control, while *Capsicum annum* repellent average (56.82 mm) came closer to the negative control. In any case, although the differences among treatments were significant, the effect size was very small (η^2 value = 0.016) with only 2.45 mm between the highest and lowest average values.

Treatments are noted as: C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control; N, number of repetitions; HSD,

Table 2
Average yield per tree achieved in each treatment with the Kruskal-Wallis rank sum test values.

Treatment	N	Mean	KW	sd	se	skew	kurtosis	Shapiro
C-	22	2.15	c	3.59	0.77	1.80	1.92	4.14804E-06
CuGA	22	7.30	a	7.09	1.51	0.82	−0.48	0.011678169
ZnRep	22	6.89	ab	11.26	2.40	3.16	11.48	1.69647E-06
CapRep	22	3.31	bc	4.59	0.98	1.61	2.07	0.000100594
C+	22	4.94	abc	5.89	1.26	1.04	0.07	0.001003215

honestly significant difference, different letters mean significant differences for alpha = 0.05; sd, standard deviation; se, standard error; p-value for the Shapiro-Wilk normality test, p-value below 0.05 indicates a non normal distribution; ANOVA values: Df = 763; F-value = 3.18; p-value = 0.013;.

Fruit diameter correlated positively with the seed number (Fig. 9), but weakly (R^2 = 0.15) and was mainly triggered by a few large fruits with many seeds (7.5–10 seeds), and a few very small fruits with no

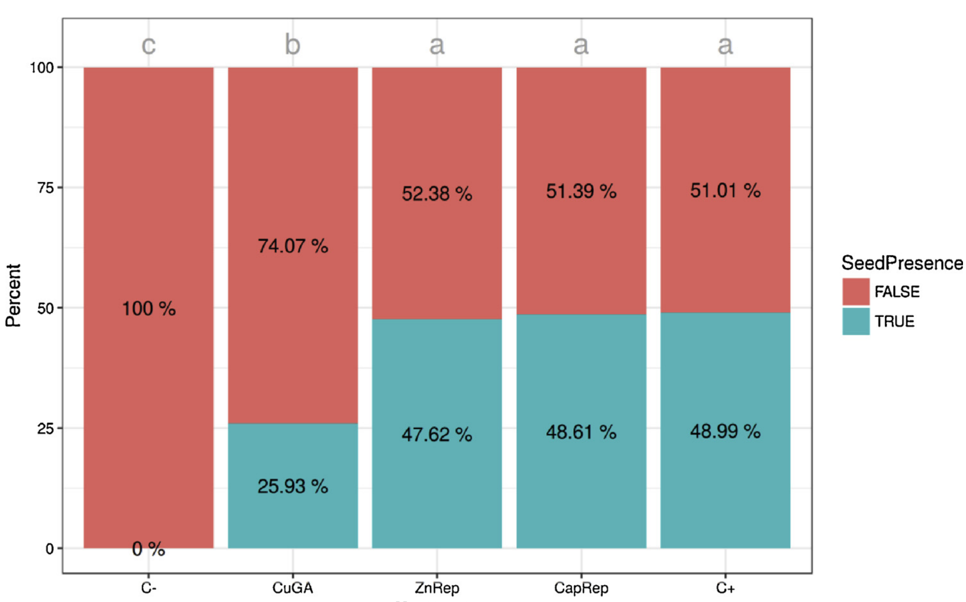

Fig. 5. Percentage of the seeded and seedless fruits by treatment. Blue (true) mean fruits with one seed or more; red (false) denotes fruits without seeds. Different gray letters mean significant differences in the chi-square by Fisher test with Holm's correction *post hoc* test. Total chisq p = 5e-22. Chisq = 106.06. Treatments and the total number of fruits per treatment were C- negative control (n = 118); CuGA copper sulfate + GA_3 (n = 189); ZnRep zinc-based repellent (n = 168); CapRep *Capsicum annuum*-based repellent (n = 144); C + positive control (n = 149). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

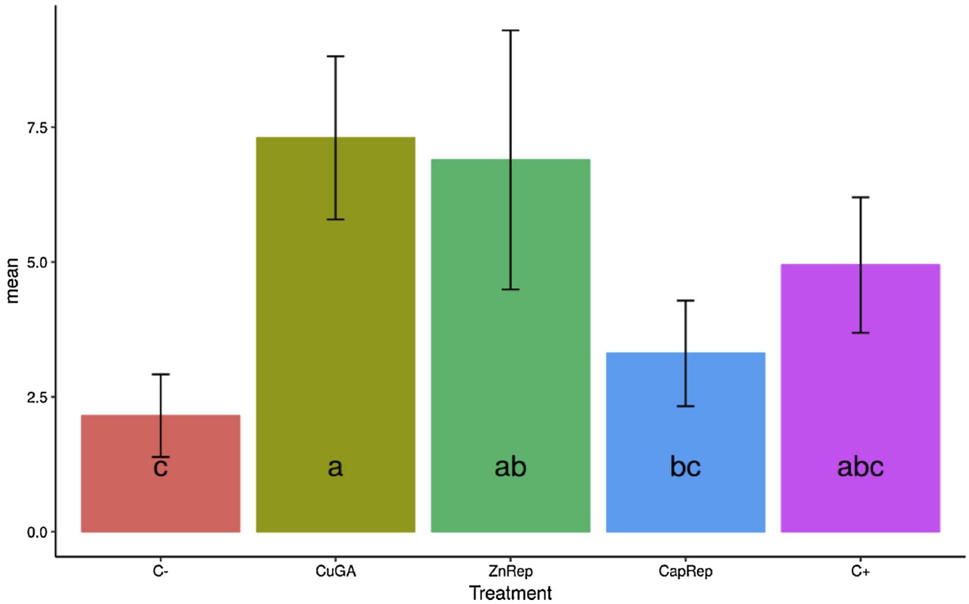

Fig. 6. Barplot for the effect of treatments on yield. Different letters mean significant differences in the Kruskal-Wallis test by ranks. Error lines indicate standard errors. Treatments are noted as C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control.

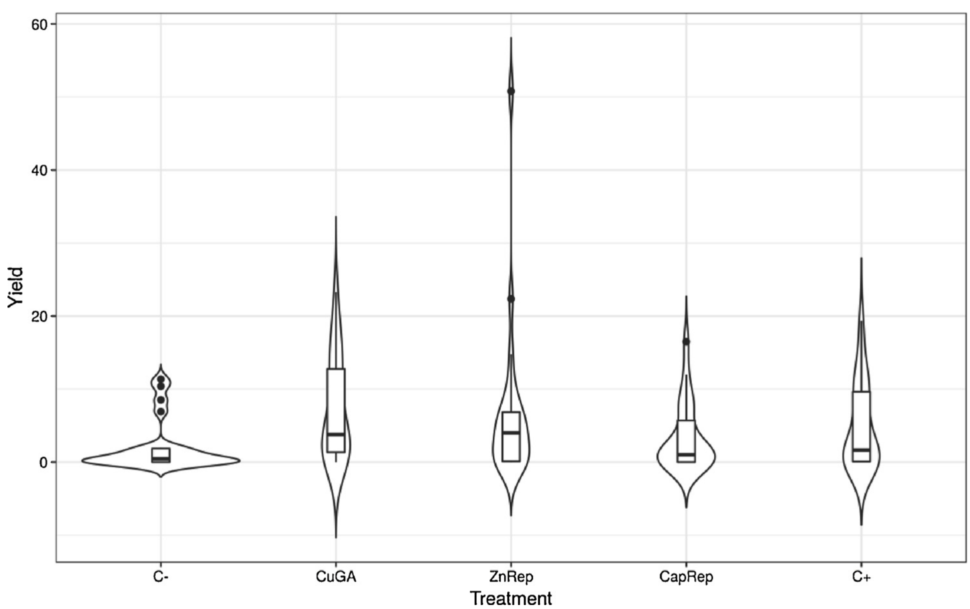

Fig. 7. Violin plot for the effect of treatments on yield by treatment. Treatments are noted as C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-basef repellent; CapRep *Capsicum annuum*-based repellent; C + positive control. Violin plot combines a density plot (kernel density estimate) and a boxplot (with hinges and outlying points).

Table 3
Average fruit diameter per treatment with the ANOVA and *post hoc* Tukey test.

Treatment	N	mean	HSD	sd	se	skew	kurtosis	Shapiro
C-	118	56.56	b	7.54	0.69	−0.14	0.45	0.56
CuGA	189	57.96	ab	7.13	0.52	−0.04	1.73	0.01
ZnRep	168	58.58	ab	7.07	0.55	0.24	−0.24	0.17
CapRep	144	56.82	ab	6.52	0.54	−0.11	0.23	0.43
C +	149	59.02	a	7.31	0.60	0.07	−0.07	0.94

Treatments are noted as: CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-basef repellent; C + positive control; N, number of repetitions; HSD, honestly significant difference, different letters mean significant differences for alpha = 0.05; sd, standard deviation; se, standard error; p-value for the Shapiro-Wilk normality test; ANOVA values: Df = 763; F-value = 12.7; p-value = 3.7E-8.

seeds (diameters 30–40 mm).

3.4. Influence of treatments on style length

Style length was measured in 10 flowers per tree with a total of 1069 measures (Fig. S7). The styles of the negative control treatment were not measured because trees were covered by nets. The ANOVA for the effect of treatments on style length showed significant differences among treatments (F-value = 12.7, p = 3.7e-8). The flowers from the trees treated with $CuSO_4$ + GA_3 had the longest styles (6.51 ± 0.05 mm) with significant differences compared with the styles of the control treatment flowers (6.18 ± 0.05 mm) (Table 4; Fig. 10). No significant differences in style length were found among the flowers that belonged to both the repellent and control treatments. Levene's test indicated that standard deviations were homogenous among treatments and the eta square value (η^2 = 0.034) indicated a small effect size with 0.35 mm between the highest and the lowest average values.

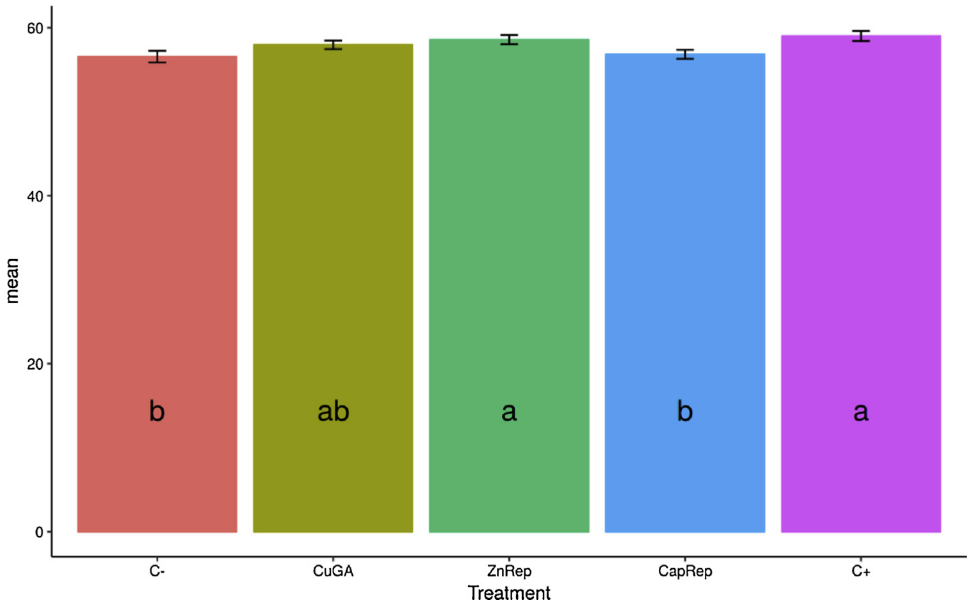

Fig. 8. Barplot for the effect of treatments on fruit diameter. Different letters mean significant differences in the ANOVA. Error lines indicate standard errors. Treatments are noted as: C- negative control; CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control.

3.5. Effect on the presence of aphids

Ten young buds per tree were observed 3 times with a total of 3300 observations. At moment one, chi-square test ($p < 0.001$, chi-sq = 49.3) displayed significant differences among observations with more aphids present in the buds of the trees treated with $CuSO_4$ + GA_3 (Table 5 and Fig. 11). At moment two, differences were also significant ($p < 0.001$, chi-sq = 59.1), but there were fewer aphids in the buds of net-covered trees (0.18%) and more aphids present in the buds of trees treated with $CuSO_4$ + GA_3 (3.36%). This pattern was confirmed at moment 3 once again and with significant differences ($p < 0.001$, chi-sq = 60.7). At this time, the buds of trees of the negative control treatment had no aphids (0%), whereas the presence of aphids in the buds of trees treated with $CuSO_4$ + GA_3 was the highest (3.64%) (Table 5 and Fig. 11).

Table 4
Average style length for each treatment with the ANOVA and *post hoc* Tukey test.

Treatment	N	mean	HSD	sd	se	skew	kurtosis	Shapiro
CuGA	281	6.51	a	0.82	0.05	−0.37	0.26	0.04
ZnRep	297	6.16	b	0.78	0.05	−0.15	0.30	0.57
CapRep	227	6.18	b	0.76	0.05	−0.39	0.34	0.09
C+	264	6.18	b	0.78	0.05	−0.33	0.41	0.03

4. Discussion

4.1. Seed reduction by nets

The most effective method to reduce the seed number per fruit in ‘Afourer’ mandarins was to cover with nets, which agrees with Gravina et al. (2016). We did not find seeds in the net-covered trees, while 0.99 seeds per fruit were found in the positive control treatment. The net-

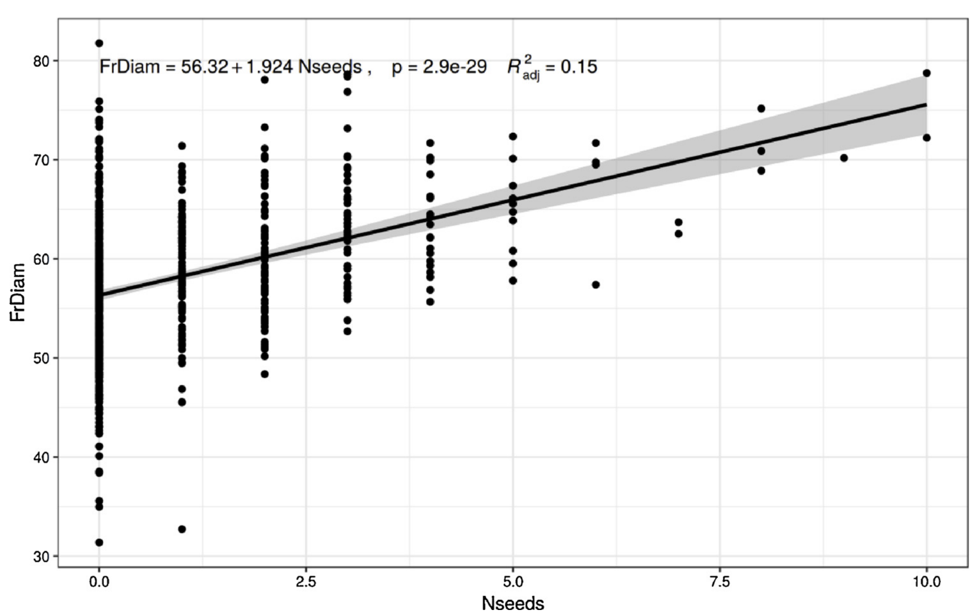

Fig. 9. Linear model of the effect of the seed number on fruit diameter. In gray, 0.95 confidence levels.

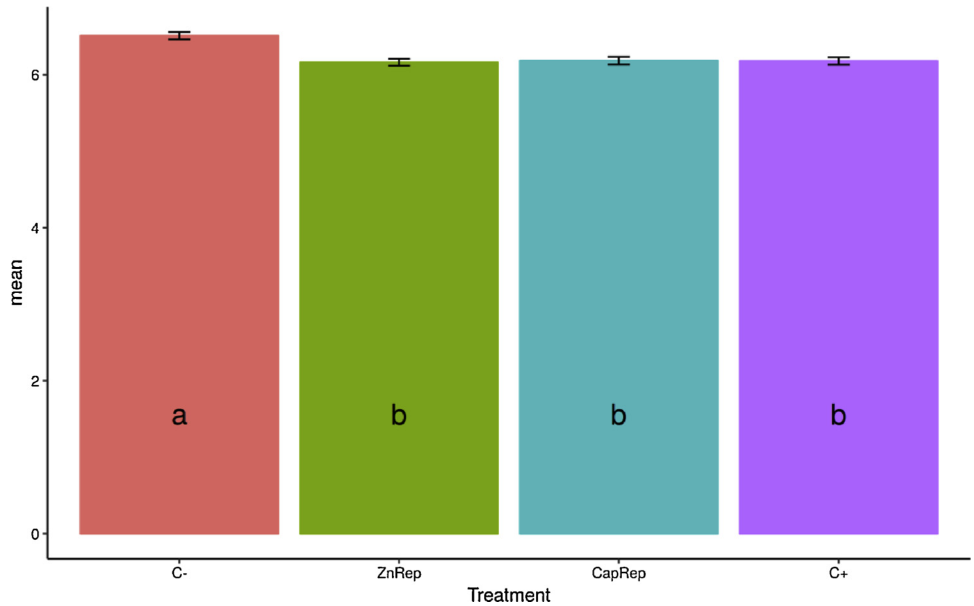

Fig. 10. Barplot for the effect of treatments on style length. Different letters mean significant differences in the ANOVA. Error lines indicate standard errors. Treatments are noted as: CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control.

covered 'Afourer' trees usually presented around 0.03 to 0.07 seeds per fruit and between 3–7% of fruits with no seeds, while the usual number of seeds in the open pollination treatment was around 2–4 seeds per fruits and between 85–100% of fruits with no seeds (Gambetta et al., 2013; Gravina et al., 2016; Otero and Rivas, 2017). Therefore compared with similar studies, this experiment was conducted under low cross-pollination conditions, which was probably due to the low winter and spring rainfall and to other climatic factors, with less available pollen and fewer pollinators.

4.2. $CuSO_4$ + GA_3

The copper sulfate + GA_3 treatment only reduced the seed number per fruit by 35% compared with the open pollination treatment. While Mesejo et al. (2006) indicated better efficiencies for the copper treatment of up to 55–81% seed reduction, the latest studies agree more with our results, and have provided efficiencies of around 35% (Gambetta et al., 2013). Otero and Rivas (2017) reduced seedy fruits by between 33–40% compared to controls with a single spray of GA_3 plus six subsequent $CuSO_4$ sprays. These efficiencies are clearly not enough to reduce the seed number for commercial purposes and performing more than three applications during the flowering period is probably too much under field conditions.

4.3. Bee repellents

Biologically-based products are increasingly available on the market to improve yields, protect plants from diseases or to deter pests. Nevertheless, more independent scientific-based studies are needed to validate the efficacy of these products (Gagic et al., 2017; Garmendia et al., 2018). The two bee repellents tested here did not significantly reduce the seed number per fruit. The trees treated with the zinc-based repellent gave an average of 1.11 seeds per fruit, while that of the trees treated with the *Capsicum annuum* repellent gave an average of 1.15 seeds per fruit. Both were over 0.99 seeds per fruit found in the positive control treatment. The *Capsicum annuum* repellent is currently sold to farmers in Spain as a promising natural product to prevent bees and, therefore, seeds. Similarly, the zinc-based repellent is being used by farmers and is undergoing a registration process named 'Fitolex Repellent'. These repellents were tested on the 'tree scale' and showed no effect. Perhaps on a larger "field scale" a stronger effect would be found. Therefore, although this first study indicated no effect, more studies are desirable to unravel whether these repellents are useful or not.

4.4. Effects on yield

The influence of treatments on yield was difficult to assess given the

Table 5
Observed and expected values for the presence of aphids per treatments.

Treatment	Date 1			Date 2			Date 3			Total		
	0	1	Fisher	0	1	Fisher	0	1	Fisher	0	1	Fisher
C-	213	7	b	218	2	c	220	0	c	651	9	d
	19.36%	0.64 %		19.82 %	0.18 %		20 %	0%		19.73%	0.27%	
CuGA	181	39	a	183	37	a	180	40	a	544	116	a
	16.45%	3.55%		16.64%	3.36%		16.36%	3.64 %		16.48%	3.52%	
ZnRep	213	7	b	209	11	bc	211	9	b	633	27	c
	19.36%	0.64 %		19 %	1 %		19.18 %	0.82 %		19.18%	0.82	
CapRep	208	12	b	216	4	c	207	13	b	631	29	bc
	18.91 %	1.09 %		19.64 %	0.36%		18.82 %	1.18 %		19.12%	0.88	
C +	207	13	b	201	19	ab	203	17	b	611	49	b
	18.82%	1.18%		18.27%	1.73%		18.45%	1.55%		18.52%	1.48%	
Test	Statistic	Df	P-value	Statistic	Df	P-value	Statistic	Df	P-value	Statistic	Df	P-value
Chi-square	49,34	4	< 0,001	59,071	4	< 0,001	60,742	4	< 0,001	161,892	4	< 0,001

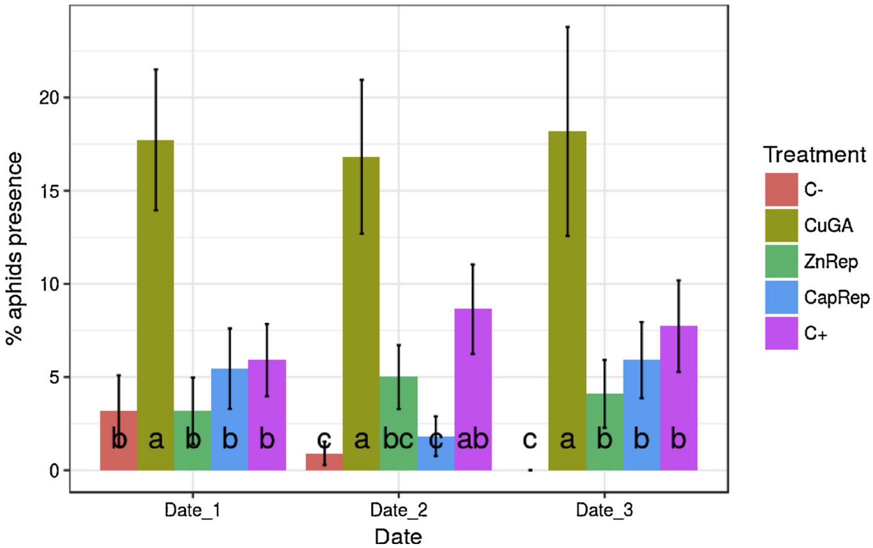

Fig. 11. Percentage of aphids present per treatment. Different letters mean significant differences in the chi-square by the Fisher test with Holm's correction *post hoc* test. Total chisq p = 5e-22. Chisq = 161.89. Treatments are noted as: CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-based repellent; C + positive control.

wide variability among individuals. Citrus yield depends on many exogenous factors, such as irrigation, water quality, soil fertility, and so on (Shalhevet et al., 1974; Romero et al., 2006; Molin et al., 2012), but also depends on many endogenous factors, such as tree vitality and previous yields (Moss, 1971; Martínez-Alcántara et al., 2015). Alternate bearing is common in late *Citrus* (Moss and Bevington, 1977), and 'Afourer' mandarin trees can present severely alternate bearing with some erratic behavior (Stander et al., 2017). If fruit load is too high and harvest is delayed, 'Afourer' trees can weaken (Stander et al., 2017). Therefore, an 'individual history' is essential for current yields, and an 'individual effect' can mask the influence of treatments on yield. Sometimes non significant differences among treatments do not necessarily mean lack of effect, but the need for more data due to wide variability.

Despite these limitations, the net-covered 'Afourer' trees presented the lowest yields, which agrees with other studies (Gambetta et al., 2013; Gravina et al., 2016; Otero and Rivas, 2017). No clear consensus on the magnitude of this yield reduction caused by nets has been reached. While, Otero and Rivas (2017) observed a reduction up to 66% in the number of fruits of net-covered trees, Gravina et al. (2011) reported no reduction in cold areas of Uruguay; Gambetta et al. (2013) observed a reduction of 30% of fruit set in a bagged flower treatment, while Otero and Rivas (2010) reported a clear fruit set reduction of whole net-covered trees. In our experiment, the net-covered trees yielded an average of 2.15 kg, while the positive control trees yielded an average of 4.94 kg; that is, a yield reduction of 56%, but with no significant differences due to wide data variability.

Different causes have been suggested for reduced yields. Exogenous factors, such as a higher air temperature and humidity changes, can reduce leaf photosynthesis and, therefore, increase fruit drop under nets (Cary and Weerts, 1980; Jifon and Syvertsen, 2003). The absence of seeds in relation to low endogenous GA levels has also been highlighted as a main cause (Ben-Cheikh et al., 1997, p.). Accumulated evidence strongly supports the notion that seeds can improve yield and fruit diameter, even in parthenocarpy varieties, and that GAs, rather than auxin, are particularly essential for citrus fruit development (Vardi et al., 2008).

Therefore, the seedless varieties or horticultural techniques to reduce seeds (e.g. covering with nets) can alternatively lead to fruit set problems and, consequently, to reduced yields. Exogenous GA treatments have often been suggested to improve fruit set in numerous citrus cultivars (García-Martínez and García-Papí, 1979).

4.5. Fruit diameter

Fruit diameter is an important commercial trait. While Chao (2005) found a moderate relationship between fruit size and the seed number per fruit (R = 0.59), Gravina et al. (2011) found no relationship between these two parameters. In our experiment, fruit diameter correlated positively with the seed number. However, it was a weak relationship as the seed number explained only 15% of fruit size variability ($R^2 = 0.15$). A few large fruits with many seeds and some very small fruits with no seeds lie behind this positive correlation. Although seed can improve fruit diameter by a direct hormonal effect, an opposite side effect through total yield per tree should be taken into account. Total yield = fruit set *x* fruit size; thus if fruit set per tree diminished by seed reduction, fruit size can improve as there are fewer fruits per tree that compete with one another.

4.6. Tissue elongation by GA

It is well-known that GA induces organ elongation (Sachs et al., 1959; Kende and Zeevaart, 1997). This growth response is the combined result of enhanced cell division activity in the subapical meristem and increased cell elongation (Kende and Zeevaart, 1997). The flowers treated with $CuSO_4$ + GA_3 had the longest styles with significant differences compared with the control treatment. It has been suggested that such enhanced pistil growth can be related with the impaired fertilization caused by GA as pollen tubes will take longer to reach ovules (Mesejo et al., 2008). In any case, whether the GA effect is caused by enhancing ovule abortion, reducing pollen tube growth or stimulating pistil growth, impaired fertilization is not enough to properly reduce the seed number for commercial purposes.

Finally, the same elongation effect could act on young buds. The buds of the trees treated with $CuSO_4$ + GA_3 presented significantly more aphids. Gibberellic acid could enhance sprouting and could, therefore, probably attract more aphids.

5. Conclusions

Under these experimental conditions, bee repellents were not useful for reducing the seed number per fruit in mandarin 'Afourer'. The $CuSO_4$ + GA_3 treatment lowered the average number of seeds per fruit by 35%, but this reduction is not enough for commercial purposes. The only effective horticultural technique that reduced the seed number was covering with nets. This technique is expensive and can impair fruit set. Therefore, reducing the seed number in highly valuable well-established mandarins remains an unmet objective.

Treatments are noted as: CuGA copper sulfate + GA_3; ZnRep zinc-based repellent; CapRep *Capsicum annuum*-basef repellent; C + positive control; N, number of repetitions; Fisher: different letters mean significant differences in the chi-square by the Fisher test with Holm's correction *post hoc* test

Acknowledgements

Authors thank Explotaciones Agrícolas Serrano S.A. (Valencia, Spain) for providing technical assistance and the orchards.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2018.11.025.

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