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Agronomic treatments to avoid seed presence in 'Nadorcott' mandarin I. Effect on in vivo pollen tube growth

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ABSTRACT

Fresh market demands high quality fruit and, therefore, citrus growers and researchers are constantly looking for solutions to avoid seed presence. Current horticultural techniques have low effectiveness or high cost. The objective of this study is to evaluate the inhibition effect of seven products on the *in vivo* pollen tube growth in Nadorcott mandarin, which is a high-value seedy variety. To achieve this main objective, three inorganic fertilizers (ammonium nitrate, potassium nitrate, sulfur), and four saccharides (saccharose, methyl cellulose, callose, chitosan) were applied to Nadorcott stigmas 24 h before and after hand pollination. Pollen tubes were counted 1, 3, and 5 days after treatment in longitudinal blue violet autofluorescence-stained sections. Of the seven evaluated products, only sulfur had a strong inhibitory effect. Elemental sulfur (S_8) inhibited pollen tube growth by 94-100%. This strong effect was observed regardless of sulfur being applied 24 h before or after pollination, and on fixed flowers 1, 3 or 5 days after applications. Saccharose treatment seemed to have the opposite effect: stimulated pollen tube growth, but the difference with the positive control was small and non-significant. The sulfur effect could be useful for designing agronomic applications capable of preventing seed presence in Nadorcott mandarin.

1. Introduction

Citrus is one of the most important crops worldwide with a world production of more than 157 million tons in 2019 ("FAOSTAT," n.d.). Most of this production goes to the fresh market, which demands high-quality fruit ("FAOSTAT," n.d.). One quality characteristic is seedlessness, which is very much demanded by consumers, especially in mandarins, and is a very important economic trait (Gambetta et al., 2013; Ye et al., 2009). Therefore, citrus growers and researchers are constantly looking for solutions to avoid seed presence.

Seeds in mandarins are usually caused by cross pollination with compatible varieties (Cronje et al., 2014). Most mandarin varieties are parthenocarpic and self-incompatible, and do not produce seeds if foreign pollens are lacking (Soost, 1965). However, farmers tend to diversify their production to meet seasonal demands and consumer preferences, which causes variety mosaics and, hence, cross-pollination (Bono et al., 2000). In fact several studies have indicated a decline in seedy fruit because the distance from the pollen source increased (Otero and Rivas, 2017).

Techniques to avoid seeds in mandarins can be grouped into two kinds: (i) those that focus on finding new seedless varieties; (ii) agronomic practices followed to lower seed numbers on the commercial orchards of seedy varieties. Conventional breeding, triploid hybrids, mutations induced by gamma rays and new biotechnological approaches are some of the techniques followed to obtain seedless varieties (Cimen et al., 2021; Cuenca et al., 2020; Garavello et al., 2020; Grouh et al., 2011; Neves et al., 2018; Rattanpal et al., 2019; Zhang et al., 2017).

The success of a new variety depends on many factors, and not just on being seedless. Some triploids have shown adaptation problems to climate conditions and other varieties have presented low fruit set and yields (AVA-ASAJA, 2015; Lladró, 2013). Therefore, changing to a new

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variety is risky for citrus growers, who need to wait 5-8 years to obtain full production and without any guarantee that the new variety will meet market preferences. This is why the farmers with a productive and already successful variety look for horticultural techniques to avoid seeds. According to Gravina et al. (2016), net covering is one of the most effective practices used to reduce seeds, but its main disadvantages are its high cost, and several studies have indicated a reduction of yields of around 15-30% (Garmendia et al., 2019a; Gravina et al., 2016).

Gibberellic acid (GA) is useful to control many citrus development processes (Duarte et al., 2006; Garmendia et al., 2019b). Treatments with GA and copper sulfate ($CuSO_4 \bullet 5H_2O$) have been proposed (Mesejo et al., 2008, 2006). However, the latest reports show barely any effectiveness of these treatments to reduce the average number of seeds per fruit (Gambetta et al., 2013; Garmendia et al., 2019a), and this reduction (around 30%) is not enough for commercial purposes.

Finally, another research line involves acting on pollinators. Citrus pollination is generally entomophilous and bees (*Apis mellifera* L.) are the main pollinator (Pons et al., 1996). As pollinators are strongly protected by national and international regulations, treatments are based on respectful repellents. In a recent study, two bee repellents, one based on *Capsicum annuum* L. and one on zinc, were tested and proved useless to lower the number of seeds per fruit (Garmendia et al., 2019a).

'Nadorcott' tangor is one of those varieties with organoleptic and ripening characteristics that are very much appreciated by the fresh market. Nadorcott is reported to be self-incompatible and produces seedless fruit if grown in isolation, however contains seeds when cross-pollinated (Chao, 2005; Gambetta et al., 2013). The usual number of seeds under open pollination conditions is around 2 to 4 seeds per fruit, but this will depend on how close compatible varieties are (Gravina et al., 2016; Otero and Rivas, 2017). This variety, also known as 'Afourer', 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). The flowering period of cv. 'Nadorcott' overlaps many other pollen-compatible varieties, such as cv. 'Nova' (a 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).

In this context, we aimed to study new agronomic treatments capable of lowering the number of seeds per fruit in Nadorcott mandarin. To fulfill this main objective, we set up the study in two consecutive phases: (i) in a first report, we address the effect of treatments on pollen tube growth when applied to individual flowers; (ii) in a second report, we will address the effect of the same treatments on the number of seeds per fruit, yield and fruit quality when applied to whole trees. Two reports were needed to more clearly present the results given the large number of analyses. Consequently, the objective of this first report was to evaluate the effect of seven products on *in vivo* pollen tube growth in Nadorcott mandarin flowers. To fulfill this main objective, we performed two experiments: (1) pre-pollination experiment: flowers were treated and were hand-pollinated 24 h later; (2) post-pollination experiment: flowers were pollinated and treated 24 h later.

2. Materials and methods

2.1. Experimental site and plant material

The experiment was performed on flowers of adult cv. 'Nadorcott' trees growing in a commercial orchard in the municipal district of Montserrat in the Valencia province, Spain (39° 21' 35'' N 0° 32' 44'' W; altitude 150 m) during the flowering period of April 2017. 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 (weather station at 39° 22' N 0° 27' W). Soil was calcareous sandy clay loam with a pH of 8.06 and 5.2% limestone.

Orchard management was carried out under standard cultural conditions with no other treatments during the flowering period except for the experimental treatments. Rootstock was *Citrus macrophylla* Wester and trees with drip irrigation stood 6 × 4 m apart.

2.2. Treatments

Three adult cv. Nadorcott trees were randomly selected from the orchard to use flowers for the experiments. At the beginning of the flowering period, the three trees were covered with nets to avoid unwanted pollinations. Upon anthesis, two experiments were performed: (1) flowers were treated and pollinated after 24 h; (2) flowers were pollinated and treated 24 h later. For pollinations, the flowers of a compatible variety (cv. Nova) growing close to the experimental plot (less than 150 m) were collected on the same day and donor anthers were brushed gently against cv. Nadorcott stigmas.

The initial hypotheses led us to propose products that could modify the physico-chemical characteristics of stigma secretion, such as viscosity or pH, and thus prevent pollen tube growth. Therefore, we selected products from the two main groups: saccharides/polysaccharides and inorganic salts. Nine treatments were included in the experiment (Table 1): a positive control (where stigmas were gently rubbed with a clean brush with no product); a negative control (where flowers were individually bagged in semipermeable nylon bags to avoid pollination); three inorganic fertilizers (ammonium nitrate, potassium nitrate, sulfur); four saccharides/polysaccharides (saccharose, methyl cellulose, callose, chitosan). Chitosan is a polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. Treatments were performed with a small brush to apply products on stigma surfaces. Approximately 0.5 g of pure solid product was applied to each stigma.

Fifteen flowers per treatment on the three trees (coded as A, B or C) and two experiments were treated. Each tree was treated with a 3-day difference, and each one represented a complete repetition of experiments. Therefore, 135 flowers per tree and experiment (9 × 15), 405 flowers per experiment (135 × 3) and 810 flowers in the two experiments (405 × 2) were treated.

2.3. Measurements

Flowers were collected 1, 3 and 5 days after the last action (pollination or treatment). On all these days, five flowers per treatment, tree and experiment were sampled early in the morning. Anthers and perianths were removed, and pistils were immediately fixed in Formalin-Aceto-Alcohol (FAA) solution (10 mL of 37% formaldehyde, 50 mL of 95% ethyl alcohol, 5 mL of glacial acetic acid, 35 mL of distilled water). The vials with the fixed material were transported to the laboratory in an insulated container, and subsequently stored at 4°C in the refrigerator until analyzed.

For the autofluorescence-stained sections, flowers were sectioned longitudinally on ca. 20 μm with a freezing microtome (Leica CM 1325). Sections were stained with aniline blue fluorochrome (0.1% in PBS buffer) for 15 minutes, subsequently washed with distilled water and

Table 1
Treatments with code, name and formula.

	Code	Name	Formula / observations
1	C-	Negative control	Bagged flowers without pollination or product
2	Sulfur	Sulfur	S_8
3	A_Nitrat	Ammonium nitrate	NH_4NO_3
4	K_Nitrat	Potassium nitrate	KNO_3
5	Chitosan	Chitosan	β- D-($C_6H_{13}NO_5$)
6	M_Cellul	Methyl cellulose	$C_6H_7O_2(OH)_x(OCH_3)_y$
7	Callose	Callose	$(C_6H_{10}O_5)_n$
8	Sacchar	Saccharose	$C_{12}H_{22}O_{11}$
9	C+	Positive control	Pollinated, but no product applied

mounted on microscope slides to be observed under an epifluorescence microscope. Sections were examined with light microscopy (LM) and epifluorescence microscopy (EFM) to analyze stigmas, and style, and to count the number of pollen tubes. All the microscopy photographs were coded with four positions: 'experiment 1 or 2′, 'treatment e.g., C+', 'tree A, B or C', 'sampling day 1, 3, or 5, and 'Flower' (e.g., in Fig. 1, 1C+C1F1).

Pollen tubes were counted in four zones (Fig. 1): zone 1, papillary cells and surrounding area of the stigma; zone 2, inner part of the stigma; zone 3, neck (union between the stigma and style); zone 4, first third of the style. All the LM and EFM observations were made by an Olympus Provis AX 70 fluorescence microscope equipped with an Infinity 2–3 C Lumenera® digital camera and analyzed by the "Infinity Analyze" software, v.6.4.1. For fluorescence microscopy, an Olympus U-ULS 100 HG epifluorescence system with a U-MWBV cube (excitation filter 400–440 nm, dichroic mirror 455 nm, barrier filter 475 nm) was used.

Additionally, the squash technique was applied to several flowers to observe pollen grains on stigma surfaces.

2.4. Statistical analysis

The average, standard error, skew, and kurtosis were assessed per treatment. The Shapiro-Wilk test was used to check for normality requirements. When residues met normality, an ANOVA was applied with the Tukey *post hoc* honestly significant difference test (HSD). When residues did not meet 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, respectively. All the statistical analyses were performed by R (R Core Team, 2020) with RStudio (RStudio Team, 2020) and some extra packages: agricolae (Mendiburu, 2020); ggplot2 (Wickham et al., 2019a); dplyr (Wickham et al., 2019b); tidyr (Wickham and Henry, 2019); cowplot (Wilke, 2019).

3. Results

3.1. Effect of treatments on the number of pollen tubes for the pre-pollination experiment

Flowers were treated and pollinated after 24 h. There were no differences in the number of pollen tubes among the flowers of the three trees (A, B and C) or their interaction with treatments (SM1). Pollen tubes were counted in four pistil zones, but the results indicated fewer pollen tubes in zones 3 and 4. Fewer tubes reached the style canals, and it was more difficult to observe tubes by microscope techniques. Therefore, although tendencies were similar in the four zones, the differences between treatments were better studied in zones 1 and 2 (SM2).

Flowers were fixed 1, 3 or 5 days after pollination. Similar trends among treatments were observed over the 3 days, and were more well-established on day 5 (Fig. 2).

An ANOVA for the effect of treatments on the pollen tube number showed significant differences among treatments (F-value = 4.62; p-value = 0.00028; Table 2). Remarkably, the sulfur treatment inhibited 100% pollen tube growth, while the positive control (C+) showed 62.2±12.1 tubes in zone 1 (Fig. 3). This result was also obtained for the flowers fixed on days 1 and 3 after pollination (Fig. 2), and in all the evaluated zones (z1 to z4; SM2).

The saccharose treatment produced more pollen tubes in zone 1 than the C+ treatment (66.0±22.4 Sacchar vs. 62.2±12.1 C+) but, in this case, the difference was not significant (Fig. 3, Table 2). The saccharose treatment consistently presented more pollen tubes than the positive control for the four zones (SM2) and for evaluation days 1 and 5 (Fig. 2). The other treatments presented non-significant differences with the positive control (Table 2). The negative control did not present pollen tubes in the flowers fixed on day 1 (Fig. 2), rather a few tubes in the flowers fixed on days 3 (8.78) and 5 (5.00), which indicated little pollen contamination.

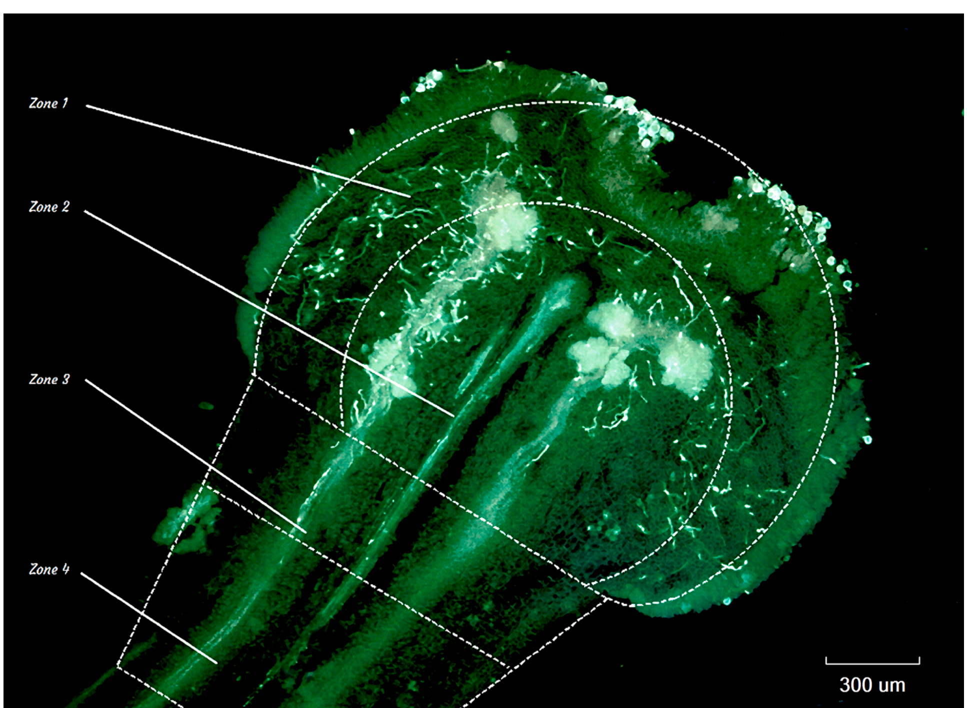

Fig. 1. Longitudinal blue violet autofluorescence-stained freeze section (20 µm) of a mandarin stigma (cv. Nadorcott). The four zones where the pollen tubes were counted are indicated: zone 1, papillary cells and surrounding area of the stigma; zone 2, inner part of the stigma; zone 3, neck or union between stigma and style; zone 4, first third of the style.

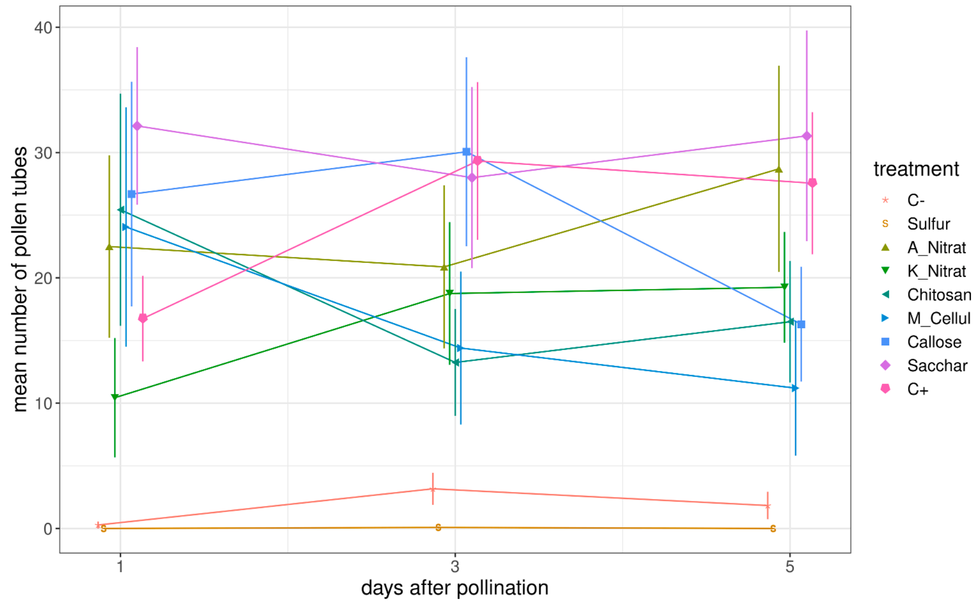

Fig. 2. Mean number of pollen tubes (all zones together) for each treatment at the three evaluation times: 1 day, 3 days and 5 days after pollination for the pre-pollination experiment. Error bars indicate the standard error.

Table 2
Average number of pollen tubes per treatment in zone 1 (stigma) 5 days after pollination in the pre-pollination experiment with the ANOVA Tukey *post hoc* test (HSD) values.

Treatment	N	Median	Mean	sd	se	skew	kurtosis	Shapiro	HSD
C-	9	0	5.0	12.0	4.0	2.70	7.42	0.00	b
Sulfur	9	0	0	0	0	NaN	NaN	NaN	b
A_Nitrat	5	62	68.6	46.5	20.8	-0.59	-0.42	0.45	a
K_Nitrat	5	45	44.2	18.9	8.5	-1.42	2.43	0.25	ab
Chitosan	6	27.5	34.2	34.9	14.2	0.27	-2.56	0.15	ab
M_Cellul	5	8	24.8	39.7	17.8	1.97	3.94	0.02	ab
Callose	5	37	39.8	24.3	10.9	-0.65	-0.21	0.51	ab
Sacchar	6	64	66.0	54.9	22.4	0.09	-2.37	0.42	a
C+	9	61	62.2	36.4	12.1	0.67	0.41	0.81	a

NB: sd, standard deviation; se, standard error; Shapiro, p-value for the Shapiro-Wilk normality test, p-value below 0.05 indicates non normal distribution; HSD, Tukey's Honestly Significant Difference test, where different letters mean significant differences in the ANOVA Tukey *post hoc* test (HSD) for alpha = 0.05 (F-value = 4.626; p-value = 0.00028). Shapiro test for residuals = 0.059.

3.2. Autofluorescence-stained freeze sections for the pre-pollination experiment

Several photographs were taken of each flower. As a result, hundreds of photos documented the experiment. Differences in treatments were observed directly on the images (Fig. 4; full-size images available at SM3). Pollen grains were clearly observed on stigma surfaces of the sulfur-treated flowers, but no pollen tubes grew inside (Fig. 4, B). The positive control and the other treatments showed pollen tubes growing inside stigmas (Fig. 4, C to I). The saccharose-treated flowers had many pollen tubes (Fig. 4, H).

Pollen grains and tubes were observed on the stigma surfaces on the squash images (Fig. 5; full-size images and more treatments at SM4). The negative control did not present any pollen grains (Fig. 5, A), while the other three treatments did. The sulfur treatment presented pollen grains, but not pollen tubes, while abundant pollen tubes were observed for saccharose and C+ (Fig. 5, B, C and D).

3.3. Effect of treatments on the number of pollen tubes for the post-pollination experiment

Flowers were pollinated and treated 24 h later. There was no difference in the number of pollen tubes among the three whole repetitions (trees A, B and C) as in the pre-pollination experiment (SM5). Once again, zones 1 and 2 showed more pollen tubes and were the best zones to study differences in treatments (SM6).

Analyses were performed directly on the flowers fixed 5 days after treatment (Fig. 6). The Kruskal-Wallis test for the effect of treatments on the number of pollen tubes showed significant differences in treatments (KW value = 23.22; p-value = 1.52 e-22; Table 3). As in the pre-pollination experiment, the sulfur treatment significantly and strongly lowered the number of tubes growing on stigmas (4.0±1.5 sulfur vs. 64.2±28.7 C+, Fig. 6). This powerful reduction was observed in all the zones (SM6).

Once more, the saccharose treatment presented more pollen tubes (76.3±10.0), but no significant differences with C+ (Table 3). This tendency was observed in all the zones (SM6). In this experiment, the ammonium nitrate treatment significantly lowered the number of tubes growing on stigmas (32.4±24.4 A_Nitrat vs. 64.2±28.7 C+). The negative control presented a few tubes on the flowers fixed on day 5 (7.7±10.4), which denotes little pollen contamination.

3.4. Autofluorescence-stained freeze sections for the post-pollination experiment

The differences in treatments for the post-pollination experiment were observed directly on the images (Fig. 7; full-size images available at SM7). Pollen grains were clearly observed on the stigma surfaces of

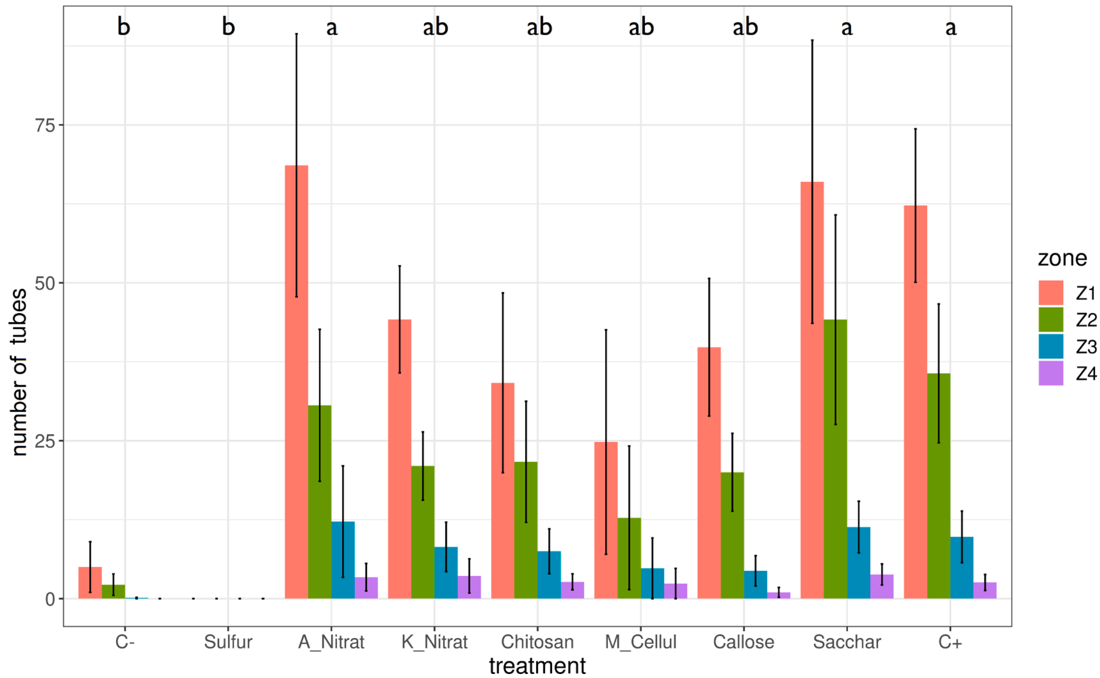

Fig. 3. Effect of treatment on the average number of tubes in the four zones 5 days after pollination for the pre-pollination experiment. Error bars indicate the standard error. For Z1: F-value = 4.626; p-value = 0.00028; Shapiro test for residuals = 0.059. Different letters mean significant differences for alpha = 0.05 (zone 1, HSD test).

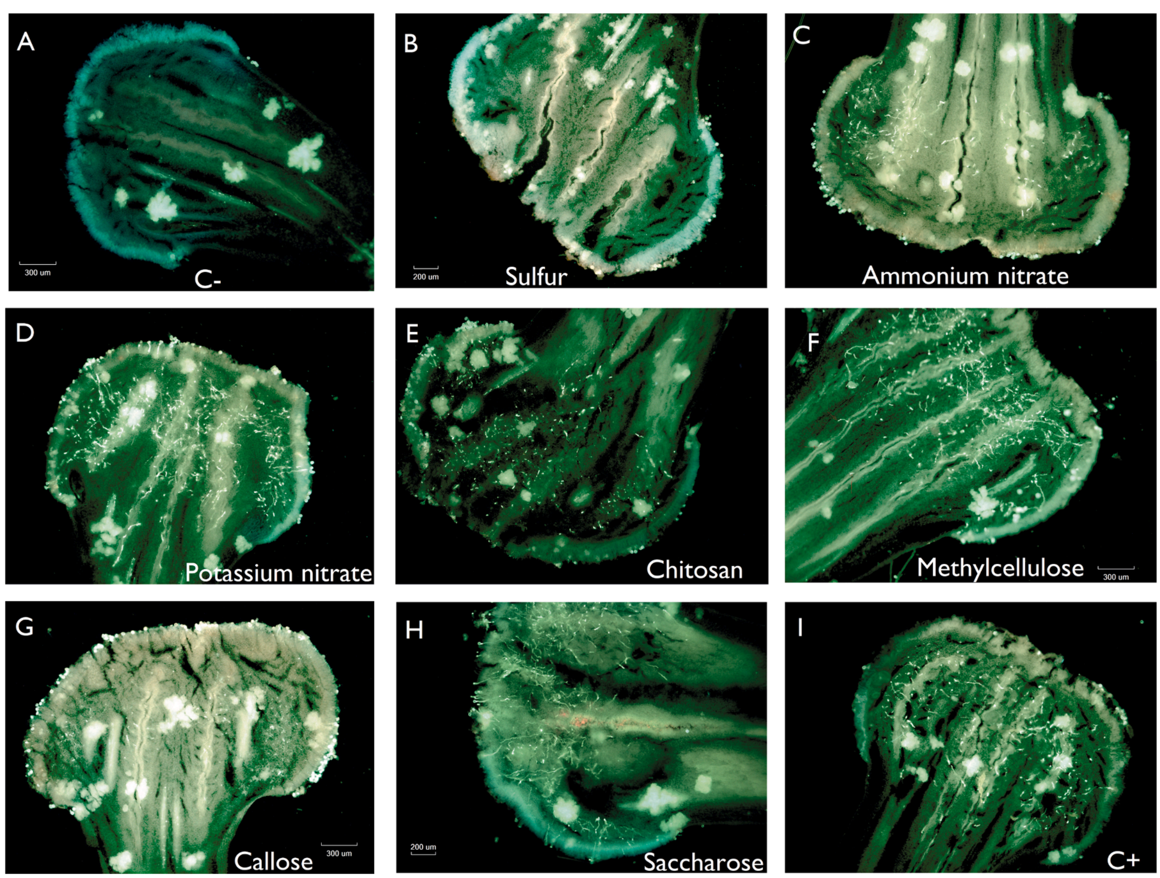

Fig. 4. Longitudinal blue violet autofluorescence-stained freeze section (20 μm) of nine mandarin cv. Nadorcott stigmas of the nine treatments for the pre-pollination experiment. Pollen grains and tubes are fluorescent. All these flowers were fixed 5 days after the last action. The final part of the style and stigma are always displayed regardless of orientation (up or upside down). A, C-; B, Sulfur; C, A_Nitrat; D, K_Nitrat; E, Chitosan; F, M_Cellul; G, Callose; H, Sacchar; I, C+.

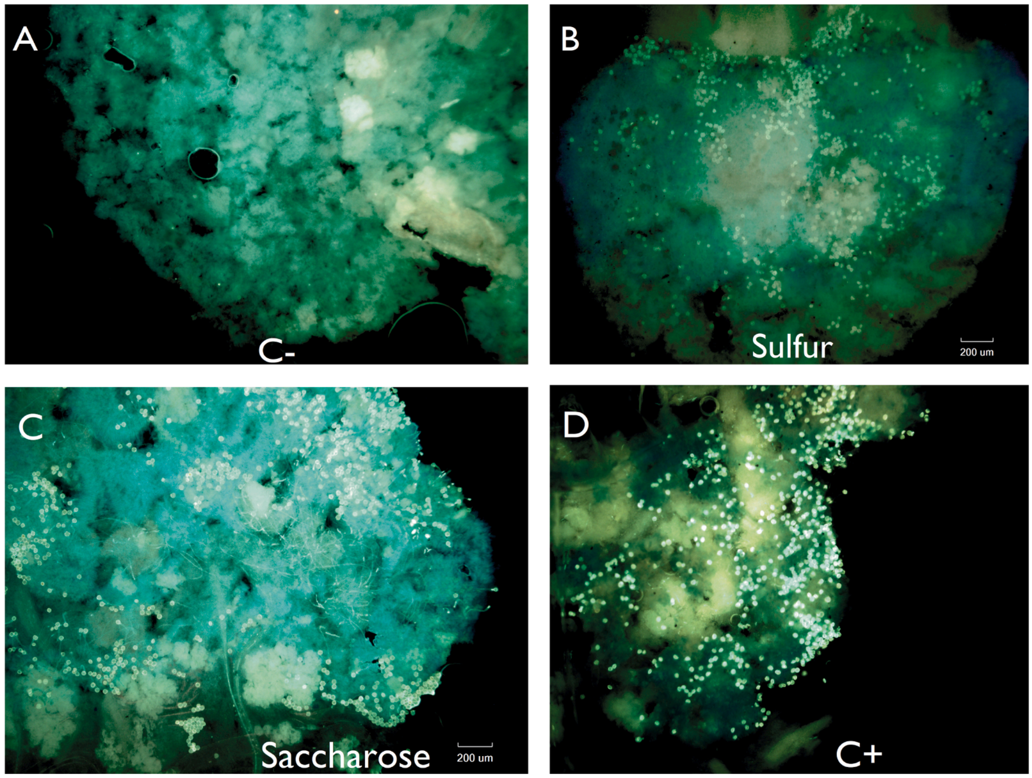

Fig. 5. Squash stained with blue violet autofluorescence made with the stigma tissue of four mandarin cv. Nadorcott in the pre-pollination experiment: A, C-; B, Sulfur; C, Sacchar; D, C+. Pollen grains and tubes are fluorescent. All these flowers were fixed 5 days after pollination.

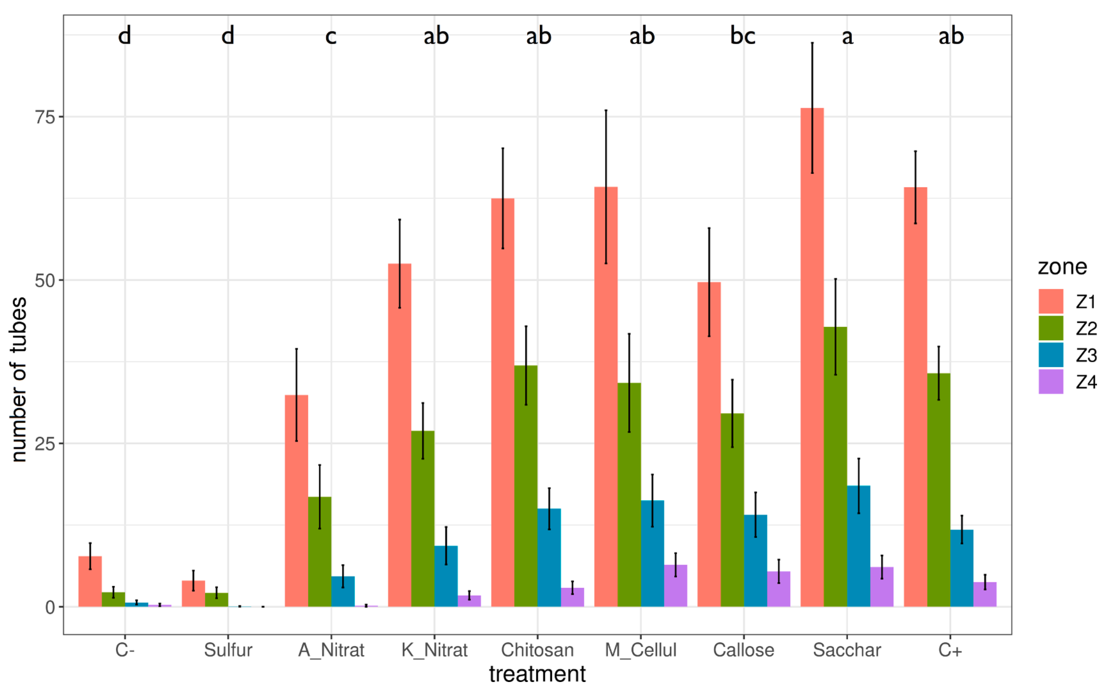

Fig. 6. Effect of treatment on the number of tubes in the four zones 5 days after treatment for the post-pollination experiment. Error bars indicate the standard error. For Z1: KW value = 23.22; p-value = 1.52 e-22; Shapiro test for residuals p = 0.0094. Different letters mean significant differences for alpha = 0.05 (zone 1).

the sulfur-treated flowers, but no pollen tubes grew inside (Fig. 7, B). The positive control and the other treatments (except C-) showed many pollen tubes growing inside stigmas (Fig. 7., C to I).

A small amount of pollen grains was observed on the stigma surface of the negative control (Fig. 8, A; full-size images and more treatments at SM8). Pollen grains were observed on the stigma surface in the other treatments but, once again, the sulfur treatment presented no tubes (Fig. 8, B, C and D).

Table 3
Average number of pollen tubes per treatment in zone 1 (stigma) 5 days after pollination for the post-pollination experiment with the Kruskal-Wallis rank sum test values.

Treatment	N	Median	Mean	sd	se	skew	kurtosis	Shapiro	KW
C-	27	0	7.7	10.4	2.0	1.13	-0.18	0.00	d
Sulfur	27	0	4.0	8.0	1.5	1.73	1.48	0.00	d
A_Nitrat	12	26	32.4	24.4	7.1	0.55	-0.92	0.33	c
K_Nitrat	12	58.5	52.5	23.3	6.7	-0.75	-0.40	0.11	ab
Chitosan	12	63	62.5	26.6	7.7	-0.22	-0.37	1.00	ab
M_Cellul	12	74	64.3	40.6	11.7	-0.18	-1.45	0.36	ab
Callose	12	49.5	49.7	28.7	8.3	0.16	0.04	1.00	bc
Sacchar	12	78.5	76.3	34.5	10.0	-0.26	-0.61	0.70	a
C+	27	57	64.2	28.7	5.5	0.95	0.81	0.09	ab

NB: sd, standard deviation; se, standard error; Shapiro, p-value for the Shapiro-Wilk normality test, p-value below 0.05 indicates non normal distribution; KW, the Kruskal-Wallis test by ranks, different letters mean significant differences for alpha=0.05 (KW value = 23.22; p-value = 1.52 e-22). Shapiro test for residuals p = 0.0094 that did not, therefore, meet normality for the ANOVA analyses.

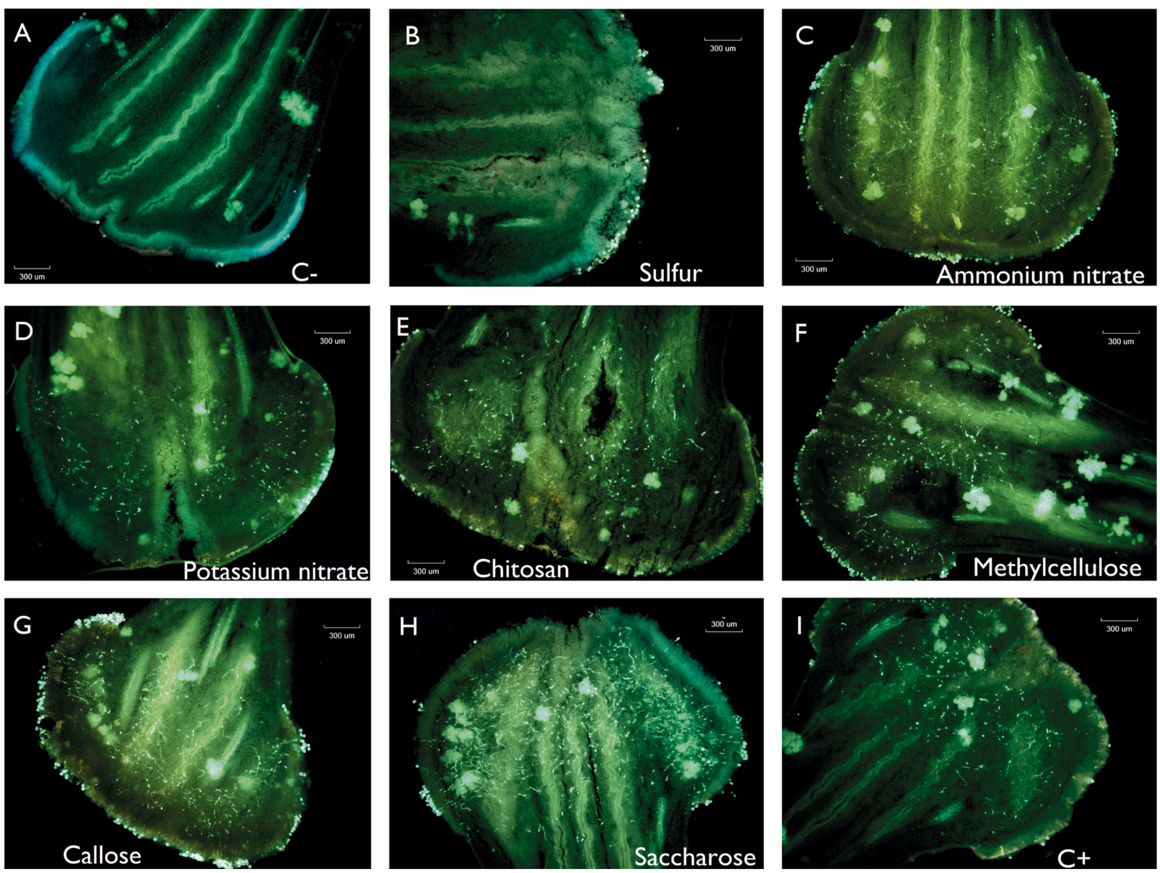

Fig. 7. Longitudinal blue violet autofluorescence-stained freeze section (20 μm) of nine mandarin cv. Nadorcott stigmas of the nine treatments for the post-pollination experiment. Pollen grains and tubes are fluorescent. All these flowers were fixed 5 days after the last action. The final part of the style and stigma are always displayed regardless orientation (up or upside down). A, C-; B, Sulfur; C, A_Nitrat; D, K_Nitrat; E, Chitosan; F, M_Cellul; G, Callose; H, Sacchar; I, C+.

4. Discussion

This is the first time that elemental sulfur (S_8) is tested to prevent pollen tube growth on mandarin stigmas, and the first time that such a powerful effect with almost 100% pollen tube growth inhibition is reported. Sulfur is probably acting on the papillary cells, but autofluorescence-stained freeze sections do not show adequate definition to study this activity in depth. Transmission electron microscopy and semi-thin sections will be needed to clarify how sulfur inhibits growth. Very few studies have performed experiments with compounds that include sulfur to prevent sexual reproduction and none with elemental sulfur. According to Mesejo et al. (2006), addition of 25 mg l^{-1} of $CuSO_4 \bullet 5H_2O$ to germination media significantly inhibits the *in vitro* pollen germination of 'Fortune' mandarin. In this study, SO_4 was used as a vehicle of copper (Cu^{2+}), and Cu^{2+} was the main tested component. The main conclusion was that copper had an inhibitory effect on pollen tube growth (Mesejo et al., 2006).

In other species like *Lepidium virginicum* L. (Brassicaceae), exposing pollen to 0.6 ppm sulfur dioxide (gas SO_2) for 8 h during flowering reduced pollen germination *in vivo* by 50% from the control, but did not affect seed set (Du Bay and Murdy, 1983). Finally in apples (*Malus* × *domestica* Borkh., Rosaceae), a combination of liquid lime sulfur (LLS) and fish oil (FO) applied during bloom inhibited pollen tube growth, but had no effect when applied 48 h after pollination (Yoder et al., 2009). The use of lime sulfur for flower thinning in apples have been reported by several studies (Chun et al., 2012; DeLong et al., 2018; Peck et al.,

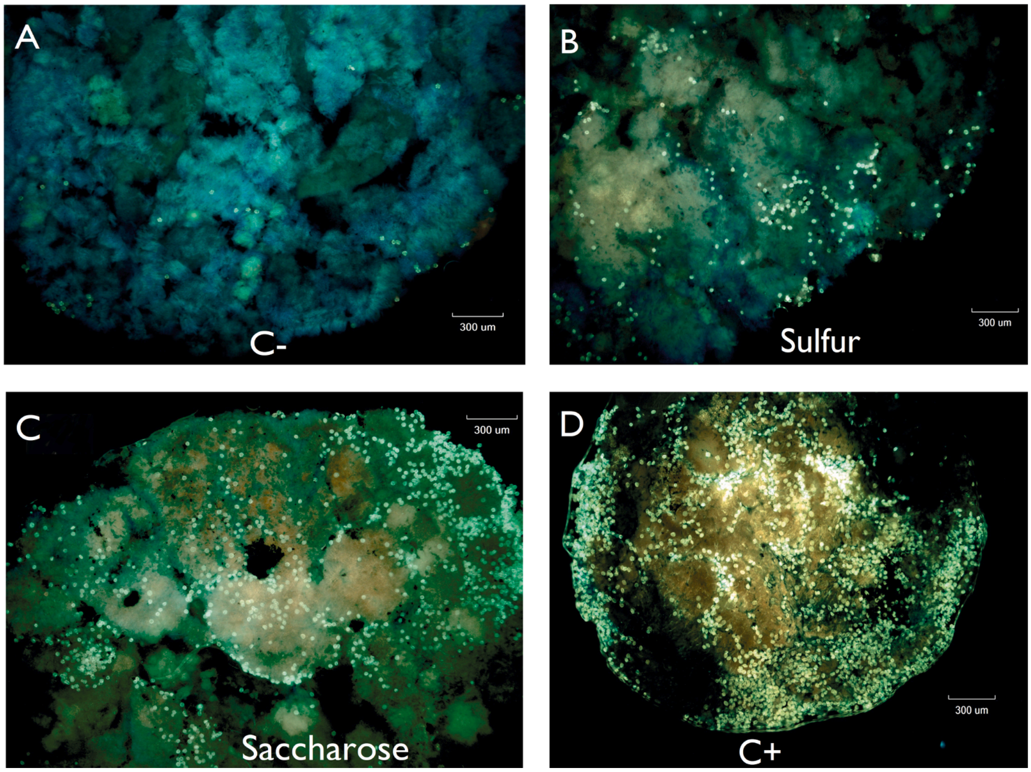

Fig. 8. Squash stained with blue violet autofluorescence made with the stigma tissue of four mandarin cv. Nadorcott in the post-pollination experiment: A, C-; B, Sulfur; C, Sacchar; D, C+. Pollen grains and tubes are fluorescent. All these flowers were fixed 5 days after pollination.

2015).

When pollination was forced, the microscopy squash images showed abundant germinated pollen grains on stigma surfaces (Fig. 5 C and D). This prolific event was completely blocked by the sulfur (S_8) treatment (Fig. 5 B). This strong effect can be useful in agronomy to develop applications that avoid seeds in mandarins, or for other breeding uses. Present horticultural techniques (bee repellents, $CuSO_4$, $CuSO_4 + GA_3$) are barely effective (around 30% seed reduction) and only net covering is effective, but very expensive (Gambetta et al., 2013; Garmendia et al., 2019a). Therefore, lowering the seed number in well-established mandarins remains an unmet objective, and the result herein presented opens up new possibilities of developing more effective applications.

Although the differences with C+ were not significant, the saccharose treatment seemed to stimulate pollen tube growth on stigmas (Fig. 4 and 7 H). On the squash images, abundant germinated pollen grains were observed (Fig. 5 C). Saccharose is usually included in *in vitro* culture media to improve the germination of many species (Hayase and Hiraizumi, 1955; Qinghua et al., 1986). Melgarejo et al. (2000) have concluded that for pomegranate (*Punica granatum* L.) pollen, the optimum saccharose concentration lies between 10% and 20%, with levels below 10% producing low pollen germination levels. Therefore, it is consistent that an external saccharose application on stigma surfaces can improve pollen germination and growth. This effect could be useful for non parthenocarpic varieties with a low fruit set, where fertilization is an important stimulus for achieving good yields.

Both experiments obtained a similar result: during pre-pollination, sulfur inhibited 100%, but inhibition was 94% in the post-pollination experiment, which suggests a strong effect when applied 24 h before or after pollination. The ammonium nitrate treatment significantly lowered the number of tubes in the post-pollination experiment, but not in the pre-pollination experiment. Autofluorescence-stained microscopy is an appropriate technique for studying the effect of treatments, as in other studies and species (García-Breijo et al., 2020; Mesejo et al., 2007). For Nadorcott mandarins, 5 days after treatment was the best time to take microscopic observations. Finally, although same tendencies were observed among treatments at the 4 studied zones, zones 1 and 2 (stigma) were the best for treatments comparison rather than zones 3 and 4 (style) where fewer tubes were observed and with more difficulty.

Citrus flowering can be influenced by many factors and last for more than 4 weeks depending on climate conditions (Distefano et al., 2018; Garmendia et al., 2019c). Therefore, finding an effective seed reduction treatment under real field conditions is no easy task. With this effort, the present study represents a first step. The next steps will include applying the same products to entire trees during the flowering period and study the ultrastructure action of sulfur to prevent pollen tube development in mandarin stigmas.

5. Conclusions

The effect of seven products on *in vivo* pollen tube growth when applied to Nadorcott flowers was evaluated. Of those seven products, only sulfur had a strong inhibitory effect. Elemental sulfur (S_8) inhibited pollen tube growth by 94-100%. This effect could be useful for designing agronomic applications capable of preventing seed presence in Nadorcott mandarin. The saccharose treatment seemed to have the opposite effect as it stimulated pollen tube growth, but the difference with the positive control was small and non-significant.

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CRediT authorship contribution statement

A. Garmendia: Investigation, Writing – review & editing, Formal analysis, Conceptualization, Validation, Resources, Visualization, Supervision. **F. García-Breijo:** Investigation, Writing – review & editing, Validation, Resources, Visualization, Supervision. **J. Reig:** Investigation, Writing – review & editing, Validation, Resources, Visualization. **M.D. Raigón:** Investigation, Writing – review & editing, Validation, Resources, Supervision. **R. Beltrán:** Investigation, Writing – review & editing, Validation. **C. Zornoza:** Investigation, Writing – review & editing. **N. Cebrián:** Investigation, Visualization. **H. Merle:** Investigation, Writing – review & editing, Formal analysis, Conceptualization, Validation, Resources, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

Part of the results reported in this study were used for the international patent application PCT/ES2019/070509.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2021.110760.

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