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The effect of three plant bioregulators on pollen germination, pollen tube growth and fruit set in almond [*Prunus dulcis* (Mill.) D.A. Webb] cvs. Non Pareil and Carmel

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A B S T R A C T

Background: In commercial almond [*Prunus dulcis* (Mill.) D.A. Webb] orchards in Chile, the percentage of fruit set is low and commonly reaches 5–30%. As almond trees bloom during a cool period and also suffer from self-incompatibility, any factor that can improve pollination, pollen germination and pollen tube growth or extend the effective pollination period, such as the application of plant bioregulators (PBRs), should be beneficial for fruit production.
Results: Three plant bioregulators (PBRs): brassinolide (BL), gibberellic acid (GA_3) and kinetin (KN) were evaluated for pollen germination and pollen tube growth *in vitro*, as well as for fruit set in almond cultivars Non Pareil and Carmel, in central Chile, during the 2013 and 2014 seasons. For pollen germination *in vitro*, the BL concentration of 10 mg L^{-1} had the highest value in Non Pareil (97.7%), after 4 h germination in 2014 growing season (the control was 90.9%). KN at a concentration of 50 μL L^{-1} induced the longest pollen tube growth of 1243.4 μm in Carmel after 8 h germination in 2013 (the control was 917.7 μm). In Non Pareil, the highest percentage of fruit set (31.0%) was achieved in 2014 by spraying during bloom at pink bud stage with KN at 50 μL L^{-1} (the control was 16.7%).
Conclusions: A significant favorable effect of the tested PBRs was observed in pollen germination and pollen tube growth *in vitro*, as well as on fruit set in Non Pareil and Carmel almonds.

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1. Introduction

Pollen germination and pollen tube growth are key events in the sexual reproduction of plants [1]. Currently, it is accepted that both fruit set and fruit development are regulated by the coordinated action of hormones produced in the ovary after pollination or fertilization [2]. Pollination and subsequent fertilization lead to a strong shift in the balance of phytohormones and development the ovule [3].

After anthesis and pollination, pollen development continues in the stigma with rehydration, germination and pollen tube growth. Both pollen germination and pollen tube growth are dependent on gibberellins synthesized *in situ* [4]. Gibberellins have long been recognized as endogenous hormones controlling stem elongation, root and fruit growth, leaf shape, de-etiolation, flowering and flower development [5]. The influence of gibberellins on pollen germination and pollen tube growth has been verified by application to rice flowers (*Oryza sativa*) [6,7]. Exogenous applications of gibberellins have also been reported to promote pollen germination and increase pollen tube length *in vitro* in apricot (*Prunus armeniaca*) [8]. Huang et al. [9] showed that gibberellin application completely restored the fertility and morphology of gibberellin-deficient mutant flowers in both Arabidopsis (*Arabidopsis thaliana*) and tomato (*Lycopersicon esculentum*).

Brassinosteroids are naturally-occurring plant hormones that regulate growth and development in plants [10]. These hormones significantly promote both pollen germination and pollen tube growth [11]. Like other plant hormones, brassinosteroids are involved in processes such as division and cell elongation, synthesis of DNA, RNA and proteins, growth and development of plant organs, senescence and stress responses [12]. Vogler et al. [11] demonstrated that brassinosteroids promoted both pollen germination and pollen tube growth *in vitro* in *A. thaliana*. Also, brassinosteroids have been reported to enhance pollen germination and pollen tube growth in *Prunus avium*, *Camellia japonica* [13] and *Prunus dulcis* [14]. Brassinosteroids were also effective for increasing pollen viability and reducing its loss under heat stress, and improved both pollen germination *in vivo* and fruit set in rice [10]. Fu et al. [15] in experiments on *Cucumis sativus* found that brassinosteroids play a role in the early development of the fruit.

Cytokinins are plant hormones promoting cell division and differentiation [16]. They regulate cell division, morphogenesis and meristematic tissue activity, delaying the onset of senescence involved in apical dominance. They have also been reported to influence the

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development of flowers and seeds, seed germination, nutrient uptake and demand, and organ assimilation [17]. Bolat and Pirlak [8] found that the exogenous application of kinetin at low concentrations *in vitro* improved pollen germination in apricot (*P. armeniaca*) cv. Hasanbey, and significantly improved pollen tube lengths in the Hasanbey, Karacabey and Mahmudun Erigi cultivars. They also reported that high concentrations of kinetin can inhibit pollen tube growth. In some species, the application of cytokinins to flowers before fertilization, originates the beginning of fruit growth [18]. Also, exogenous treatment with cytokinins delays flower senescence in several plant species [19]. As cytokinins regulate cell division, they are associated with fruit cytokinesis. Moreover, a correlation between levels of cytokinins and cell division activities has been observed in tomato (*L. esculentum*) [18].

The almond (*P. dulcis*) is not only an early fruit in breaking dormancy, but also shows the widest range of flowering time among all deciduous fruit trees and nut species [20]. The determination of fruit set has been used in almond as a consistent indicator of the real productivity level [21]. In commercial orchards, the percentage of fruit set is commonly only 5–30% of the possible total [22]. Most almond cultivars are self-incompatible, requiring orchards to have at least two cultivars blooming synchronously to obtain adequate yields [23]. Pollination is especially critical for fruit set in infertile species [24]. As almond trees bloom during a cool period and also suffer from self-incompatibility, any factor that can extend the effective pollination period, such as the application of plant bioregulators (PBRs), should be beneficial for fruit production [14]. The establishment of yield models in apple and almond show that fruit set is determined by the number of flowers per tree and the effective pollination period [25]. Pollen maturation and fertilization are affected by environmental conditions such as light, temperature and relative humidity. Consequently, unfavorable environmental conditions can drastically reduce fertilization and fruit development. The development processes of seeds and fruit are connected and synchronized closely and are controlled by phytohormones [18].

PBRs are an important part of current agricultural technology. In recent years, the biological efficiency and action mechanisms have been studied. These PBRs have multifunctional effects and low toxicity, characterized by having no negative impacts on the environment. Their physiological effects include stimulation of growth, increasing the resistance of plants to abiotic stress and diseases, and increased crop yields [26]. In this study, the effects of three commercially available PBRs were evaluated on pollen germination and pollen tube growth in almond (*P. dulcis*) cvs. Non Pareil and Carmel *in vitro*, as well as on fruit set when applied in the field.

2. Materials and methods

The field experiments and the collection of pollen, were done in an almond orchard located in the area of Paine, Metropolitan Region of Chile (latitude 33°46′21.3″ S–longitude 70°38′12.5″ W). The *in vitro* experiments were done at the Deciduous Fruit Crops Laboratory of the Pontificia Universidad Católica de Chile, San Joaquín Campus, in Santiago.

2.1. Pollen collection

Flowers were collected in anthesis when the anthers were still closed, from the almond cultivars Non Pareil and Carmel. The flowers were kept in perforated zip bags, put into a portable cooler, and transported to the laboratory. Closed anthers from 100 flowers were extracted and placed on Petri dishes (90 mm diameter) and dried in an incubation chamber (Velp Scientifica, FTC 90E, Italy) at 24°C for 24 h, during which time the anthers opened and liberated pollen. The extracted pollen and anthers were then kept in closed plastic containers in a refrigerated chamber at -20°C and 60% relative humidity.

2.2. Viability test using fluorochromatic reaction

Pollen viability was evaluated using the fluorochromatic reaction test (FCR) with the application of fluorescein diacetate (FDA) following the method proposed by Heslop-Harrison and Heslop-Harrison [27] and adapted by Li [28]. A BK buffer solution of S15 MOPS pH7.5 ($Ca(NO_3)_2$ $4H_2O$, 30 mg L^{-1}; $MgSO_4$ $7H_2O$, 20 mg L^{-1}; KNO_3, 10 mg L^{-1}; MOPS, 10 mM and 15% sucrose) was prepared in distilled water and also a stock solution of FDA with 2 mg of FDA per ml of acetone that was maintained at -20°C in an Eppendorf tube. One microliter of the FDA stock solution was added to 1 ml of the BK S15 MOPS pH 7.5 buffer solution. A drop of the FDA-buffer solution was placed on a clean microscope slide and almond pollen was added using a sterile paintbrush, then a coverslip was placed on top. After 5 min the slide was examined for fluorescence under an optical microscope (Olympus CX31, Japan) set at 10× with blue light (495 nm). The viable pollen grains showed intense fluorescence. Four replications were used for each cultivar (each microscope slide was considered an experimental unit), and 3 fields from each replication were photographed at random (ProgRes®C3 camera with the program Capture-Pro v2.8.8) having a final total of 12 fields from each cultivar. One hundred pollen grains in each field were counted at random (with or without fluorescence), evaluating a total of 1200 grains per cultivar. To calculate the percentage of pollen viability, the number of high fluorescence-emitting grains in each field was divided by 100 and this quotient was multiplied by 100.

2.3. Germination of pollen and growth of pollen tube in vitro

Three PBRs were applied for evaluation of on pollen *in vitro* and on the almond tree cultivars Non Pareil and Carmel in the field.

Brassinolide 0.1%, wettable powder (WP) with the active ingredient 24-epibrassinolide (chemical formula: 22R, 23R, 24R)-2α,3α,22,23-tetrahydroxy-24-methyl-β-homo-7-oxa-5-cholestan-6-one.

ProGibb® 4%, soluble concentrate (SL), active ingredient gibberellic acid 3.2% p/v, GA_3, (chemical formula: 3S, 3aR, 4S, 4aS, 7S, 9aR, 9bR, 12S)-7, 12- dihydroxy-3-methyl-6-methylene-oxoperhydro-4, 7-methano-9b, 3-propenoazuleno (1,2-b) furan-4-carboxylic acid.

X-Cyte®, soluble concentrate (SL), whose active ingredient is cytokinin, as kinetin, with a concentration of 0.04% p/v (chemical formula: 6-furfurylamino-9H-purine).

Both the experiments in the field and *in vitro* used the same three PBRs: Brassinolide (BL), ProGibb® (GA_3) and X-Cyte® (KN) at concentrations of 10, 30 and 50 mg L^{-1}, in the case of BL which is a wettable powder, and 10, 30 and 50 μL L^{-1} in the cases of GA_3 and KN which are soluble concentrates. All of the concentrations used in this study were commercial products.

To evaluate these PBRs on almond pollen germination and on the growth of pollen tubes, an agar medium was prepared with 1% agar, 10% sucrose and 50 ppm of boric acid (H_3BO_3) (formula proposed by Sutyemez [29] with modification), plus the addition of the PBRs at their different concentrations of commercial product. The media were deposited in Petri dishes (50 mm diameter) and after solidification, pollen was scattered over them using a sterile paintbrush. Plates were then placed in a dark incubation chamber (Velp Scientifica, FTC 90E, Italy) at 24°C [30] for 4 and 8 h. This experiment used a completely randomized design with four replications, considering each Petri dish as an experimental unit. The controls used agar medium prepared with no PBRs.

The observations were done using an optical microscope (Olympus CX31, Japan) with the objective at 4×. In each replication three fields were photographed at random (ProgRes®C3 camera with the program Capture-Pro v2.8.8) allowing clear observation of germination and growth of the pollen tubes. The percentage of germination was evaluated after 4 h and calculated with the number of germinated

grains divided by the total number of grains by 100. Pollen grains were considered germinated if the length of the pollen tube was equal to or greater than the diameter of the pollen grain [31,30,32]. The length of the pollen tubes was measured using the computational program Qcad Professional v 2.2.2.0 on the photos taken after 8 h of incubation. The pollen tubes were selected and measured at random, for a total of 300 tubes for each treatment, 25 for each photographed field.

2.4. Effect of the plant bioregulators on fruit set in almond

Twelve 10-year-old almonds trees (Non Pareil and Carmel), were selected from alternating rows. From each tree, 10 uniform branches were selected (experimental unit), for the treatments at the phenological stage of pink bud [33] and 10 uniform branches were also selected for treatment application at the phenological stage of fallen petals [33]. The nine treatments and the control were applied to the branches following a random block design. Treatments were sprayed onto the flowers using a hand pump in the months of August, 2013 and 2014. The controls were only sprayed with water. Before the application, the number of flowers was counted on each branch, and at 60 days after full bloom, the number of fruit that had been set was counted. The fruit set was converted to percentages for comparison among the treatments using the following formula:

$$\%\ \text{Fruit Set} = \frac{\text{N}^\circ\ \text{of fruit set per branch at 60 DAFB}}{\text{N}^\circ\ \text{of flowers per branch}} \times 100.$$

2.5. Statistic analysis

An analysis of variance was done for all of the variables evaluated. In the case of the quantitative discrete variables such as the percentage of germinated pollen grains and the percentage of fruit set, the data was transformed to arcsine $\sqrt{x}$ prior to analysis. The averages were compared using Tukey–Kramer test at $\alpha = 0.05$. SAS (SAS v.9.1.3) software was used for the statistical analysis.

3. Results

3.1. Viability test using fluorochromatic reaction

The pollen viability test using the fluorochromatic reaction procedure (FCR), was done in 2013 and 2014 seasons using pollen from Non Pareil and Carmel almond cultivars. The fluorochromatic reaction was evaluated in 1200 pollen grains from each cultivar. In the case of Non Pareil, the percentages of grains that showed intense fluorescence, and were thus considered viable, were 86.5 and 91.8%, in 2013 and 2014, respectively. In Carmel, the percentages of grains with intense fluorescence were 92.3 and 93.5% in 2013 and 2014 respectively.

3.2. Pollen germination and pollen tube growth in vitro in the presence of plant bioregulators

The effects of the PBRs on pollen germination were measured at 4 h after placement on the germination media containing the PBRs at the previously mentioned concentrations. The results are shown in Table 1.

In the Non Pareil cultivar, a statistically significant effect of the PBRs was observed in both seasons (2013 and 2014) ($p < 0.0001$ and $p < 0.0009$). For Brassinolide (BL) the lowest concentration (10 mg L^{-1}) had the highest percentage of germination with 95.3 and 97.7% respectively in the 2013 and 2014 seasons. For ProGibb® (GA_3), the highest concentration (50 μL L^{-1}) showed the best effect, reaching 95.1 and 96.6% germination in 2013 and 2014, respectively. X-Cyte® (KN) was similar to GA_3, in that the highest concentration showed the best effect, with 94.1 and 95.9% germination, in 2013 and 2014, respectively. The averages from the controls were 90.0 and 90.9% germination in 2013 and 2014.

Table 1
Percentage of pollen germination *in vitro* on Non Pareil and Carmel almond cultivars after 4 h, in the presence of plant bioregulators in the 2013 and 2014 growing seasons.

Treatments	cv. Non Pareil				cv. Carmel			
	2013		2014		2013		2014	
Control	90.0	d	90.9	c	89.2	c	91.9	b
BL 10 mg L^{-1}	95.3	a	97.7	a	95.5	ab	95.1	a
BL 30 mg L^{-1}	92.4	cd	94.4	abc	92.6	bc	94.0	ab
BL 50 mg L^{-1}	91.0	d	91.4	bc	90.4	c	93.6	ab
GA_3 10 μL L^{-1}	90.9	d	91.3	bc	92.4	bc	94.3	ab
GA_3 30 μL L^{-1}	92.6	bcd	95.2	abc	96.7	ab	94.8	ab
GA_3 50 μL L^{-1}	95.1	ab	96.6	ab	96.9	a	95.1	a
KN 10 μL L^{-1}	90.7	d	92.8	abc	92.9	abc	92.8	ab
KN 30 μL L^{-1}	92.0	cd	94.5	abc	93.3	abc	94.3	ab
KN 50 μL L^{-1}	94.1	abc	95.9	abc	94.4	abc	94.7	ab

Means followed by the same letter are not statistically different according to the Tukey–Kramer test ($p \leq 0.05$).

The effect of PBRs on Carmel pollen was statistically significant in both years ($p < 0.0001$ and $p < 0.0184$). GA_3 at the highest concentration (50 μL L^{-1}) showed the highest germination with 96.9 and 95.1% respectively, in 2013 and 2014. BL at the lowest concentration (10 mg L^{-1}) showed the greatest effect on pollen germination with 95.5 and 95.1%, in 2013 and 2014, respectively. KN showed the highest germination percentages at the highest concentration of 50 μL L^{-1} with 94.4 and 94.7%, in 2013 and 2014, respectively. The averages of the controls in Carmel were 89.2 and 91.9% in 2013 and 2014, respectively.

The length of the pollen tube was measured at 8 h after placement on the germination media. The length of the tube varied significantly due to the presence of the PBRs ($p < 0.0001$ in both cultivars and 2013 and 2014 seasons). The results are shown in Table 2.

In cv. Non Pareil, GA_3 showed the greatest pollen tube length, at the highest concentration (50 μL L^{-1}), with 1100.6 and 1096.0 μm, in the 2013 and 2014 seasons, respectively. At the lowest concentration, BL induced longer pollen tube lengths with 1067.4 and 1078.8 μm, in 2013 and 2014, respectively. KN at the highest concentration showed longer pollen tube lengths with 1056.8 and 1066.9 μm, in 2013 and 2014, respectively. Pollen tube length in the controls was 937.1 and 945.0 μm, in the years 2013 and 2014, respectively.

In cv. Carmel, KN at the highest concentration (50 μL L^{-1}) got the greatest pollen tube length with 1243.4 and 1215.9 μm in 2013 and 2014 seasons, respectively. GA_3 at the highest concentration (50 μL L^{-1}) reached 1226.6 and 1183.5 μm in the 2013 and 2014 seasons, respectively. BL at the lowest concentration (10 μL L^{-1}) reached important lengths with 1117.0 and 1100.1 μm in both seasons. All these values were significantly higher than the controls.

Table 2
Pollen tube length in Non Pareil and Carmel almond cultivars after 8 h, in the presence of plant bioregulators in the 2013 and 2014 growing seasons (values in μm).

Treatments	cv. Non Pareil				cv. Carmel			
	2013		2014		2013		2014	
Control	937.1	f	945.0	h	917.7	e	921.3	g
BL 10 mg L^{-1}	1067.4	b	1078.8	b	1117.0	c	1100.1	e
BL 30 mg L^{-1}	1032.6	c	1043.0	d	1059.7	d	973.1	f
BL 50 mg L^{-1}	963.9	e	971.6	f	921.3	e	964.0	f
GA_3 10 μL L^{-1}	977.0	e	971.7	f	1183.0	b	1144.6	d
GA_3 30 μL L^{-1}	1000.0	d	997.7	e	1199.4	b	1168.1	c
GA_3 50 μL L^{-1}	1100.6	a	1096.0	a	1226.6	ab	1183.5	b
KN 10 μL L^{-1}	942.0	f	947.3	h	1198.4	b	1179.9	bc
KN 30 μL L^{-1}	965.1	e	960.6	g	1212.5	ab	1186.2	b
KN 50 μL L^{-1}	1056.8	b	1066.9	c	1243.4	a	1215.9	a

Means followed by the same letter are not statistically different according to the Tukey-Kramer test ($p \leq 0.05$).

Table 3
Percentage of fruit set in Non Pareil almond cultivar at 60 days after full bloom, with plant bioregulators treatments at two phenological stages (2013 and 2014).

Treatments	Pink Bud				Fallen Petals			
	2013		2014		2013		2014	
Control	17.1	d	16.7	d	15.6	c	16.5	c
BL 10 mg L^{-1}	24.6	ab	22.3	bcd	21.7	abc	22.6	ab
BL 30 mg L^{-1}	22.5	abcd	19.2	cd	19.5	abc	20.4	abc
BL 50 mg L^{-1}	22.1	abcd	18.9	cd	17.8	bc	16.7	bc
GA_3 10 μL L^{-1}	23.7	ab	26.2	ab	19.8	abc	22.5	abc
GA_3 30 μL L^{-1}	27.1	a	28.0	ab	26.2	a	22.7	ab
GA_3 50 μL L^{-1}	18.0	cd	22.7	bcd	20.6	abc	19.8	abc
KN 10 μL L^{-1}	20.1	bcd	22.7	bcd	22.1	ab	19.8	abc
KN 30 μL L^{-1}	23.5	abc	24.8	abc	23.7	ab	25.6	a
KN 50 μL L^{-1}	25.8	ab	31.0	a	22.1	ab	24.0	a

Means followed by the same letter are not statistically different according to the Tukey–Kramer test ($p \le 0.05$).

Control values were 917.7 and 921.3 μm pollen tube lengths in the 2013 and 2014 seasons, respectively.

3.3. Evaluation of plant bioregulators on fruit set in almond trees

Three PBRs were sprayed on the flowers of Non Pareil and Carmel almond trees in August of 2013 and August of 2014 at the phenological stages of pink bud and fallen petals. The results obtained with the application of the PBRs 60 days after full bloom are shown in Table 3 and Table 4.

The application of the PBRs on Non Pareil showed statistically significant differences at the two phenological stages in both seasons ($p < 0.0001$) (Table 3).

GA_3 (30 μL L^{-1}) showed the highest percentage of fruit set in the 2013 season, at both phenological stages with 27.1% at pink bud and 26.2% at fallen petals. KN reached important values at concentrations of 30 and 50 μL L^{-1} at the two phenological stages in 2013, with 23.5 and 25.8% of fruit set at pink bud and 23.7 and 22.1% of fruit set at fallen petals, respectively. BL (10 μL L^{-1}) improved fruit set at pink bud with 24.6% of fruit set and 21.7% of fruit set at the stage of fallen petals. The control values were 17.1 and 15.6% of fruit set at pink bud and fallen petals, respectively, in the same season.

In the 2014 season, in Non Pareil, KN at the concentrations of 30 and 50 μL L^{-1} significantly improved fruit set. KN in concentration 50 μL L^{-1} reached the highest percentage of fruit set with 31.0% at pink bud stage. At the fallen petals stage KN in concentration 30 μL L^{-1} obtained the better value with 25.6% of fruit set. GA_3 (30 μL L^{-1}) also showed a positive effect on fruit set, with 28.0 and 22.7% at pink bud and fallen petals stages, respectively. BL (10 μL L^{-1}) enhanced fruit set at pink bud stage with 22.3% of fruit set and 22.6% of fruit set at fallen petals stage. The control values were 16.7 and 16.5% of fruit set at pink bud and fallen petals respectively in the 2014 season.

Table 4
Percentage of fruit set in Carmel almond cultivar at 60 days after full bloom, with plant bioregulators treatments at two phenological stages (2013 and 2014).

Treatments	Pink bud				Fallen petals			
	2013		2014		2013		2014	
Control	11.8	b	12.2	b	13.8	c	13.1	b
BL 10 mg L^{-1}	19.5	a	20.4	a	20.0	ab	20.1	a
BL 30 mg L^{-1}	17.7	a	15.6	ab	15.2	abc	16.4	ab
BL 50 mg L^{-1}	15.2	ab	14.1	b	14.0	bc	13.7	b
GA_3 10 μL L^{-1}	17.6	a	16.6	ab	15.5	abc	17.2	ab
GA_3 30 μL L^{-1}	19.4	a	17.5	abs	18.6	abc	19.7	a
GA_3 50 μL L^{-1}	15.4	ab	13.0	b	16.4	abc	17.8	ab
KN 10 μL L^{-1}	17.2	ab	14.4	b	15.3	abc	15.6	ab
KN 30 μL L^{-1}	19.8	a	20.3	a	21.0	a	20.4	a
KN 50 μL L^{-1}	18.2	a	20.2	a	20.6	a	20.0	a

Means followed by the same letter are not statistically different according to the Tukey–Kramer test ($p \le 0.05$).

For the Carmel cultivar, the results of fruit set show statistically significant differences between treatments with application of PBRs during the 2013 and 2014 growing seasons at both phenological stages of pink bud and fallen petals ($p < 0.0001$) (Table 4).

In the 2013 season, the application of KN at concentration of 30 μL L^{-1} had the best effect on fruit set, with 19.8 and 21.0% of fruit set at the phenological stages of pink bud and fallen petals, respectively. BL at a concentration of 10 mg L^{-1} improved fruit set, reaching 19.5 and 20.0% at the stages of pink bud and fallen petals, respectively. GA_3 applied at a concentration of 30 μL L^{-1} got 19.4 and 18.6% at the stages of pink bud and fallen petals, respectively. Control values were 11.8% at pink bud and 13.8% at fallen petals in the 2013 season.

In the 2014 season, BL at a concentration of 10 mg L^{-1} showed the highest percentage of fruit set in cv. Carmel with 20.4% at pink bud stage and 20.1% at fallen petals. KN at a concentration of 30 μL L^{-1} likewise had a high percentage of fruit set with 20.4% at fallen petals stage and 20.3% at pink bud stage. KN at concentration of 50 μL L^{-1} showed values statistically equivalent to treatments previously analyzed, with 20.2 and 20.0% at pink bud and fallen petals, respectively. GA_3 (30 μL L^{-1}) obtained values statistically equivalent to previous results with 17.5 and 19.7% of fruit set at pink bud and fallen petals, respectively. Control values were 12.2 and 13.1% at pink bud and fallen petals, respectively in the 2014 season.

4. Discussion

4.1. Viability test using fluorochromatic reaction

The results obtained in the evaluation of pollen viability of Non Pareil and Carmel almond cultivars *in vitro* using the fluorochromatic reaction test (FCR) with fluorescein diacetate (DAF) in two years (2013–2014), show high reliability compared with the *in vitro* germination test for *P. dulcis* in the Non Pareil and Carmel cultivars. It is both easier and faster than the germination test [34,35]. The percentage of viability obtained with the FCR test, in the case of cv. Non Pareil, is high and similar to the results of Bayazit et al. [36] and higher than Bazayit et al. [24]. In the case of cv. Carmel the viability percentage was also high. There are no reports of pollen viability using FCR testing in cv. Carmel. These results indicate that the pollen quality of these two almond cultivars is high.

4.2. Pollen germination and pollen tube growth in vitro in the presence of plant bioregulators

Statistically significant differences are observed in pollen germination and pollen tube growth. The results of the evaluation of these two variables demonstrate clear trends in both seasons. BL at low concentrations had the best response and GA_3 and KN at high concentrations significantly improved the percentage of germination as well as pollen tube lengths.

In the cv. Non Pareil, BL at 10 mg L^{-1} reaches the highest germination percentage (Table 1). There are no reported studies using BL, GA_3 and KN in Non Pareil almond pollen. In other species, such as *A. thaliana*, the addition of 10 and 20 μM of BL in the pollen germination media showed a beneficial effect for pollen germination, while for pollen tube growth, 10 μM of BL was beneficial. Therefore, 10 μM of BL is recommended to optimize the conditions of *in vitro* growth and improve pollen germination rates of *A. thaliana* [11], similar to the results of this study with Non Pareil almond pollen. Bolat and Pirlak [8] reported that for GA_3 used in the germination of apricot pollen, the maximum beneficial effects were observed with concentrations between 0.05 and 0.5 ppm (81.45%) depending on the cultivar (the control was 49.93%). In addition, they found that KN at low concentrations significantly improved the percent of germination in

one of the cultivars they evaluated; reaching 73.78% (the control was 56.36%).

With regard to the length of the pollen tube, in Non Pareil, GA_3 at 50 $\mu L\ L^{-1}$ resulted in greater lengths (Table 2). No previous studies were found using BL, GA_3 and KN in Non Pareil almond pollen *in vitro*. There is evidence that BL improved pollen tube growth in *P. avium*, surpassing the control [13]. In experiments on *A. thaliana*, Singh et al. [37] provided genetic evidence for gibberellins promoting growth of pollen tubes in these plants. The development of anthers and pollen tube growth require gibberellins, however, superoptimal levels of gibberellins are also inhibitory. Wu et al. [1] in experiments on *Torenia fournieri* pollen found that GA_3 stimulated pollen tube growth *in vitro* and significantly increased the quantity of pollinated styles. These results suggest that GA_3 plays an essential role in pollen tube growth and pollen–pistil interactions in *T. fournieri*. Gibberellins have a high impact on pollen viability and pollen tube growth [38].

In Carmel, GA_3 at 50 $\mu L\ L^{-1}$ reached the highest germination percentage (Table 1). These values share the same range with the percentages obtained with BL within the same periods. These results agree with those reported by Sotomayor et al. [14] indicating that the highest percentages of Carmel almond pollen germination *in vitro*, after 4 h, were observed with BL and GA_3 that were significantly higher than the control. The percentages obtained by Sotomayor et al. [14] were 58.0% with BL and 58.4% with GA_3. It is possible that these results differ from those obtained in this experiment due to the pollen quality.

In pollen tube length for Carmel, KN at 50 $\mu L\ L^{-1}$ achieved greater lengths (Table 2). These values are extremely superior to those obtained by Sotomayor et al. [14] that used the same X-Cyte plant bioregulator (KN), probably due to the high concentrations used. However, they agree with those found in apricot (*P. armeniaca*), where low concentrations of KN significantly improved pollen tube growth, yet high concentrations inhibited pollen tube growth [8]. Wu et al. [1] after conducting experiments with Zeatin (Zt), a cytokinin, found that there was no apparent effect on pollen germination and pollen tube growth *in vitro* in *T. fournieri*.

4.3. Evaluation of plant bioregulators on fruit set in almond trees

The application of PBRs improves fruit set in NonPareil and Carmel almonds when applied at the pink bud and fallen petals phenological stages during the 2013 and 2014 seasons.

In Non Pareil, all treatments outperformed the control (Table 3). It should be noted that the effect of GA_3 at 30 $\mu L\ L^{-1}$ in 2013 reached a percentage of 27.1% of fruit set when it was sprayed at the phenological stage of pink bud and 26.2% when it was sprayed at fallen petals phenological stage. These results are similar to those obtained by Sotomayor [39] with the application of GA_3 in the concentration of 50 ppm applied at full bloom in cv. Non Pareil and agree with those reported by Joolka et al. [40] where maximum fruit retention in Non Pareil was observed with GA_3 at a concentration of 200 ppm. In the 2014 season, KN at 50 $\mu L\ L^{-1}$ during pink bud and 30 $\mu L\ L^{-1}$ during fallen petals reached 31.0 and 25.6% of fruit set, respectively, being significantly higher than the control. No other studies of PBRs applied to Non Pareil almond trees have been reported, to date. In other species, such as *O. sativa* the application of BL significantly improved seed formation [10].

In Carmel, all treatments exceeded the control in both phenological stages, in the two seasons (Table 4). KN at 30 $\mu L\ L^{-1}$ achieved the greatest percentage of fruit set in the year 2013 with 19.8 and 21.0% of fruit set in both phenological stages respectively. In 2014, BL at 10 $\mu L\ L^{-1}$ and KN at 30 $\mu L\ L^{-1}$ showed the highest percentages of fruit set with 20.4% of fruit set at both phenological stages. Treatments that were statistically equal included KN at 30 and 50 $\mu L\ L^{-1}$, BL at 10 $mg\ L^{-1}$ and GA_3 at 30 $\mu L\ L^{-1}$, in both phenological stages. These results partially agree with those obtained by Sotomayor et al. [14] who found improved fruit set in Carmel almonds with application of BL (Brassinosteroid 0.2%) at 0.1 $g\ L^{-1}$, with 46.1% of fruit set while the control was 38.6%. In this same experiment X-Cyte (KN) and GA_3 were also applied, obtaining fruit set values lower than the controls, possibly due to the high concentrations used.

5. Conclusion

The evaluation of pollen viability of Non Pareil and Carmel almonds *in vitro* using the fluorochromatic reaction (FCR) with fluorescein diacetate (DAF) is a fast and reliable method. The pollen from the Non Pareil and Carmel almond cultivars used in this study can be considered of high quality.

It is evident that these PBRs had significant effects on pollen germination and pollen tube length in Non Pareil and Carmel almond (*P. dulcis*) pollen. Fruit set was significantly affected by the action of these PBRs. The greatest percentages of fruit set with the application of PBRs in this study are considered high for Chile, where the average percent of fruit set is approximately 15%.

Significant effects were not observed between the two phenological stages of flowering selected for the application of PBRs during the 2013 and 2014 seasons in both cultivars.

Conflict of interest

The authors declare that they have no conflict of interest.

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