Improvement of fruit set in almonds [Prunus dulcis (Miller) D.A. Webb] by applying three plant bioregulators in flowering

Segundo Maita Supliguicha

2015
Improvement of fruit set in almonds \([Prunus dulcis\) (Miller) D.A. Webb\] by applying three plant bioregulators in flowering

Segundo Maita Supliguicha

Thesis
to obtain the degree of

Doctor
en Ciencias de la Agricultura

Santiago, Chile, August 2015
Thesis presented as part of the requirements to obtain the degree of Doctor en Ciencias de la Agricultura, approved by the

Thesis Committee

__________________________________________________________
Dr. Carlos Sotomayor, Advisor

__________________________________________________________
Prof. Thomas Fichet

__________________________________________________________
Prof. Claudia Bonomelli

Santiago, September 3rd 2015
In memory of my son and my father

To my entire family
Acknowledgements

To Government of the Republic of Ecuador, for financing my PhD, scholarship granted by National Secretary of Higher Education, Science, Technology and Innovation.

To Universidad Católica de Cuenca, Republic of Ecuador, for the support provided during my PhD studies.

I specially thank the Thesis Committee, compounded by Dr. Carlos Sotomayor, Dr. Thomas Fichet and Dr. Claudia Bonomelli, for their appreciated support on attaining this thesis.

To Facultad de Agronomía e Ingeniería Forestal (FAIF) of Pontificia Universidad Católica de Chile, and the Professors, for their high-level-academic training.

To current and preceding Directors in FAIF’s Board of Research and Postgraduate Studies, for their support and assistance during my PhD studies.

To Departamento de Fruticultura y Enología, Director Dr. Claudia Bonomelli and Professors and Technicians of the Section Deciduous Fruit Trees, for their support during the attainment of this thesis.

To my family, for their constant support and understanding.
Content

Chapter 1. General Introduction ................................................................. 1

Chapter 2. Hypothesis and Objectives ...................................................... 35


Chapter 4. Original Article: Using three plant bioregulators in flowering almonds cvs. Non Pareil and Carmel affects the weight and size of kernels at harvest ................................................................. 68

Chapter 5. General Discussion and Conclusions ........................................ 95
List of figures

Chapter 1

Figure 1. The molecular mechanism of self-incompatible recognition in fruit trees of the family *Rosaceae* ................................................................. 8

Chapter 4

Figure 1. Relation between variables kernel dry weight according to length and width of cv. Non Pareil and cv. Carmel. In all the cases in the phenological stage pink bud in 2015 season................................. 88
List of tables

Chapter 1

Table 1. Proposed cross-incompatibility groups in almond................................. 9

Table 2. Almond cultivars grouped by approximate bloom periods......................11

Chapter 3

Table 1. Percentage of pollen germination in vitro on Non Pareil and Carmel almond cultivars after 4 hours, in the presence of plant bioregulators in the 2013 and 2014 growing seasons.................................................. 49

Table 2. Pollen tube length in Non Pareil and Carmel almond cultivars after 8 hours, in the presence of plant bioregulators in the 2013 and 2014 growing seasons................................................................. 51

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)................................................................. 53

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)................................................................. 54
Chapter 4

Table 1. Effect of plant bioregulators on kernel dry weight (g) of cv. Non Pareil almonds in two phonological stages during two seasons............ 79

Table 2. Effect of the plant bioregulators on kernel dry weight (g) in cv. Carmel almond in two phonological stages and two seasons......................... 81

Table 3. Effect of the plant bioregulators on kernels length and width (mm) in the cv. Non Pareil almond in two phonological stages, season 2014..... 82

Table 4. Effect of the plant bioregulators on kernel length and width (mm) of cv. Non Pareil almonds in two phonological stages in 2015 season...... 83

Table 5. Effect of the plant bioregulators on kernel length and width (mm) of cv. Carmel almonds in two phonological stages in 2014 season......... 85

Table 6. Effect of plant bioregulators on kernels length and width (mm) on almond cv. Carmel in two phonological stages in 2015 season.......... 86

Table 7. Summary of the regression analysis of the variable kernels dry weight according to the variables length and width of almonds cvs. Non Pareil and Carmel in 2014 and 2015 seasons......................... 8
Chapter 1

General Introduction

Almond [(Prunus dulcis (Miller) D.A. Webb] is a fruit tree with a characteristical species diversity in Central and Southeast Asia (Zaurov et al., 2015). Molecular studies show almond cultivations spreading from Central Asia to Eastern Mediterranean and, subsequently to the Western Mediterranean, North America, and finally to the South Hemisphere, including South America and Australia (Zeinalabedini et al., 2010; Fernandez i Marti et al., 2015). Due to the origin, almond is a fruit tree requiring low chilling conditions; therefore, it has a relatively early flowering. This trait determines a flowering stage coinciding with late winter season, and consequently with low temperature and precipitation that exert negative effects on pollination (Sutyemez, 2011).

In Chile, almond cultivation extends between the Regions of Coquimbo and Maule, with larger surfaces in the Metropolitan and O’Higgins Regions (ODEPA, 2014). In Central Chile, almond flowering occurs early in the season, commonly from late July to mid-August. Therefore, climate conditions during these months contribute against adequate pollination. Characteristic rain and cold in this season may also affect the work of bees, and delay pollen germination and pollen growth tube. Thus, the number of flowers producing final fruit or almonds (fruit set) is affected. These conditions partly explain average low yield in Chilean almond orchards (Sotomayor, 2013).
Almond is a gametophytic self-incompatibility species, with a genetic control allowing flower styles to reject their own pollen. This prevents self-pollination and promotes cross-pollination (Wu et al., 2013). Gametophytic self-incompatibility in almond is varietal, with varieties with different allele gene composition. Most of the varieties planted in Chile are self-incompatible; therefore, more than one cultivar compatible with the main cultivar is required, and additionally to flower simultaneously to achieve a successful cross-pollination.

After a successful pollination in almond, optimal conditions for pollen germination, growth of the pollen tube and pollination of the embryo-sac should be expected. Fruits and seeds experience an associated development after flower pollination; however, seed development depends strictly on a successful pollination, in contrast to the fruit developing in absence of pollination. Seed development includes endosperm proliferation and embryo growth, and both processes are multihormonally regulated by auxins, cytokinins, gibberellins and brassinosteroids (Ruan et al., 2012). Apparently, plant hormones have a prominent role on pollination synchronization and fruit development. Gibberellins, auxins and, in some cases, cytokinins have been particularly efficient to trigger fruit growth in many species (Dorcey et al., 2009).

Currently, scientific research on almond in Chile is focused on solving low fruit set; any action in order to solve this problem, like the exogenous application of plant bioregulators, will contribute to improve competitiveness on this crop.
1. Almond flowering, pollination and fruiting

1.1. Flowering

Flowering is one of the most important events on life cycle in plants, with synchronization as a crucial factor. Flowering is controlled by the interaction of several endogenous and environmental signals, in order to secure appropriate conditions for seed production (Wang et al., 2015).

Floral induction occurs by biochemical signals causing tissues changing from the vegetative state of a bud to the reproductive state, and it is affected by an integrated signal of genes involves on flowering, in response to the environment and endogenous factors (Chandler, 2011). Balance on hormones like auxins, gibberellins, cytokinins and ethylene is essential in this process (Imani et al., 2012). In Chile, floral induction on almond buds (P. dulcis) occurs on early summer (December), followed immediately by differentiation in January, with visible anatomical and morphological changes. Then, a slow and gradual development of flower parts begins in fall, continuing even in dormancy bud. Therefore, the future compressed flower contained in the bud presents sepals, petals, stamens and ovary in early winter, although sexual cells (gametophytes) are still absent (Sotomayor, 2013).

Almond crops probably present the widest date range on flowering among all deciduous fruit trees and nut fruit species (Socias i Company and Felipe, 1992).
Flowering date depends on the progression of winter temperature affecting the different dormancy states. However, flowering sequence of different almond crops is maintained with slight changes through time (Alonso et al., 2005). During winter, buds must be subject to cold season in order to break dormancy and to flower in spring. Chilling requirements vary according to the almond crop, between 400 and 600 hours under 7°C. However, recent studies have found that for the Californian varieties grown in Chile are convenient to accumulate more than 800 hours to get a uniform bloom of short duration, which favors cross-pollination. If chilling requirement is not satisfactorily accomplished, flowering is irregular, long and of low quality. When cold season is long and followed by warm temperature, flowering is uniform, short and of good quality (Sotomayor, 2013). Flowering mainly depends on temperature, associated with specific cold and heat requirements of the different cultivars (Ramirez et al., 2010).

After cold season, the plant must receive some amount of heat to form, male gametophytes (pollen) and female gametophytes (embryo sac/egg cell), by meiosis (cell division producing haploid sex cells). This stage occurs when buds are already swollen (approximately July-August in Central Chile) (Sotomayor, 2013).

Date of occurrence of flowering depends on the almond cultivar and climate conditions in winter/spring. For example, orchard flowering may last between 10 and 30 days (or even more), according to environmental conditions. In Central Chile, almonds usually flowering in August (Sotomayor, 2013).
1.2. Pollination

Pollination in fruit trees involves release, transport and deposition of pollen from the anthers on the stigma (Kodad and Socias i Company, 2013). Pollination is essential for fruit production; however, interaction of pollination with vegetative and reproductive traits of plants has still not completely studied (Klein et al., 2015).

Almond flowering is mainly self-incompatible and, therefore requires cross-pollination (Yi et al., 2006). Thus, almond orchards must be planted with at least two compatible cultivars and of simultaneous flowering, requiring also pollinator insects for pollen transfer (Vezvaei and Jackson, 1995). Pollination may be limiting factor in some production areas; for example, in Chile fruit set percentage lower than 20% are obtained in years with irregular spring climate (Sotomayor, 2013). Lack of pollination vector or bad climate conditions may hinder pollen transference. Even getting adequate pollen transport, not always flowers are pollinated the first day and it could occur in the few days that the flower remains receptive. Therefore, a short period of a receptive flower often limits potential productivity of trees. In 1966, Williams introduced a new concept, in order to evaluate flower receptivity. The Effective pollination period (EPP) was defined as the period when pollination is effective for fruit production. This period is determined by egg cell longevity less the period between pollination and fertilization, as long as this value does not exceed the value of stigma receptivity period (Sanzol and Herrero, 2001).
It has been proved that almond flowers with cross-pollination reach 30% of fruit set one day after opening; 21% of fruit set 3 days after opening; and only 1% 5 days after opening. Although the number of days observed might not be relevant to the conditions in all the orchards every year, these results show the importance of timely pollination (Polito et al., 1996).

The effective pollination period varies with climate conditions from year to year, and it is generally shorter when temperatures are high during the bloom season (Polito et al., 1996). In California, effective pollination period (EPP) for Non Pareil almond cultivar flowers is between 3 and 7 days for each flower (Vezvaei and Jackson, 1995).

Yi et al. (2006) found that stigma is still receptive, and fruit set maintains even in flowers at the stage when petals are abscising. This information is particularly useful for producers deciding the period for effective bee pollination. Pollen production in almond is considered high, with an estimated average value of 1.500 grains of pollen per anther, which guarantees a successful pollination, with appropriate climate conditions and presence of bees (Martinez-Garcia et al., 2011).

As almond flowers bloom during cold weather and also present gametophytic self-incompatibility, any factor extending the effective pollination period (EPP), as the application of plant bioregulators, should bring benefits for fruit production (Sotomayor et al., 2012).
1.3. Gametophytic self-incompatibility in almond

Most species of fruit trees in the *Rosaceae* family present gametophytic self-incompatibility. This is a genetically-controlled-mechanism allowing floral styles to reject their own pollen in fruit crops like pear, apple, almond, cherry tree, among others. Rejecting their own pollen prevents self-pollination and promotes cross-pollination in these species (Wu *et al*., 2013).

Most almond crops present gametophytic self-incompatibility, which is controlled by only a unique *S* gene, with codominant multiple alleles (Sanchez-Perez *et al*., 2004; Lopez *et al*., 2006). Twenty six incompatible alleles (from *S*₁ to *S*₂₅ and *S*₇₄) and one part of dominant self-compatible alleles *S*₁ have been identified so far in cultivated almonds. Eighteen cross-incompatibility groups formed by crops with the same self-incompatible genotypes have been reported (Groups I to XVII and Group O of crops with unique genotype) (Lopez *et al*., 2006). Ortega *et al*. (2006) propose new cross-incompatibility groups, after of RNasas analysis in almond (Table 1).

Self-incompatibility is expressed in flower styles as *S*-RNases glycoproteins, which are responsible of stopping their own pollen tube growth (Sánchez-Pérez *et al*., 2004; López *et al*., 2006). Figure 1 shows that *S* locus encodes *S* determinants in the style and the pollen tube, called *S*-RNasas and *SLF/SFB*, respectively. Figure 1 on top (A) shows that cross-pollination of pollen *S*₁ and the style *S*₂, incompatible *S*₂-RNasa of the style interacts with SFB/SLF, and it is finally ubiquitinized. Therefore, pollen *S*₁ growing in the style is not affected. Figure 1 on bottom (B)
shows self-pollination; the style $S_1$-RNase is ubiquitinization-free. Thus, it degrades RNA of the $S_1$ pollen tube that finally stops the growth (Wu et al., 2013).

**Figure 1.** The molecular mechanisms of self-incompatible recognition in fruit trees of the family *Rosaceae* (SFB: *S* haplotype-specific F-box protein gene; SLF: *S* locus F-box) (Source: Wu et al., 2013).

Information provided on self-incompatibility by genotypes from almond crops is essential for designing commercial orchards (choosing a compatible cultivar is essential for production) (Lopez et al., 2006). Although self-incompatibility has limited cultivation of commercial crops, availability of useful characteristics has widened, producing options for the development of improved almond crops (Woolley et al., 2000).
Table 1. Proposed cross-incompatibility groups in almond.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cultivars</th>
<th>S-genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Nonpareil, IXL, Long IXL, Riedenhoure, Tardy Nonpareil</td>
<td>$S_7S_8$</td>
</tr>
<tr>
<td>II</td>
<td>Texas (=Mission), Ballico, Garbi, Glorieta, Languedoc, Wawona</td>
<td>$S_1S_5$</td>
</tr>
<tr>
<td>III</td>
<td>Thompson, Woods Colony, Granada, Harvey, Mono, Robson, Sauret 2</td>
<td>$S_2S_7$</td>
</tr>
<tr>
<td>IV</td>
<td>Aldrich, Merced, Nec Plus Ultra, Norman, Pearl, Price, Ripon</td>
<td>$S_1S_7$</td>
</tr>
<tr>
<td>V</td>
<td>Carmel, Carrion, Jubilee, Livingston, Monarch, Reams, Sauret 1</td>
<td>$S_2S_8$</td>
</tr>
<tr>
<td>VI</td>
<td>Butte, Monterrey, Bigelow, Dottie Won, Grace, Kutsch, Northland, Rivers Nonpareil, Sultana</td>
<td>$S_1S_8$</td>
</tr>
<tr>
<td>VII</td>
<td>Solano, Eureka, Kapareil, Sonora, Vesta</td>
<td>$S_8S_{13}$</td>
</tr>
<tr>
<td>VIII</td>
<td>Ferragnes, Ferralise</td>
<td>$S_1S_3$</td>
</tr>
<tr>
<td>IX</td>
<td>Jeffries</td>
<td>$S_7A_{S_8}$</td>
</tr>
<tr>
<td>X</td>
<td>Harpareil, Jordanolo</td>
<td>$S_7S_{14}$</td>
</tr>
<tr>
<td>XIII</td>
<td>Drake, Smith XL</td>
<td>$S_8S_8$</td>
</tr>
<tr>
<td>XIV</td>
<td>Fritz, Peerless, Ruby, Rumbeta-2</td>
<td>$S_1S_6$</td>
</tr>
<tr>
<td>XV</td>
<td>Anxaneta, Tarragones</td>
<td>$S_2S_9$</td>
</tr>
<tr>
<td>XVI</td>
<td>Ardechoise, Desmayo Largueta</td>
<td>$S_1S_{10}$</td>
</tr>
<tr>
<td>XVII</td>
<td>Achaak, Ferrarstar</td>
<td>$S_2S_{10}$</td>
</tr>
<tr>
<td>XVIII</td>
<td>Pajarera-2, Pestanhieta</td>
<td>$S_{12}S_{23}$</td>
</tr>
<tr>
<td>XIX</td>
<td>Malagueña, Planeta Fina, Planeta Roja</td>
<td>$S_{22}S_{23}$</td>
</tr>
<tr>
<td>XX</td>
<td>Garrigues, Pajarera-1</td>
<td>$S_{13}S_{27}$</td>
</tr>
<tr>
<td>O*</td>
<td>Ai ($S_3S_4$), Atascada ($S_5S_{22}$), Atocha ($S_{13}S_{22}$), Avellanera Gruesa ($S_{22}S_{26}$), Bertina ($S_6S_{11}$), Carretas Bajas ($S_4S_{12}$), CEBAS-I ($S_4S_{13}$), Colorada ($S_{12}S_{28}$), Cristomorto ($S_1S_2$), Del Cid ($S_{12}S_{22}$), Ferraduel ($S_1S_4$), Fina del Alto ($S_{28}S_{29}$), Fournat de Brezenaud ($S_{29}S_{27}$), Gabaix ($S_{10}S_{24}$), Jimenez Salazar ($S_{21}S_{26}$), Jordi ($S_5S_8$), La Mona ($S_{23}S_{28}$), Marcona ($S_{11}S_{12}$), Marcona Flota ($S_8S_{12}$), Marcona de San Joy ($S_{22}S_{27}$), Masbovera ($S_1S_9$), Milow ($S_7S_{13}$), Padre ($S_1S_{18}$), Peraleja ($S_3S_{23}$), Pestañeta ($S_{11}S_{22}$), Primorskiy ($S_2S_9$), Ramillete ($S_8S_{23}$), Rumbeta ($S_{11}S_{21}$), Tio Martin ($S_{23}S_{27}$), Tioga ($S_5S_8$), Titan ($S_8S_{14}$), Tokyo ($S_8S_7$), Verruga ($S_8S_{10}$), Yosemite ($S_8S_{10}$)</td>
<td>$S_{13}S_{27}$</td>
</tr>
</tbody>
</table>

*Self-compatible and compatible with all the other groups

Source: Ortega et al., 2006.
1.4. Flowering coincidence

In addition to cultivars compatibility, flowering coincidence is required in almond in order to reach good pollination. Ideally, cultivars flowering at the same time or with some days away, should be planted in an orchard. However, compatible cultivars and with simultaneous flowering should be chosen, along cultivars with similar cold and heat requirements, in order to achieve maximum coincidence of the flowering period, separately from temperature regime before flowering. Therefore, possibilities for cross-pollination are optimized (Alonso et al., 2005). This is not always possible, so that is another reason to include more than two cultivars on a plantation. In relation to main cultivar, it can be planting for example, other cultivars with slightly earlier or later flowering (Sotomayor, 2013). According to the flowering season, almond cultivars may be classified in three types: early cultivars, medium cultivars and late cultivars (Imani and Mehr-Abadi, 2012) (Table 2). On the other hand, requirement of winter cold for the different varieties affects significantly floral coincidence. Generally, coincidence is better in all the cultivars when quantity accumulated cold is high (> 800 hours) (Sotomayor, 2013). Almonds, and other fruit and nut species of temperate climate, show a clear influence of spring temperature on flowers development. Low spring temperature widens the flowering period, while high temperature shortens it (Bernad and Socias i Company, 1995).
Table 2. Almond cultivars grouped by approximate bloom periods (The numbers at the top of the columns indicate the days before (-) or after (+) the flowering peak of cv. Nonpareil bloom)

<table>
<thead>
<tr>
<th>Early (-6 and earlier)</th>
<th>Early mid (-5 to -1)</th>
<th>Mid (0 to +2)</th>
<th>Late mid (+3 to +4)</th>
<th>Late (+5 to +7)</th>
<th>Very late (+8 and later)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jordanolo</td>
<td>Milow</td>
<td>Aldrich</td>
<td>Butte</td>
<td>Livingston</td>
<td>Planada</td>
</tr>
<tr>
<td>Ne Plus Ultra</td>
<td>Peerless</td>
<td>Carmel</td>
<td>Carrion</td>
<td>Mission</td>
<td>Ripon</td>
</tr>
<tr>
<td>Sonora</td>
<td></td>
<td>Fritz</td>
<td>Drake</td>
<td>Mono</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Harvey</td>
<td>LeGrand</td>
<td>Padre</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jeffries</td>
<td>Monarch</td>
<td>Ruby</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Merced</td>
<td>Monterey</td>
<td>Thompson</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonpareil</td>
<td>Norman</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Price</td>
<td>Sauret #2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tokyo</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sauret #1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solano</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Woods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colony</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Although indicated in Table 2 may be valid in Chile, it is not always necessarily so, it depends largely on the chilling accumulation during winter dormancy. For example, Solano usually overtakes to Non Pareil and Carmel delays some days in slightly cold winters (Sotomayor, 2013). Thus, studies should be made under local conditions, because experiences in other latitudes have proven to be unsatisfactory (Ramirez, 2010).
1.5. Fruit set

Pollination and subsequent fertilization lead to a dramatic change in the balance of plant hormones, and to the activation of egg cell development (Obroucheva, 2014). Fruit set is the transition from egg cell to a developing fruit, and from egg cell to seeds; it begins with pollination and it continues some time after, determining seeds, the number of fruits, final size, and subsequently, potential yield (Ruan et al., 2012; McAtee et al., 2013). This transition includes two different but coordinate processes, called egg cell pollination and growth of pistil tissues, and other structure around the egg cell to contribute to seeds development (Dорsey et al., 2009). These processes are highly sensitive to biotic and abiotic stress. Stress includes insufficient nutrients supply, drought, heat or cold, which often induce to a substantial flower, seed and fruit abortion, and subsequently, to an irreversible yield loss (Ruan et al., 2012). Fruit set depends fundamentally on the action of three hormones: auxins, gibberellins, and/or cytokinins. Sole application of these hormones may trigger fruit development in many plant species, which indicates their necessary interrelation for fruit set and fruit development (McAtee et al., 2013). Brassinosteroids might exert an important role on fruit set (Fu et al., 2008). However, the interaction with other hormones in the fruit set has not been investigated yet (McAtee et al., 2013).
1.6. Seed development (kernels)

After fertilization, the hormonal stimulation of the developing embryo tends to prevent abscission of the fruit and allow growth of the ovary and surrounding tissues to form the fruit (Dorcey et al., 2009). Then, embryo development and endosperm formation are a pre-requisite for normal fruit development (Sotomayor, 2013).

The almond flower ovary has two egg cells and only one (normally the best developed) does cause a seed (Gonzalez et al., 2005; Ortega et al., 2010). In some cases, however, both egg cells may be fertilized, producing double almonds. This trait is partly varietal and stimulated by flower size and low temperature during pollination (Gonzalez et al., 2005).

Forty five days after pollination, the fertilized triploid nucleus is actively divided by mitosis to form the seed endosperm (Gonzalez et al., 2005; Martinez-Gomez et al., 2008). On the other hand, the diploid zygote also begins slowly to form the embryo by mitotic division (Sotomayor, 2013).

After 70 days, the embryo begins to grow rapidly, absorbing the endosperm. At 100 days, the cotyledons (as part of the embryo, with the plumule, radicle and hypocotyl) already occupy most of the seed, representing the edible portion of kernels. This process is known as almond filling (Martinez-Gomez et al., 2008; Sotomayor, 2013).
1.7. Fruit development (drupe)

After pollination, the flower ovary thickens, becoming fruit (drupe). The outer layer of cells gives rise to exocarp usually pubescent, together to layer middle cells form the mesocarp or fleshy part of the fruit, known as hull (exo + mesocarp). Finally innermost layers ovary cells originate endocarp or shell, which encloses the seed or kernel (Sotomayor, 2013).

In studies on fruit development in Spain (cvs. Ramillete, Desmayo Largeta, Antoñeta and Wawona), the main states have been identified on fruits and seeds development: 1) growth in fruit size (mesocarp and endocarp) and seed (kernel) during March and April; 2) growth stabilization of fruit and seed, the embryo grows and the shell hardens during May, June and July, and 3) stable fruit growth with seeds increasing their dry weight in August and September (Martinez-Gomez et al., 2006).

The edges of the ovary suture remain marked, which allows their opening and release until they ripe, where the exocarp and mesocarp tissues slowly dehydrate, opening half way and showing the shell containing the seed or kernel. This is the moment for harvest, when the hull is still more or less attached, depending on the variety, and subsequently splits leaving the almond free (Sotomayor, 2013).
1.8. Climate as limiting factor in almond production

Stress by heat or cold during reproductive phase may impact seriously and negatively leading to an abnormal gamete formation, tapetal dysfunction in anthers, reduced pollen viability, asynchronous development of male and female reproductive organs, growth alteration of the pollen tube and fertilization signaling, low fruit set, and in severe cases, lack of seed formation (Bahuguna and Jagadish, 2015).

Among temperate climate species, almond is normally the first fruit tree in flowering, especially among some crops with low chilling requirement. As result, in most regions where almond is cultivated, flowering occurs when climate conditions are unfavorable for pollination and fruit set, causing yield important losses due to the frost (Bernad and Socias i Company, 1995; Egea et al., 2003; Sanchez-Perez et al., 2014).

1.9. Main almond cultivars

1.9.1. Non Pareil

Non Pareil cultivar derived from a unique plant, planted in an orchard by A.T. Hatch de Suisun, California in 1879, known as “Hatch” cultivar. Non Pareil became rapidly the main cultivar in California, due to the good plant quality, and high kernel quality (Bartolozzi et al., 1998). Almond size is medium, with high and consistent
yield, with variations due to adverse climate conditions in specific years. The consistent yield reflects a production practice, in both vegetative buds and shoots of the year, with good ability to renew production centers. This species is relatively resistant to frost damage and is vigorous but generally easy to train (Asai et al., 1996).

In California, the surface with Non Pareil cultivar reaches 47% of the area planted with almonds (Bartolozzi et al., 1998), while in Chile the surface planted is 55% (ODEPA, 2014), being the most important cultivar in the United States and Chile.

1.9.2. Carmel

This cultivar was discovered as a unique tree in a commercial orchard near Le Grand, California. Although originally considered a mutation from Non Pareil, genetic evidence indicates that Carmel originates from a ‘Non Pareil’ x ‘Mission’ cross (Kester et al., 1994; Asai et al., 1996). The tree is straighter Non Pareil and medium size. Carmel was introduced in 1966 and a good pollinator for Non Pareil, highly productive in the first years, but declining in yield of plant vigor through time. Kernel is large, elongate and of good quality (Asai et al., 1996).

In California, the surface planted with Carmel cultivar, in comparison to the total area with almonds, reaches 17% (Bartolozzi et al., 1998), while in Chile, the surface planted is 19% (ODEPA, 2014). Therefore, it is the second most important cultivar in the United States and Chile.
1.10. Plant hormones

Plant development and growth are regulated by several plant hormones interacting in complex nets, and also exerting a direct and indirect effect on the immunological system of plants, most of which have been demonstrated in recent years (Lozano-Duran and Zipfel, 2015). Plant hormones such as abscisic acid, ethylene, gibberellins, auxins, cytokinins and brassinosteroids act directly on initiation and seeds development. There is synergic action of auxins and gibberellins in initiation and seeds development, a key role of cytokinins in cell division and endosperm differentiation during the early phase of seeds development, and a role of brassinosteroids on cell elongation and the regulatory action of ethylene and abscisic acid on sugar metabolism and seed ripening (Bahuguna and Jagadish, 2015). Discovering plant hormones and their capacity to regulate all aspects in growth and development in agriculture was crucial (Greene, 2010).

Plants response to phytohormones signaling varies depending on the phenological states and still represents a challenge in research. However, new technology on hormonal profiles is promising for holistic comprehension of interactions induced by temperature and proportional changes between hormones, and between hormones and metabolites (Bahuguna and Jagadish, 2015).
1.10.1. Gibberellins

There are currently 136 gibberellins (GA$_n$) isolated from plants, but also produced by microorganisms such as fungi and bacteria or obtained synthetically. Gibberellins are designed by GA$_n$ where $n$ corresponds, approximately, order of their discovery (Hedden and Thomas, 2012; Rodrigues et al., 2012). Gibberellins are involved on multiple aspects of growth and development, including shoots elongation, ripening and seed germination, floral induction, pollen germination and pollen tube growth (Cheng et al., 2015).

Relatively, few gibberellins have an intrinsic biological activity in higher plants, where the most common are GA$_1$, GA$_3$, and GA$_4$. Gibberellin A$_3$, also known as gibberellic acid, is the most common and the main gibberellic acid resulting from *Fusarium fujikuroi*, obtained for commercial application, but also endogenously in some higher plants species (Hedden and Thomas, 2012).

Gibberellins also have a significant role on fertility, as they are necessary for pollen development, release and germination and growth of the pollen tube, in addition to allow stamen elongation. As they work, gibberellins act in response to environmental and development signals, which may regulate their biosynthesis, activation (deactivation), perception or transduction of signals, sometimes acting in several points of the route of signal transduction (Hedden and Thomas, 2012).
1.10.2. Brassinosteroids

Brassinosteroids (BRs) are a class of plant-specific steroid hormones, and are key regulators of growth and development and play an essential role on almost all stages of life cycle of plants, controlling processes as seed germination, etiolation, vegetative growth, development stomata, flowering and fertility (Symons et al., 2008; Lozano-Duran and Zipfel, 2015; Wang et al., 2015). Brassinosteroids also confer tolerance to plants against different abiotic and biotic stress, including stress caused by salinity, cold, heat, drought and pathogens (Naz et al., 2015). The highest levels of bioactive brassinosteroids generally occur in reproductive organs such as pollen [(5 – 1,000 ng·g$^{-1}$ of fresh weight (FW))] (from where brassinosteroids were originally isolated by Mitchel and collaborators in 1970), seeds (0.3 – 1,600 ng·g$^{-1}$ FW), and fruits (0.2 – 3.5 ng·g$^{-1}$ FW). This is coherent with the important roles proposed for brassinosteroids in processes such as fruit development and ripening (Symons et al., 2008).

The net of brassinosteroids biosynthesis in Arabidopsis derives from campesterol to brassinolide (BL), which is biologically the most active brassinosteroid. The biosynthesis route of brassinosteroids originally proposed considered the initiation with campesterol conversión to campestanol. However, recent studies have shown these stages as secondary, and that an independent campestanol route for brassinosteroids biosynthesis is the main path based on substrate preferences of enzymes (Wang et al., 2015).
1.10.3. Cytokinins

Cytokinins (CKs) are low-molecular-weight components belonging to a group of phytohormones with crucial roles for the life cycle in plants. They regulate cell division and morphogenesis; therefore, take part on the meristematic activity of tissues; they delay senescence; they are involved in apical dominance, and affect the development of flowers and seeds, seeds germination and consumption of nutrients and assimilated in sink organs (Zalabak et al., 2013). Additionally, advanced molecular, genetic, biochemical and genomic approaches have shown the different roles of cytokinins signaling on proliferation and cell differentiation, circadian rhythm, responses to light, transition to flowering, immunity, stress, and senescence. High cytokinin levels are considered to be involved on the accumulation of osmolites, photorespiration, sugars distribution, nitrogen allocation, root viability and constant and efficient water use. This suggests that cytokinins affect different physiological processes and plant metabolism by tolerating stress in a complex manner (Hwang et al., 2012).

Structurally, cytokinins derive from adenine, substituted in position N6, where isopentenyladenine [iP, N6-(2-isopentenyl)adenine] and trans-zeatin [tZ, N6-(4-Hydroxy-3-methyl-trans-2-butenyl)adenine] are the most important. The cytokinin molecule joins to membrane receptors and begins a fall of signal translation leading to the activation of a primary hormone response. Many genes affecting synthesis, transport, metabolism and cytokinin perception have been identified in the last decade (Zalabak et al., 2013).
The increased cytokinins content after pollination suggests that this hormone also contributes to a regulation net required for a precise and coordinated fruit growth (Mariotti et al., 2011).

1.11. Plant bioregulators

Since 1940s, natural and synthetic plant bioregulators have been used with constant increase for modifying plant cultivation, by change in rates or patterns, or both, of responses to internal and external factors that govern the development, from germination to vegetative growth, reproductive development, ripening, senescence or aging, and postharvest preservation, as well as the defense against biotic and abiotic stress (Lu et al., 2014).

Many plant bioregulators are hormones or substances similar to hormones promoting, inhibiting or affecting biological or biochemical processes in plants. They are singular, as in some cases the same active ingredient may induce different responses, depending on the period of application and used concentration. Many factor as the cultivar, vigor and tree yield, climate factors during, before and after applying and the application method affect their effectiveness (Dussi, 2011).

Plant bioregulators were soon identified as improving yield, quality and postharvest life, reaching their biggest impact precisely in fruit production. Fruit trees are considered as high value cultivations and even small changes in production
efficiency, product quality or improvement on esthetics and have the potential for a significant increase on product value (Greene, 2010). Plant bioregulators have become essential in modern agriculture, horticulture and viticulture (Rademacher, 2010).

Research on molecular biology for identifying regulation genes will allow new uses for plant bioregulators. A holistic approach for plant bioregulators usage should consider the physiological status of the plant, irrigation management, fertilization, pruning, conduction, perception and light distribution in the tree, and climate conditions. Therefore, the models should be developed to predict and explain the trees response (Dussi, 2011). Future usage will certainly arise for plant bioregulators (Greene, 2010).

1.11.1. Technical gibberellic acid

Gibberellic acid (GA₃) is an important member of the gibberellin family, acting as a natural hormone in plants, controlling numerous development processes. Industrially, GA₃ is produced by submerged fermentation (SmF), using the ascomycota fungus Gibberella fujikuroi, renamed as Fusarium fujikuroi. It is also possible to produce GA₃ by chemical synthesis or extraction from plants, but these methods are unfeasible economically. Chemically, GA₃ is tetracyclic dihydroxy γ-lactonic acid containing two ethylene links and a group of free-carboxilic acid. The molecular formula is C₁₉H₂₂O₆ (Uthandi et al., 2010; Rodrigues et al., 2012).
In the 1950s, the British company Imperial Chemical Industry (ICI) began a program of strain selection of *Fusarium miniliforme*, with a big ability to produce gibberellins. Attempts to optimize studies on liquid and superficial fermentation were made. After some purification steps, one gibberellin was isolated and called “gibberellic acid”. These gibberellins had the molecular formula $\text{C}_{19}\text{H}_{22}\text{O}_6$, with several physiological properties from the Japanese gibberellin A. This stimulated Japanese researchers to produce gibberellins and separate them into three products: gibberellins $A_1$, $A_2$ and $A_3$. Gibberellin $A_3$ was identified as gibberellic acid produced by ICI (Rodrigues *et al.*, 2012).

Effects from gibberellic acid on growth and plant development are interceded by genes expression, as well as inhibitor of ARN synthesis and proteins interfering on these processes. A better understanding of the molecular mechanism commanding how gibberellic acid regulates growth and plant development requires the identification and analysis of more genes controlled by gibberellic acid (Yamaguchi, 2008; Bomke and Tudzynski, 2009).

Gibberellins are tetracyclic diterpenoids controlling a wide range of processes during plant development, including seed germination, leaves expansion, shoots and roots elongation, floral induction and flower development. Studies also indicate that gibberellins are a key factor for fruit set and fruit development (Gallego-Giraldo *et al.*, 2014).
1.11.2. 24-Epibrassinolide

Discovery of brassinolide, a vegetal hormone of the brassinosteroids group, has led to a renewed interest on natural chemical products from steroids. Many similar hormones have been isolated from a wide plant variety, and the synthesis of these brassinosteroids has been significant, especially promising on agriculture (Rao et al., 2002).

The 24-epibrassinolide synthesized from ergosterol is deemed the most practical brassinosteroid on applications, because it presents a combination of important traits, like a biologically strong activity, similar to brassinolide (McMorris and Patil, 1993).

Currently, 24-epibrassinolide is used as an active ingredient for officially registered preparations, with large scale application due to a fundamental role on growth and plant development. The 24-epibrassinolide apparently protects plants from a series of environmental stress, including stress by high and low temperature, drought, salinity, herbicide damage and pathogens attacks (Khripach et al., 2000; Kagale et al., 2007).

Exogen 24-epibrassinolide applications have been reported to improve substantially wheat yield and the tolerance to stress by inducing cell changes related to that tolerance, like the synthesis stimulation of nucleic acid and proteins, activation of the ATPasas pump, increased activity of antioxidant enzymes and
osmoprotectants accumulation, induce other hormone responses, regulates the stress gene expression and induce photosynthetic efficiency and photosynthates translocation to the sink organs (Naz et al., 2015).

1.11.3. Kinetin

Kinetin (N<sub>6</sub>-fururyladenine) was the first natural cytokinin to be isolated and identified, in 1955 (Mik et al., 2011). Kinetin and N<sub>6</sub>-benzyladenin (BA) are the most recognized cytokinins, by the ring substitution in the position N<sub>6</sub> (Mok and Mok, 2001; Mik et al., 2011). Kinetin induces plant growth, delays leaf senescence, regulates the plant response to stress caused by environmental factors (Wyszko et al., 2003).

Kinetin affects mainly genes expression, inhibits auxins action, stimulates calcium flow, acts on the cell cycle, acting as an anti-stress and anti-aging molecule. rRNA transcription is similarly induced on roots, floral tissues and the entire plant, which suggests that the kinetin action is non-tissue-specific (Barciszewski et al., 1999). Kinetin findings in DNA and cell extracts brought in the obvious question on the synthetic route. Furfural has been suggested as an assumed precursor for kinetin. Furfural has been identified forming during oxidation from hydroxyl radical from C5' from deoxyribose in the DNA (Wyszko et al., 2003).

Kinetin might be regarded for inducing systemic acquired resistance and anti-stress hormone in plants (Shah, 2011). A potential complexity in the mode of action
of two growth regulators, kinetin and ABA, interacting antagonistically in many cases, could suggest that kinetin works as an anti-stress agent (Barciszewski et al., 1999).

Singh and Prasad (2014), indicate that foliar kinetin applications improves the antioxidant system, photosynthesis and mitigates the harmful effects from metals like cadmium on *Solanum melongena* seedlings, and that it may generally improve cultivation yields.

Kinetin functions at transcriptional, translational, posttranslational and metabolic level are clear, according to a wide-range-effect analysis. Kinetin ability to Foster the transcription beginning has been proven in *Arabidopsis thaliana*, directly on the promoter gene rARN (Gaudino and Pikaard, 1997; Barciszewski et al., 1999).

**References**


Naz, F., M. Yasuf, T. Khan, Q. Fariduddin, and A. Ahmad. 2015. Low level of selenium increases the efficacy of 24-epibrassinolide through altered physiological and biochemical traits of *Brassica juncea* plants. Food Chem. 185:441-448.


Chapter 2

2. Hypothesis and Objectives

2.1. Hypothesis


2.1.2. Exogenous application of brassinosteroids, gibberellins and cytokinins during flowering of Non Pareil and Carmel almond trees increase final fruit set.

2.1.3. Exogenous application of brassinosteroids, gibberellins and cytokinins during flowering of Non Pareil and Carmel almond trees improves kernels traits at harvest.

2.2. Objectives

2.2.1. General Objective

To evaluate the effect of brassinosteroids, gibberellins and cytokinins on pollen, fruit set and kernels characteristics of Non Pareil and Carmel almonds.
2.2.2. **Specific Objectives**

2.2.2.1. To determine *in vitro* the influence of brassinosteroids, gibberellins and cytokinins on pollen germination and pollen tube growth in cvs. Non Pareil and Carmel almonds.

2.2.2.2. To evaluate final fruit set of almonds Non Pareil and Carmel of applying brassinosteroids, gibberellins and cytokinins in two phenological stages during flowering.

2.2.2.3. To measure the influence of exogenous brassinosteroids, gibberellins and cytokinins in the dry weight, length and width of kernels in Non Pareil and Carmel almond trees.
Chapter 3


Segundo Maita\textsuperscript{a} Carlos Sotomayor\textsuperscript{b}

\textsuperscript{a}Department Fruitculture and Enology, Faculty of Agronomy and Forestry Engineering, Pontificia Universidad Católica de Chile, 4860 Vicuña Mackenna Ave., Santiago, Chile. Ecuador Government Scholarship.
\textsuperscript{b}Department Fruitculture and Enology, Faculty of Agronomy and Forestry Engineering, Pontificia Universidad Católica de Chile, 4860 Vicuña Mackenna Ave., Santiago, Chile.

This paper was accepted to be published in Electronic Journal of Biotechnology

(July, 2015)
3.1. Abstract

*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 hours 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 hours 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.
Keywords: Fruit set ∙ Plant bioregulators ∙ Pollen germination ∙ Pollen tube growth ∙ Prunus dulcis

3.2. 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 leads 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,
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 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.

3.3. 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.
3.3.1. 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 zipp 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 hours, 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.

3.3.2. 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(NO₃)₂ 4H₂O, 30 mg L⁻¹; MgSO₄ 7H₂O, 20 mg L⁻¹; KNO₃, 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 µL of the FDA stock solution was added to 1 ml of the BK S15 MOPS pH7.5 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 minutes the slide was examined for fluorescence under an optical microscope (Olympus CX31,
Japan) set at 10x 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.

3.3.3. 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, GA₃, (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® (GA₃) 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 GA₃ 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 (H₃BO₃) (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 hours. 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 4x. In each replication three fields were photographed at random (ProRes®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 hours 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 hours 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.

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

Twelve 10-year-old almond 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 amongst the treatments using the following formula:
\[
\% \text{ Fruit Set} = \frac{N \text{ of fruit set per branch at 60 DAFB}}{N \text{ of flowers per branch}} \times 100
\]

3.3.5. 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.

3.4. Results

3.4.1. 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.
3.4.2. 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 hours 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® (GA₃), 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 GA₃, 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.

The effect of PBRs on Carmel pollen was statistically significant in both years (p<0.0001 and p<0.0184). GA₃ 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.

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

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.0 d</td>
<td>90.9 c</td>
<td>89.2 c</td>
<td>91.9 b</td>
</tr>
<tr>
<td>BL 10 mg L⁻¹</td>
<td>95.3 a</td>
<td>97.7 a</td>
<td>95.5 ab</td>
<td>95.1 a</td>
</tr>
<tr>
<td>BL 30 mg L⁻¹</td>
<td>92.4 cd</td>
<td>94.4 abc</td>
<td>92.6 bc</td>
<td>94.0 ab</td>
</tr>
<tr>
<td>BL 50 mg L⁻¹</td>
<td>91.0 d</td>
<td>91.4 bc</td>
<td>90.4 c</td>
<td>93.6 ab</td>
</tr>
<tr>
<td>GA₃ 10 µL L⁻¹</td>
<td>90.9 d</td>
<td>91.3 bc</td>
<td>92.4 bc</td>
<td>94.3 ab</td>
</tr>
<tr>
<td>GA₃ 30 µL L⁻¹</td>
<td>92.6 bcd</td>
<td>95.2 abc</td>
<td>96.7 ab</td>
<td>94.8 ab</td>
</tr>
<tr>
<td>GA₃ 50 µL L⁻¹</td>
<td>95.1 ab</td>
<td>96.6 ab</td>
<td>96.9 a</td>
<td>95.1 a</td>
</tr>
<tr>
<td>KN 10 µL L⁻¹</td>
<td>90.7 d</td>
<td>92.8 abc</td>
<td>92.9 abc</td>
<td>92.8 ab</td>
</tr>
<tr>
<td>KN 30 µL L⁻¹</td>
<td>92.0 cd</td>
<td>94.5 abc</td>
<td>93.3 abc</td>
<td>94.3 ab</td>
</tr>
<tr>
<td>KN 50 µL L⁻¹</td>
<td>94.1 abc</td>
<td>95.9 abc</td>
<td>94.4 abc</td>
<td>94.7 ab</td>
</tr>
</tbody>
</table>

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

The length of the pollen tube was measured at 8 hours 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, GA₃ 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. Control values were 917.7 and 921.3 µm pollen tube lengths in the 2013 and 2014 seasons, respectively.
Table 2. Pollen tube length in Non Pareil and Carmel almond cultivars after 8 hours, in the presence of plant bioregulators in the 2013 and 2014 growing seasons (values in µm)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>cv. Non Pareil</th>
<th></th>
<th>cv. Carmel</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2013</td>
<td>2014</td>
<td>2013</td>
<td>2014</td>
</tr>
<tr>
<td>Control</td>
<td>937.1 f</td>
<td>945.0 h</td>
<td>917.7 e</td>
<td>921.3 g</td>
</tr>
<tr>
<td>BL 10 mg L⁻¹</td>
<td>1067.4 b</td>
<td>1078.8 b</td>
<td>1117.0 c</td>
<td>1100.1 e</td>
</tr>
<tr>
<td>BL 30 mg L⁻¹</td>
<td>1032.6 c</td>
<td>1043.0 d</td>
<td>1059.7 d</td>
<td>973.1 f</td>
</tr>
<tr>
<td>BL 50 mg L⁻¹</td>
<td>963.9 e</td>
<td>971.6 f</td>
<td>921.3 e</td>
<td>964.0 f</td>
</tr>
<tr>
<td>GA₃ 10 µL L⁻¹</td>
<td>977.0 e</td>
<td>971.7 f</td>
<td>1183.0 b</td>
<td>1144.6 d</td>
</tr>
<tr>
<td>GA₃ 30 µL L⁻¹</td>
<td>1000.0 d</td>
<td>997.7 e</td>
<td>1199.4 b</td>
<td>1168.1 c</td>
</tr>
<tr>
<td>GA₃ 50 µL L⁻¹</td>
<td>1100.6 a</td>
<td>1096.0 a</td>
<td>1226.6 ab</td>
<td>1183.5 b</td>
</tr>
<tr>
<td>KN 10 µL L⁻¹</td>
<td>942.0 f</td>
<td>947.3 h</td>
<td>1198.4 b</td>
<td>1179.9 bc</td>
</tr>
<tr>
<td>KN 30 µL L⁻¹</td>
<td>965.1 e</td>
<td>960.6 g</td>
<td>1212.5 ab</td>
<td>1186.2 b</td>
</tr>
<tr>
<td>KN 50 µL L⁻¹</td>
<td>1056.8 b</td>
<td>1066.9 c</td>
<td>1243.4 a</td>
<td>1215.9 a</td>
</tr>
</tbody>
</table>

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

3.4.3. 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 Tables 3 and 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 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)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Pink Bud 2013</th>
<th>Pink Bud 2014</th>
<th>Fallen Petals 2013</th>
<th>Fallen Petals 2014</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.1 d</td>
<td>16.7 d</td>
<td>15.6 c</td>
<td>16.5 c</td>
</tr>
<tr>
<td>BL 10 mg L⁻¹</td>
<td>24.6 ab</td>
<td>22.3 bcd</td>
<td>21.7 abc</td>
<td>22.6 ab</td>
</tr>
<tr>
<td>BL 30 mg L⁻¹</td>
<td>22.5 abcd</td>
<td>19.2 cd</td>
<td>19.5 abc</td>
<td>20.4 abc</td>
</tr>
<tr>
<td>BL 50 mg L⁻¹</td>
<td>22.1 abcd</td>
<td>18.9 cd</td>
<td>17.8 bc</td>
<td>16.7 bc</td>
</tr>
<tr>
<td>GA₃ 10 µL L⁻¹</td>
<td>23.7 ab</td>
<td>26.2 ab</td>
<td>19.8 abc</td>
<td>22.5 abc</td>
</tr>
<tr>
<td>GA₃ 30 µL L⁻¹</td>
<td>27.1 a</td>
<td>28.0 ab</td>
<td>26.2 a</td>
<td>22.7 ab</td>
</tr>
<tr>
<td>GA₃ 50 µL L⁻¹</td>
<td>18.0 cd</td>
<td>22.7 bcd</td>
<td>20.6 abc</td>
<td>19.8 abc</td>
</tr>
<tr>
<td>KN 10 µL L⁻¹</td>
<td>20.1 bcd</td>
<td>22.7 bcd</td>
<td>22.1 ab</td>
<td>19.8 abc</td>
</tr>
<tr>
<td>KN 30 µL L⁻¹</td>
<td>23.5 abc</td>
<td>24.8 abc</td>
<td>23.7 ab</td>
<td>25.6 a</td>
</tr>
<tr>
<td>KN 50 µL L⁻¹</td>
<td>25.8 ab</td>
<td>31.0 a</td>
<td>22.1 ab</td>
<td>24.0 a</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not statistically different according to the Tukey-Kramer test (p≤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. GA₃ 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.

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)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Pink Bud</th>
<th></th>
<th>Fallen Petals</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2013</td>
<td>2014</td>
<td>2013</td>
<td>2014</td>
</tr>
<tr>
<td>Control</td>
<td>11.8 b</td>
<td>12.2 b</td>
<td>13.8 c</td>
<td>13.1 b</td>
</tr>
<tr>
<td>BL 10 mg L(^{-1})</td>
<td>19.5 a</td>
<td>20.4 a</td>
<td>20.0 ab</td>
<td>20.1 a</td>
</tr>
<tr>
<td>BL 30 mg L(^{-1})</td>
<td>17.7 a</td>
<td>15.6 ab</td>
<td>15.2 abc</td>
<td>16.4 ab</td>
</tr>
<tr>
<td>BL 50 mg L(^{-1})</td>
<td>15.2 ab</td>
<td>14.1 b</td>
<td>14.0 bc</td>
<td>13.7 b</td>
</tr>
<tr>
<td>GA(_3) 10 µL L(^{-1})</td>
<td>17.6 a</td>
<td>16.6 ab</td>
<td>15.5 abc</td>
<td>17.2 ab</td>
</tr>
<tr>
<td>GA(_3) 30 µL L(^{-1})</td>
<td>19.4 a</td>
<td>17.5 ab</td>
<td>18.6 abc</td>
<td>19.7 a</td>
</tr>
<tr>
<td>GA(_3) 50 µL L(^{-1})</td>
<td>15.4 ab</td>
<td>13.0 b</td>
<td>16.4 abc</td>
<td>17.8 ab</td>
</tr>
<tr>
<td>KN 10 µL L(^{-1})</td>
<td>17.2 ab</td>
<td>14.4 b</td>
<td>15.3 abc</td>
<td>15.6 ab</td>
</tr>
<tr>
<td>KN 30 µL L(^{-1})</td>
<td>19.8 a</td>
<td>20.3 a</td>
<td>21.0 a</td>
<td>20.4 a</td>
</tr>
<tr>
<td>KN 50 µL L(^{-1})</td>
<td>18.2 a</td>
<td>20.2 a</td>
<td>20.6 a</td>
<td>20.0 a</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not statistically different according to the Tukey-Kramer test (p≤0.05).
3.5. Discussion

3.5.1. 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.

3.5.2. 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, GA₃ 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 GA₃ 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, GA₃ at 50 µL L⁻¹ resulted in greater lengths (Table 2). No previous studies were found using BL, GA₃ 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 GA₃ stimulated pollen tube growth *in vitro* and significantly increased the quantity of pollinated styles. These results suggest that GA₃ 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 µL 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 hours, 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 µL 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*. 
3.5.3. Evaluation of plant bioregulators on fruit set in almond trees

The application of PBRs improve 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 µL 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 µL L$^{-1}$ during pink bud and 30 µL 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 µL 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 µL L$^{-1}$ and KN at 30 µL 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 µL L⁻¹, BL at 10 mg L⁻¹ and GA₃ at 30 µL 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 GA₃ were also applied, obtaining fruit set values lower than the controls, possibly due to the high concentrations used.

3.6. Conclusions

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%. Two limiting factors of note here are the cold climate, and the gametophytic self-incompatibility typical of this species.
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.

Acknowledgments

To Agricola Pomes Andrade y Cia. Ltda. (Paine) for field support in the development of this research.

References


Chapter 4

Using three plant bioregulators in flowering almonds cvs. Non Pareil and Carmel affects the weight and size of kernels at harvest

Segundo Maita$^1$ and Carlos Sotomayor$^2$

$^1$Ecuador Government Scholarship. Email address: smmaita@uc.cl

$^2$Department of Fruitculture and Enology, Faculty of Agronomy and Forestry Engineering, Pontificia Universidad Católica de Chile, 4860 Vicuna Mackenna Ave., Santiago, Chile.

Submitted for publication in HortScience (July, 2015)
4.1. Abstract

Three plant bioregulators: 24-epibrassinolide (BL), gibberellic acid (GA$_3$) and kinetin (KN) in three concentrations were sprayed on almonds trees \textit{Prunus dulcis} (Mill.) D.A. Webb] cvs. Non Pareil and Carmel in two phenological stages of flowering: pink bud and fallen petals, in order to evaluate their effect on the final dry weight, length and width of kernels (almonds), during seasons 2014 and 2015.

The treatments applied were BL in 10, 30 and 50 mg L$^{-1}$ concentrations, GA$_3$ and KN both in 10, 30 and 50 µL L$^{-1}$ concentrations, and water aspersion as control. The results show statistical differences ($p < 0.0001$) with all the treatments applied on the three variables evaluated. In the variable almond dry weight in cv. Non Pareil, the best treatment was BL (30 mg L$^{-1}$), weighing 1.45 g in average, while the control reached 1.30 g at pink bud phenological stage, in season 2015. In the case of cv. Carmel, the best dry weight was 1.23 g, with the BL treatment (30 mg L$^{-1}$) at fallen petals stage, in the two seasons. The same value was obtained applying KN and GA$_3$ (50 µL L$^{-1}$), at fallen petals and pink bud phenological stages, during 2014 and 2015 seasons respectively. Dry weight in control in cv. Carmel varied between 1.13 and 1.18 g depending on the seasons and phenological stages evaluated. The highest values on almond length and width in the cv. Non Pareil was 24.98 mm and 15.05 mm, with the BL treatment (30 mg L$^{-1}$) and KN treatment (50 µL L$^{-1}$), respectively, at pink bud phenological stage, in season 2015. The control reached 23.91 mm of length and 14.71 mm of width in corresponding phenological stage and season. In the cv. Carmel, the maximum length and width
were obtained with the BL treatment (30 mg L\(^{-1}\)), with 24.38 mm, and 13.44 mm respectively, at pink bud and fallen petals phenological stages, in season 2015. The control reached 23.20 mm of length and 12.93 mm of width in corresponding phenological stage and season.

In conclusion, a significant statistical effect occurred with all the plant bioregulators tested favoring dry weight, length and width on almond kernels at harvest in the cvs. Non Pareil and Carmel in two consecutive seasons.

**Keywords:** Brassinolide ∙ Gibberellic Acid ∙ Kinetin ∙ Kernels ∙ *Prunus dulcis*

### 4.2. Introduction

Almond [(*Prunus dulcis* (Miller) D.A. Webb)] is a fruit tree with low chilling requirements; therefore, it shows early flowering, which coincides with the end of winter season, and that low temperature, mist and rains have negative effect on pollination, fruit set and yield (Sutyemez, 2011). Additionally and importantly, almond flowers are mainly self-incompatible; thus, cross pollination is required (Yi *et al.*, 2006). Therefore, almond orchards must be planted with at least two compatible cultivars with simultaneous flowering, requiring also pollinator insects for pollen transference (Vezvaei and Jackson, 1995). Pollination can be limiting in some production areas of Chile, for example, fruit set lower than 20% will be an impact on cultivation yield, and this may occur in years with irregular spring climate (Sotomayor, 2013).
In some cases, unfavorable climate conditions during almond flowering, plus the lack of adequate technology on cultivation management, make low yields in Chile, reaching 1400 kg ha\(^{-1}\) in average, if compared with average yields in California, which easily reach 2800 kg ha\(^{-1}\) of almond kernels (Sotomayor et al., 2012).

Pollen germination and fertilization are affected by environmental factors such as light, temperature and relative humidity. Unfavorable environmental conditions may dramatically reduce ovule fertilization and subsequently fructification. Seed and fruit development are intimately linked and synchronized to each other and controlled by phytohormones (Pandolfini, 2009). After flower fertilization, fruits and seeds experiment a concomitant development. However, seeds depend strictly on a successful fertilization, contrarily to fruits, which may sometimes develop under pollination absence. Seed development includes endosperm proliferation and embryo growth; both processes show multihormonal regulation by auxins, cytokinins, gibberellins and brassinosteroids (Ruan et al., 2012). In the case of almond, a successful fertilization is particularly important for optimal yield, as seed is the edible portion (Kodad and Socias i Company, 2013).

Cytokinins are a group of phytohormones regulating cell division and affecting numerous physiological processes and plant development, including leaf senescence, vascular development, cell differentiation in apical meristems into branches and roots, nutrient consumption and distribution, responses to biotic and abiotic stress and regulation of the source-sink relation. Recent studies have revealed that cytokinins are key regulators for seed production (Zhao et al., 2015).
Stimulation of organ growth is the main physiological function of gibberellins in higher plants, by improving cell elongation and, in some cases, cell division. Additionally, gibberellins promote some changes between dormancy and seeds germination, between juvenile and adult growth, and vegetative and reproductive development. In the performance of their functions, gibberellins act in response to development and environmental signals (Hedden and Thomas, 2012). Gallego-Giraldo et al. (2014) also indicate that gibberellins are a key factor for fruit set and fruit development.

The brassinosteroids are plant hormones that act through the entire plant, including the roots. These plant hormones cause a wide range of physiological responses, including stem elongation, growth of the pollen tube, leaf bending and epinasty, inhibition of root growth, induction of ethylene synthesis, activation of the proton pump, xylem differentiation, synthesis of nucleic acids and proteins, enzyme activation and photosynthesis. Additionally, brassinosteroids are also known by having palliative role in plants that are subject to biotic and abiotic stress (Hayat et al., 2007).

Phytohormones play a crucial role on modulating multiple development processes and cell responses to different kinds of stress. Several natural vegetal hormones like gibberellins, salicylic acid, ethylene, and brassinosteroids, have been associated to the response to stress by cold in fruits (Ding et al., 2015).
Currently, the discovery and use of chemical substances for replace or mimic the action of vegetal hormones, called plant bioregulators, they have allowed farmers to correct some deficiencies, in order to prevent economical loss (Wertheim and Webster, 2005). Plant bioregulators were soon identified as yield, life quality and post-harvest improvers, reaching their higher impact precisely on fruit production (Greene, 2010).

Swain et al. (1997) suggest that gibberellins, presumably GA$_1$ and GA$_3$ synthesized in the embryo and/or endosperm are required for seeds development in the first days after fertilization. Gibberellins application and, subsequently, high levels of endogenous gibberellins, are causally associated with early growth of small fruits and ripe fruit in *Pyrus pyrifolia* (Zhang et al., 2010). Gibberellic acid (GA$_3$) improved fruit set in almond and maximum fruit retention was observed with a concentration of 200 ppm (Joolka et al., 1991). Swain et al. (1997) suggest two models to explain the role of gibberellins on assimilates distribution; gibberellins might directly promote assimilates consumption for seeds development or indirectly by changes in seeds growth.

Exogenous applications of synthetic cytokinins may induce fruit set and fruit development in several crop species (Matsuo et al., 2012). Zhao et al., (2015), evaluating the effect of cytokinins on cotton, found that a moderate concentration promotes seeds development, but an overdose inhibits development. Application of adequate concentrations promoted the development and enlarged seeds. Kinetin,
a synthetic cytokinin, applied to plants of transgenic tobacco with male sterility, resulted on normal fertilization and seed development (Huang et al. 2003).

Experimenting on live vegetal cells, Vogler et al. (2014) observed an over five time increase on rates of cell elongation when the germination medium was supplemented with 10 µM of epibrassinolide. Brassinosteroids treatment on rice, tomato, maize and cucumber plants improved their ability to resist low temperature (Hayat et al., 2007). On the other hand, Thussagunpanit et al. (2013) suggest that applying brassinosteroids should be useful for increasing rice yields under high temperature in field conditions.

Yield is the most important component in plant cultivation, always requiring improvement to satisfy growing food demand. Therefore, yield improvement is an essential topic in agricultural agenda in the world (Bartrina et al., 2011). In this study, the effect of three plant bioregulators commercially availables, on final weight, length and width of almond kernels of cvs. Non Pareil and Carmel was evaluated in an orchard, in the Metropolitan Region of Chile.

4.3. Materials and Methods

The study was carried out during seasons 2013-2014 and 2014-2015 in a commercial orchard located in Paine, Metropolitan Region of Chile (latitude 33°46′21.3″ S – longitude 70°38′12.5″ W). Non Pareil and Carmel almond plants had ten years old and were grafted on Nemaguard rootstock; these plants were in
full production and planted at 6 m x 4 m. For treatments, 12 cv. Non Pareil and 12 cv. Carmel plants were selected and these plants were planted in alternate lines with North-South orientation.

4.3.1. Treatments

Three plant bioregulators commercially availables were applied to evaluate their effect on dry weight, length and width of kernels (almonds) in cvs. Non Pareil and Carmel plants at field:

a) Brassinolide 0.1%, wet powder (WP), with active ingredient 24-Epibrassinolide (chemical formula: 22R, 23R, 24R)-2α,3α,22,23-tetrahydroxy-24-methyl-β-homo-7-oxa-5-cholen-6-one) produced and commercialized by Green Plantchem Company Limited, Republic of China.

b) ProGibb® 4%, soluble concentrate (SL), the active ingredient is gibberellic acid (GA₃) 3.2% p/v (chemical formula: 3S, 3aR, 4S, 4aS, 7S, 9aR, 9bR, 12S)-7, 12- dihydroxi-3-methyl-6-methylene-oxoperhydro-4, 7-methane-9b, 3-propenoazulene (1,2-b) furan-4-carboxilic), produced by Valent BioSciences Corporation of the United States of America, and imported and distributed by Valent BioSciences, Chile S.A.

c) X-Cyte®, soluble concentrate (SL), the active ingredient is kinetin, a cytokinin, with a 0.04% p/v concentration (chemical formula: 6-furfurilamino-
9H-purina), made by Stoller Enterprises Inc. of the United States of America and imported and distributed by Stoller of Chile S.A.

These three plant bioregulators (PBRs): Brassinolide (BL), ProGibb® (GA₃) and X-Cyte® (KN) were sprayed at 10, 30 and 50 mg L⁻¹ concentrations in the case of BL (wettable powder), and 10, 30 and 50 µL L⁻¹ in the case of GA₃ and KN (concentrated solutions). All the concentrations used in this study correspond to the commercial products.

For this experiment, a randomized block design was used, with 12 replications. For treatment application, 12 cv. Non Pareil plants and 12 cv. Carmel plants were selected; then, 20 uniform branches in the medium portion of the canopy in each plant were selected. Ten branches selected at random were used for treatment, in the phenological stage of pink bud and 10 branches selected at random for treatment in the phenological stage of fallen petals. The phenological stages were selected using the scale of almond floral development proposed by Yi et al. (2006), and when 50% of the flowers were in the stages mentioned above (Martinez-Gomez et al., 2006).

The treatments were assigned on these branches at random, including a control consisting only of water spraying. Each branch was considered as an experimental unit.
4.3.2. Kernel dry weight

When fruits were at least with 80% of open mesocarp (Ledbetter and Sisterson, 2010) they were harvested and taken to the laboratory of the Faculty of Agronomy and Forest Engineering of Pontificia Universidad Católica de Chile. Then, fruits were weighed (fresh weight), hull and shell were separated, and kernels were obtained. The fresh weight of 10 randomly selected kernels was measured in each replication (120 kernels per treatment), subsequently dried in a drying chamber at 72°C during 24 hours, until their weight was constant. The dry weight of the kernels was obtained after the dry process, with a digital scale calibrated with 0.01 g precision.

4.3.3. Kernel length and width

After the kernels (almonds) were dried and weighed, the length (polar diameter) and width (equatorial diameter) of 10 almonds were measured for each repetition (120 per each treatment). A digital caliper with 0.01 mm precision was used.

4.3.4. Statistical analysis

An analysis of variance was made for all the evaluated variables. The average values were compared through the Tukey-Kramer test with $\alpha=0.05$. A linear regression was applied for measuring the relation between the variable dry weight
with the variables length and width of kernels. The software SAS was used for the statistical analysis (SAS v.9.1.3).

4.4. Results and discussion

4.4.1. Kernel dry weight

The results obtained are presented in Tables 1 and 2, and they correspond to the average values of 120 kernels per treatment.

Application of plant bioregulators in the cv. Non Pareil showed statistically significant differences \( (p<0.0001) \) in the two phenological stages and the two seasons (Table 1). KN treatment \( (50 \, \mu\text{L L}^{-1}) \) presented the maximum dry weight in the two phenological stages in season 2014, with 1.33 g in pink bud and 1.31 g in fallen petals. Similar studies show that cytokinins application may improve yield in cotton seeds (Zhao et al., 2015), which is agree with reported by Bartrina et al. (2011), who improved seeds yield in Arabidopsis thaliana by applying cytokinins. The control reached 1.27 g of dry weight in the two phenological stages. It is noteworthy the effect of the BL treatment \( (30 \, \text{mg L}^{-1}) \), which was statistically equal to the KN treatment \( (50 \, \mu\text{L L}^{-1}) \), reaching 1.32 g in pink bud and 1.31 g in fallen petals. This is coherent to reports by Zhu et al. (2015), who indicate that brassinosteroids increase weight grain in rice. \( \text{GA}_3 \) in low and medium concentrations reached a significant improvement of kernels weight, with 1.32 g and 1.31 g in the phenological stages pink bud and fallen petals, respectively. This
coincides with reports by Huang et al. (2014), who improved seeds weight, in raps (Brassica napus) with GA$_3$ application.

In season 2015, in the cv. Non Pareil, BL in the 30 mg L$^{-1}$ concentration increased significantly the dry weight, reaching 1.45 g and 1.43 g in the phenological stages pink bud and fallen petals, respectively. Similar studies report that applying brassinosteroids improve filling in rice seeds, and subsequently their weight (Wu et al., 2008). The KN treatments in high concentration (50 µL L$^{-1}$) and GA$_3$ in low concentration (10 µL L$^{-1}$) improved significantly the dry weight of kernels in the two phenological stages used. The control reached 1.30 g and 1.34 g of average dry weight in the phenological stages pink bud and fallen petals, respectively.

Table 1. Effect of plant bioregulators on kernel dry weight (g) of cv. Non Pareil almonds in two phenological stages during two seasons

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.27 c$^2$</td>
<td>1.30 f</td>
<td>1.27 b</td>
<td>1.34 e</td>
</tr>
<tr>
<td>BL 10 mg L$^{-1}$</td>
<td>1.29 bc</td>
<td>1.39 cde</td>
<td>1.28 ab</td>
<td>1.37 bcde</td>
</tr>
<tr>
<td>BL 30 mg L$^{-1}$</td>
<td>1.32 a</td>
<td>1.45 a</td>
<td>1.31 a</td>
<td>1.43 a</td>
</tr>
<tr>
<td>BL 50 mg L$^{-1}$</td>
<td>1.29 bc</td>
<td>1.41 abcd</td>
<td>1.29 ab</td>
<td>1.36 de</td>
</tr>
<tr>
<td>GA$_3$ 10 µL L$^{-1}$</td>
<td>1.32 ab</td>
<td>1.43 ab</td>
<td>1.29 ab</td>
<td>1.39 bc</td>
</tr>
<tr>
<td>GA$_3$ 30 µL L$^{-1}$</td>
<td>1.31 ab</td>
<td>1.40 bcd</td>
<td>1.31 a</td>
<td>1.37 cde</td>
</tr>
<tr>
<td>GA$_3$ 50 µL L$^{-1}$</td>
<td>1.30 abc</td>
<td>1.38 de</td>
<td>1.29 ab</td>
<td>1.35 de</td>
</tr>
<tr>
<td>KN 10 µL L$^{-1}$</td>
<td>1.29 bc</td>
<td>1.36 e</td>
<td>1.27 b</td>
<td>1.37 cde</td>
</tr>
<tr>
<td>KN 30 µL L$^{-1}$</td>
<td>1.31 ab</td>
<td>1.40 bcd</td>
<td>1.28 ab</td>
<td>1.37 bcd</td>
</tr>
<tr>
<td>KN 50 µL L$^{-1}$</td>
<td>1.33 a</td>
<td>1.42 abc</td>
<td>1.31 a</td>
<td>1.40 b</td>
</tr>
</tbody>
</table>

$^2$Means followed by the same letter are not statistically different according to the Tukey-Kramer test (p≤0.05).

In the cv. Carmel, the application of plant bioregulators presented statistically significant differences (p<0.0001) on the variable kernel dry weight in the two
phenological stages and the two seasons (Table 2). The KN treatment (50 µL L\(^{-1}\)) presented the maximum kernel dry weight of almonds in season 2014 in the two phenological stages, with 1.19 g in pink bud and 1.23 g in fallen petals. This is similar to reported by Bartrina et al. (2011), who mentioned that cytokinins improve seeds yield in *A. thaliana*. The control reached 1.13 g and 1.18 g of kernels dry weight in the phenological stages pink bud and fallen petals, respectively. It is important to highlight the effect of BL treatment (30 mg L\(^{-1}\)), which was statistically equal to the KN treatment (50 µL L\(^{-1}\)) reaching 1.18 g in pink bud and 1.23 g in fallen petals. GA\(_3\) in high concentrations reached the best kernel dry weight, with 1.18 g and 1.22 g in the phenological stages pink bud and fallen petals, respectively. In this sense, Rastogi et al. (2013) report the improvement of dry weight in linseed seeds (*Linum usitatissimum*) applying GA\(_3\).

In season 2015, GA\(_3\) treatment in high concentration in the cv. Carmel (50 µL L\(^{-1}\)) increased significantly the dry weight of kernels, reaching 1.23 g in the phenological stages pink bud and the BL treatment with medium concentration (30 mg L\(^{-1}\)) reached 1.23 g in the phenological stage fallen petals. Additionally, it is important to highlight the effect of BL treatments (30 mg L\(^{-1}\)) reaching 1.22 g and KN (30 µL L\(^{-1}\) and 50 µL L\(^{-1}\)) reaching 1.22 g in the phenological stage pink bud and de GA\(_3\) (30 µL L\(^{-1}\) and 50 µL L\(^{-1}\)) in the phenological stage fallen petals, which reached 1.22 g. The control reached 1.15 g and 1.17 g of average of kernel dry weight in the phenological stages pink bud and fallen petals, respectively. Experimental studies globally with applications of BL, GA\(_3\) or KN to improve kernel weight in almond trees cvs. Non Pareil and Carmel have not been reported so far.
Table 2. Effect of the plant bioregulators on kernel dry weight (g) in cv. Carmel almond in two phenological stages and two seasons

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.13 d</td>
<td>1.15 e</td>
<td>1.18 d</td>
<td>1.17 d</td>
</tr>
<tr>
<td>BL 10 mg L⁻¹</td>
<td>1.18 ab</td>
<td>1.21 abc</td>
<td>1.21 abcd</td>
<td>1.19 cd</td>
</tr>
<tr>
<td>BL 30 mg L⁻¹</td>
<td>1.18 ab</td>
<td>1.22 ab</td>
<td>1.23 a</td>
<td>1.23 a</td>
</tr>
<tr>
<td>BL 50 mg L⁻¹</td>
<td>1.16 abc</td>
<td>1.20 bcd</td>
<td>1.22 ab</td>
<td>1.22 abc</td>
</tr>
<tr>
<td>GA₃ 10 µL L⁻¹</td>
<td>1.14 cd</td>
<td>1.18 cd</td>
<td>1.19 cd</td>
<td>1.19 bcd</td>
</tr>
<tr>
<td>GA₃ 30 µL L⁻¹</td>
<td>1.15 bcd</td>
<td>1.21 abc</td>
<td>1.22 abc</td>
<td>1.22 abc</td>
</tr>
<tr>
<td>GA₃ 50 µL L⁻¹</td>
<td>1.18 ab</td>
<td>1.23 a</td>
<td>1.22 abc</td>
<td>1.22 ab</td>
</tr>
<tr>
<td>KN 10 µL L⁻¹</td>
<td>1.16 abc</td>
<td>1.18 d</td>
<td>1.19 bcd</td>
<td>1.18 d</td>
</tr>
<tr>
<td>KN 30 µL L⁻¹</td>
<td>1.19 a</td>
<td>1.22 ab</td>
<td>1.21 abcd</td>
<td>1.19 bcd</td>
</tr>
<tr>
<td>KN 50 µL L⁻¹</td>
<td>1.19 a</td>
<td>1.22 ab</td>
<td>1.23 a</td>
<td>1.21 abc</td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not statistically different according to the Tukey-Kramer test (p≤0.05)*

4.4.2. Kernel length and width

Treatments with plant bioregulators showed a statistically significant effect on the variables length and width at harvest, in cvs. Non Pareil and Carmel, in the phenological stages pink bud and fallen petals, for seasons 2014 and 2015.

In the Non Pareil cultivar, season 2014 (Table 3) statistically significant differences were observed (p<0.0001) between the treatments in the two phenological stages in the two variables. The KN treatment in the 50 µL L⁻¹ concentration induced a larger length (24.04 mm and 24.20 mm in pink bud and fallen petals, respectively) and larger width (13.94 mm, and 14.09 mm in pink bud and fallen petals, respectively) in harvested kernels, this shows a tendency on increasing almond length and width, as the concentration increases. This is coherent to reports by
Zhao et al. (2015), who improved the size of cotton seeds by applying cytokinins. The BL treatment in the 30 mg L\(^{-1}\) concentration presented values statistically equal to KN (50 µL L\(^{-1}\)), maintaining a better response with the intermediate concentration. Zhu et al. (2015) also reported the increase of rice seeds by applying brassinosteroids. The GA\(_3\) treatments showed a positive effect on kernels length and width, with a better response to low concentrations in the two variables in the two phenological stages. This is coherent with reports by Rastogi et al. (2013) and Huang et al. (2014), who improved seeds size of linseed (\textit{L. usitatissimum}) and raps (\textit{B. napus}) respectively by applying GA\(_3\). All the treatments surpassed the control. The values reached by the control were 23.23 mm length and 13.31 mm width, in the phenological stage pink bud and 23.49 mm length and 13.52 mm width in the phenological stage fallen petals.

Table 3. Effect of the plant bioregulators on kernel length and width (mm) in the cv. Non Pareil almonds in two phenological stages, season 2014

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Pink Bud</th>
<th></th>
<th>Fallen Petals</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
<td>Length</td>
<td>Width</td>
</tr>
<tr>
<td>Control</td>
<td>23.23 c</td>
<td>13.31 d</td>
<td>23.49 c</td>
<td>13.52 c</td>
</tr>
<tr>
<td>BL 10 mg L(^{-1})</td>
<td>23.50 bc</td>
<td>13.48 cd</td>
<td>23.68 bc</td>
<td>13.69 bc</td>
</tr>
<tr>
<td>BL 30 mg L(^{-1})</td>
<td>23.97 ab</td>
<td>13.86 ab</td>
<td>24.06 ab</td>
<td>14.08 a</td>
</tr>
<tr>
<td>BL 50 mg L(^{-1})</td>
<td>23.23 c</td>
<td>13.53 bcd</td>
<td>23.67 c</td>
<td>13.76 bc</td>
</tr>
<tr>
<td>GA(_3) 10 µL L(^{-1})</td>
<td>23.88 ab</td>
<td>13.69 abc</td>
<td>23.80 bc</td>
<td>13.86 ab</td>
</tr>
<tr>
<td>GA(_3) 30 µL L(^{-1})</td>
<td>23.86 ab</td>
<td>13.64 abcd</td>
<td>23.73 bc</td>
<td>13.83 ab</td>
</tr>
<tr>
<td>GA(_3) 50 µL L(^{-1})</td>
<td>23.67 abc</td>
<td>13.57 bcd</td>
<td>23.54 c</td>
<td>13.74 bc</td>
</tr>
<tr>
<td>KN 10 µL L(^{-1})</td>
<td>23.36 c</td>
<td>13.36 cd</td>
<td>23.66 c</td>
<td>13.63 bc</td>
</tr>
<tr>
<td>KN 30 µL L(^{-1})</td>
<td>23.61 abc</td>
<td>13.66 abc</td>
<td>23.75 bc</td>
<td>13.82 abc</td>
</tr>
<tr>
<td>KN 50 µL L(^{-1})</td>
<td>24.04 a</td>
<td>13.94 a</td>
<td>24.20 a</td>
<td>14.09 a</td>
</tr>
</tbody>
</table>

\(^{a}\)Means followed by the same letter are not statistically different according to the Tukey-Kramer test \((p\leq0.05)\)
In season 2015, the effect of the plant bioregulators on kernel length and width of harvested cv. Non Pareil almond was similar to the effect observed in season 2014 (Table 4), showing statistically significant differences ($p<0.0001$) between treatments, in the two phenological stages in the two variables. KN treatment highlight in the $50 \mu$L L$^{-1}$ concentration, which is similar to the reports by Zhao et al. (2015) in experiments with cotton seeds. The BL treatment also highlights in the $30$ mg L$^{-1}$ concentration. In this case, maintaining the tendency to increase kernels length and width in season 2014, with increase concentration in the case of KN, and maintaining the tendency of better response with the intermediate concentration, in the case of BL. All the treatments surpassed the control.

Table 4. Effect of the plant bioregulators on kernel length and width (mm) of cv. Non Pareil almonds in two phenological stages in 2015 season

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Pink Bud</th>
<th>Fallen Petals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
</tr>
<tr>
<td>Control</td>
<td>23.91</td>
<td>d$^2$</td>
</tr>
<tr>
<td>BL 10 mg L$^{-1}$</td>
<td>24.14</td>
<td>cd</td>
</tr>
<tr>
<td>BL 30 mg L$^{-1}$</td>
<td>24.98</td>
<td>a</td>
</tr>
<tr>
<td>BL 50 mg L$^{-1}$</td>
<td>24.19</td>
<td>bcd</td>
</tr>
<tr>
<td>GA$_3$ 10 µL L$^{-1}$</td>
<td>24.59</td>
<td>abc</td>
</tr>
<tr>
<td>GA$_3$ 30 µL L$^{-1}$</td>
<td>24.38</td>
<td>abcd</td>
</tr>
<tr>
<td>GA$_3$ 50 µL L$^{-1}$</td>
<td>24.23</td>
<td>bcd</td>
</tr>
<tr>
<td>KN 10 µL L$^{-1}$</td>
<td>24.31</td>
<td>bcd</td>
</tr>
<tr>
<td>KN 30 µL L$^{-1}$</td>
<td>24.53</td>
<td>abc</td>
</tr>
<tr>
<td>KN 50 µL L$^{-1}$</td>
<td>24.79</td>
<td>abcd</td>
</tr>
</tbody>
</table>

$^a$Means followed by the same letter are not statistically different according to the Tukey-Kramer test ($p\leq0.05$)

In the case of the cv. Carmel in season 2014 (Table 5) statistically significant differences are observed ($p<0.0001$) between treatments in the two phenological
stages in the two variables. KN application in the 50 µL L\(^{-1}\) concentration stimulated a larger length (23.54 mm, and 23.53 mm in pink bud and fallen petals, respectively) and larger width (12.53 mm, and 12.67 mm in pink bud and fallen petals, respectively) of harvested kernels, which shows a tendency of increased length and width by increasing the concentration. This is agrees with the reports by Zhao et al. (2015), who improved the size of cotton seeds by applying cytokinins. BL application on the 30 mg L\(^{-1}\) concentration presented values statistically equal to KN (50 µL L\(^{-1}\)), maintaining the tendency of best response to the intermediate concentration. GA\(_3\) treatments showed a positive effect on kernel length and width, with a better response to high concentrations in the two variables in the two phenological stages. This was different to the observations in the cv. Non Pareil where a better response to low GA\(_3\) concentrations was evident. These results are similar to reports by Huang et al. (2014), who improved size of rap seeds (B. napus) and Rastogi et al. (2013), who improved the size of linseed seeds (L. usitatissimum) applying GA\(_3\). The average values of the control were 22.33 mm length and 11.99 mm width in the phenological stage fallen petals and 22.69 mm length and 12.19 mm width in the phenological stage pink bud. All the treatments surpassed the control.
Table 5. Effect of the plant bioregulators on kernel length and width (mm) of cv. Carmel almonds in two phenological stages in 2014 season

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Pink Bud</th>
<th></th>
<th>Fallen Petals</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
<td>Length</td>
<td>Width</td>
</tr>
<tr>
<td>Control</td>
<td>22.33 c</td>
<td>11.99 d</td>
<td>22.69 d</td>
<td>12.19 d</td>
</tr>
<tr>
<td>BL 10 mg L⁻¹</td>
<td>22.89 abc</td>
<td>12.33 abc</td>
<td>23.21 abc</td>
<td>12.41 bcd</td>
</tr>
<tr>
<td>BL 30 mg L⁻¹</td>
<td>23.35 ab</td>
<td>12.49 ab</td>
<td>23.47 a</td>
<td>12.62 ab</td>
</tr>
<tr>
<td>BL 50 mg L⁻¹</td>
<td>22.80 bc</td>
<td>12.32 abc</td>
<td>23.20 abc</td>
<td>12.43 abcd</td>
</tr>
<tr>
<td>GA₃ 10 µL L⁻¹</td>
<td>22.92 abc</td>
<td>12.27 bc</td>
<td>22.82 cd</td>
<td>12.45 abcd</td>
</tr>
<tr>
<td>GA₃ 30 µL L⁻¹</td>
<td>22.95 ab</td>
<td>12.29 ab</td>
<td>23.06 bcd</td>
<td>12.49 abc</td>
</tr>
<tr>
<td>GA₃ 50 µL L⁻¹</td>
<td>22.96 abc</td>
<td>12.30 abc</td>
<td>23.42 ab</td>
<td>12.55 abc</td>
</tr>
<tr>
<td>KN 10 µL L⁻¹</td>
<td>22.82 abc</td>
<td>12.18 cd</td>
<td>22.92 cd</td>
<td>12.32 cd</td>
</tr>
<tr>
<td>KN 30 µL L⁻¹</td>
<td>23.18 ab</td>
<td>12.44 ab</td>
<td>23.15 abc</td>
<td>12.44 abcd</td>
</tr>
<tr>
<td>KN 50 µL L⁻¹</td>
<td>23.54 a</td>
<td>12.53 a</td>
<td>23.53 a</td>
<td>12.67 a</td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not statistically different according to the Tukey-Kramer test (p≤0.05)

In season 2015, the effect of the plant bioregulators on kernel length and width at harvest of cv. Carmel, presented statistically significant differences (p<0.0001) between treatments in the two phenological stages in the two variables (Table 6). BL application in the 30 mg L⁻¹ concentration stimulated a larger length (24.38 mm and 24.32 mm in pink bud and fallen petals, respectively) and larger width (13.34 mm and 13.44 mm in pink bud and fallen petals, respectively) of harvested kernels. This shows a tendency to increased kernel length and width with the intermediate concentration. This is coherent to reports by Zhu et al. (2015), who improved size of rice seeds applying brassinosteroids. It is noteworthy that KN treatments (50 µL L⁻¹) and GA₃ (50 µL L⁻¹) presented values statistically equal to BL (30 mg L⁻¹).
Studies on almonds with application of plant bioregulators like BL, AG$_3$ and KN to improve the kernel size of cvs. Non Pareil and Carmel have been not reported so far.

Table 6. Effect of plant bioregulators on kernel length and width (mm) on almonds cv. Carmel in two phenological stages in 2015 season

| Treatments | Pink Bud | | Fallen Petals | |
|------------|----------||----------|----------|
|            | Length   | Width   | Length   | Width   |
| Control    | 23.20    | d       | 12.78    | c        |
|            | 23.38    | c       | 12.93    | c        |
| BL 10 mg L$^{-1}$ | 24.10    | ab      | 13.15    | ab       |
|            | 24.00    | ab      | 13.17    | abc      |
| BL 30 mg L$^{-1}$ | 24.38    | a       | 13.34    | a        |
|            | 24.32    | a       | 13.44    | a        |
| BL 50 mg L$^{-1}$ | 23.53    | cd      | 12.92    | bc       |
|            | 24.09    | ab      | 13.31    | abc      |
| GA$_3$ 10 µL L$^{-1}$ | 23.77    | bc      | 13.01    | abc      |
|            | 23.82    | abc     | 13.29    | abc      |
| GA$_3$ 30 µL L$^{-1}$ | 24.07    | ab      | 13.17    | ab       |
|            | 24.10    | a       | 13.37    | ab       |
| GA$_3$ 50 µL L$^{-1}$ | 24.28    | a       | 13.31    | a        |
|            | 24.15    | a       | 13.41    | ab       |
| KN 10 µL L$^{-1}$ | 23.68    | bcd     | 13.12    | abc      |
|            | 23.57    | bc      | 13.02    | bc       |
| KN 30 µL L$^{-1}$ | 23.99    | abc     | 13.26    | ab       |
|            | 23.86    | abc     | 13.26    | abc      |
| KN 50 µL L$^{-1}$ | 24.30    | a       | 13.30    | a        |
|            | 24.24    | a       | 13.42    | ab       |

*Means followed by the same letter are not statistically different according to the Tukey-Kramer test (p≤0.05)*

The regression analysis considering kernel dry weight as dependent variable and kernel length and width as independent variables (Table 7), in order to determine the relation between kernel weight with their length and width variables in the two phenological stages in the two seasons was applied. A statistically significant relation between kernels dry weight and kernels length and width in all the cases analyzed (p<0.05) was found. A positive correlation between kernel dry weight vs length, and kernel dry weight vs width was evident, with coefficients between 0.24 and 0.58. In general, the highest values were reached with the treatments applied
in the phenological stage pink bud in the two cultivars in the two seasons as shown in Fig. 1.

Table 7. Summary of the regression analysis of the variable kernel dry weight according to the variables length and width of almonds cvs. Non Pareil and Carmel in 2014 and 2015 seasons

<table>
<thead>
<tr>
<th>Cultivars/ Traits</th>
<th>Y</th>
<th>X</th>
<th>Model</th>
<th>R²</th>
<th>Correl Coef.</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Pareil/Pink bud/ 2014</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.70092 + 0.0254737X</td>
<td>17.27%</td>
<td>0.42</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.724397 + 0.0425316X</td>
<td>21.94%</td>
<td>0.47</td>
<td>0.0000</td>
</tr>
<tr>
<td>Non Pareil/Fallen petals/ 2014</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.800161 + 0.0206254X</td>
<td>5.97%</td>
<td>0.24</td>
<td>0.0071</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.747714 + 0.0393015X</td>
<td>14.44%</td>
<td>0.38</td>
<td>0.0000</td>
</tr>
<tr>
<td>Non Pareil/Pink bud/2015</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.149792 + 0.0509873X</td>
<td>33.39%</td>
<td>0.58</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.747714 + 0.0393015X</td>
<td>28.54%</td>
<td>0.53</td>
<td>0.0000</td>
</tr>
<tr>
<td>Non Pareil/Fallen petals/ 2015</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.568651 + 0.0356439X</td>
<td>24.26%</td>
<td>0.49</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.466022 + 0.0630518X</td>
<td>21.90%</td>
<td>0.47</td>
<td>0.0000</td>
</tr>
<tr>
<td>Carmel/Pink bud/ 2014</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.60614 + 0.0243367X</td>
<td>22.73%</td>
<td>0.48</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.603446 + 0.0456238X</td>
<td>11.83%</td>
<td>0.34</td>
<td>0.0001</td>
</tr>
<tr>
<td>Carmel/Fallen petals/ 2014</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.491596 + 0.0310084X</td>
<td>18.21%</td>
<td>0.43</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.558606 + 0.0522393X</td>
<td>18.10%</td>
<td>0.43</td>
<td>0.0000</td>
</tr>
<tr>
<td>Carmel/Pink bud/ 2015</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.45957 + 0.0309842X</td>
<td>23.71%</td>
<td>0.49</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.485813 + 0.05445X</td>
<td>29.05%</td>
<td>0.54</td>
<td>0.0000</td>
</tr>
<tr>
<td>Carmel/Fallen petals/ 2015</td>
<td>Dry weight</td>
<td>Length</td>
<td>Y=0.625685 + 0.02408X</td>
<td>15.10%</td>
<td>0.39</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width</td>
<td>Y=0.690498 + 0.0386145X</td>
<td>20.21%</td>
<td>0.45</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
4.5. Conclusions

The plant bioregulators Brassinolide (BL), Gibberellic Acid (GA$_3$) and X-Cyte (KN) tested on almonds in three concentrations, and applied in flowering in seasons 2014 and 2015, they improved significantly the kernel dry weight, length and width in the cvs. Non Pareil and Carmel, compared to the control.

A clear KN tendency in the high concentration (50 µL L$^{-1}$) to improve significantly kernel dry weight, length and width at harvest was evident. Likewise, BL in the intermediate concentration (30 mg L$^{-1}$) improves significantly kernel dry weight, length and width at harvest. In the case of GA$_3$, in the cv. Non Pareil, the low
concentrations showed a positive effect in the three variables in the two phenological stages in the two seasons, while there was a better response with high concentrations of this plant bioregulator in the cv. Carmel.

A positive correlation between kernel dry weight and kernel length and width was observed at harvest, which makes evident the significant effect of plant bioregulators on kernel traits.

**Acknowledgments**

To Agricola Pomes Andrade y Cia. Ltda. (Paine) for field support in the development of this research.

**References**


Chapter 5

5.1. General Discussion and Conclusions

Several factors influence almond yields, and unfavorable climate is the most relevant in flowering. This species is the deciduous fruit tree of less chilling requirements; therefore, it presents earlier flowering. Thus, pollination coincides with unfavorable conditions for development.

Almond flowers have gametophytic self-incompatibility that represent another special relevant trait of the cultivation, where a compatible cultivar should be associated to the main cultivar showing also synchronized flowering, in addition to effective action from a pollinator agent like bees.

Implementing any measure favoring an efficient and effective development of processes involved in almond flowering, will directly influence yield improvement. Highly vulnerable processes like pollination, pollen germination, growth of the pollen tube, fertilization and fruit set should be included in studies intended to improve the cultivation yield.

In Chile, there are two main cultivars of almond: Non Pareil, which cover 55% of the area planted, and Carmel, which cover 19%, with 50% compatibility with each other, and a similar floral overlapping.
Before improving processes involved in almond flowering are implemented, it is necessary to know the pollen quality used in cross pollination and flower fertilization. Several methods have been used to evaluate pollen quality in different vegetal species. *In vitro* evaluation of pollen germination through the use of fluorochromatic reaction (FCR) with fluorescein diacetate (FDA) is the most recommended method. Fluorescein diacetate is an apolar and non-fluorescent molecule penetrating pollen and releasing fluorescein (polar and fluorescent molecule) by esterases hydrolisis. If permeability membranes properties are intact, fluorescein accumulates inside of pollen and fluoresces under blue light. Therefore, FCR test evaluates pollen viability by esterases activity and membrane integrity of pollen grains.

In this thesis, pollen viability of Non Pareil and Carmel almonds from a production orchard was evaluated, using *in vitro* germination method and also the fluorochromatic reaction test (FCR). A high viability percentage of pollen were determined; it was 86.5 and 91.8% (2014) in cv. Non Pareil, and 92.3 and 93.5% (2014) in the case of cv. Carmel. High quality pollen in the two cultivars is essential, because it allows discriminating this variable from the beginning, and focusing research on processes inherent to pollination, germination, growth of the pollen tube, fertilization and fruit set.
5.1.1. Hypothesis Validation

The first hypothesis was supported by the information collected on almond pollen, as well as by the information of the kinds and traits of plant bioregulators commercially available and their effects; this let us announce that exogenous application of plant bioregulators based on brassinosteroids, gibberellins and cytokinins in vitro on almond pollen favor or stimulates pollen germination and/or pollen tube growth. Many in vitro experiments were made applying Brassinolide (BL), ProGibb 4% (GA$_3$) and X-Cyte (KN) with three concentrations, in order to validate this hypothesis. In regard to pollen germination, statistically significant differences ($p<0.0001$ and $p<0.0009$) were found in Non Pareil cultivar, in seasons 2013 and 2014, respectively. This is coherent to reports by Vogler et al. (2014) in experiments with Arabidopsis thaliana and by Bolat and Pirlak (1999) in apricot (Prunus armeniaca). In the case of in vitro cv. Carmel experiments, statistically significant differences were evident ($p<0.0001$ and $p<0.0184$) in seasons 2013 and 2014 respectively, in pollen germination. These results partially coincide with the findings by Sotomayor et al. (2012) in this cultivar.

In regard to length of the pollen tube in both cvs. Non Pareil and Carmel, there was a statistically significant effect ($p<0.0001$ in the two seasons) in the plant bioregulators treatments, improving growth and finally length of the pollen tube. Similar studies, although in other species, have been reported by Hewitt et al. (1985), Singh et al. (2002), and Wu et al. (2008). The experimental results obtained allow to the acceptance of the stated hypothesis.
The second hypothesis indicated that exogenous application of brassinosteroids, gibberellins and cytokinins during flowering of the cvs. Non Pareil and Carmel almonds increase final fruit set. All the treatments were sprayed \textit{in situ}, in order to prove this hypothesis on uniform branches of cvs. Non Pareil and Carmel almond canopies in Paine, Metropolitan Region, Chile, in two seasons. Two phenological stages of flowering were selected for treatment application (Yi \textit{et al.}, 2006): pink bud and fallen petals. The results from these experiments showed statistically significant differences ($p<0.0001$) when fruit set was evaluated, at 60 days after flowering. All the treatments surpassed the control. Some tendencies were evident in the plant bioregulators concentrations. In the case of the BL treatment, there was a better response at low concentrations; in the case of the GA$_3$ treatment, the intermediate concentration induced a better fruit set, while the KN treatment presented a better fruit set in high concentrations. In the case of GA$_3$, the results were similar to those obtained by Sotomayor (1996) and Joolka \textit{et al.} (1991). Studies improving seed set with BL have been reported, but in cultivations like rice (Thussagunpanit \textit{et al.}, 2013). The experimental evidence allows the acceptance of the stated hypothesis.

The third hypothesis indicates that exogenous application of plant bioregulators based on brassinosteroids, gibberellins and cytokinins during flowering of the cvs. Non Pareil and Carmel almonds improve kernel traits during harvest. In order to prove this hypothesis, the plant bioregulators were sprayed \textit{in situ} on uniform branches of cvs. Non Pareil and Carmel almonds in two phenological stages, pink bud and fallen petals, during seasons 2013 and 2014. The variables evaluated in
almonds (seeds) were: dry weight, length and width. The results show statistically significant differences (p<0.0001) between treatments for the three variables evaluated, in both cultivars, in the two phenological stages, and in the two seasons. All the treatments surpassed the control. A tendency outstands in the variables in the responses to the plant bioregulators and concentrations. In the case of the BL treatment, the intermediate concentration generally reaches the highest value. In the case of the GA\textsubscript{3} treatment, the lowest concentrations showed a better response in the cv. Non Pareil, while the highest concentrations had a generally better effect in all the variables in cv. Carmel. In the case of the KN treatment, a better response to the highest concentrations was observed in all the cases. Studies with plant bioregulators used in this research almonds to evaluate the variables mentioned above have not been reported so far. However, studies on other species like cotton (Zhao et al., 2015), arabidopsis (Bartrina et al., 2011), rice (Zhu et al., 2015), raps (Huang et al., 2014) and linseed (Rastogi et al., 2013) have been reported. The results obtained in this experiment allow the acceptance of the stated hypothesis.

5.1.2. Objectives Scope

The general objective stated in this thesis was satisfactorily accomplished. Statistically significant effect from plant bioregulators on pollen germination and length of the pollen tube, final fruit set, and physical traits of cvs. Non Pareil and Carmel almonds was determined after implementing different experiments. Plant
bioregulators action showed a clear tendency in relation to their concentrations in the *in vitro* experiments, as well as in field experiments.

In regard to the specific objectives established in this thesis:

1) To determine *in vitro* the influence of brassinosteroids, gibberellins and cytokinins on pollen germination and pollen tube growth in cvs. Non Pareil and Carmel almonds: The *in vitro* influence of the plant bioregulators was determined by *in vitro* experiments in these two variables; therefore, the objective was achieved.

2) To evaluate final fruit set of almonds by applying plant bioregulators based on brassinosteroids, gibberellins and cytokinins, in two phenological stages during flowering, in a cvs. Non Pareil and Carmel production orchard: The final fruit set was evaluated with applying the plant bioregulators in a production orchard in the two cultivars, achieving the stated objective.

3) To evaluate dry weight, length and width of harvested almonds under the influence of brassinosteroids, gibberellins and cytokinins applied in two phenological stages in flowering in a cvs. Non Pareil and Carmel orchard: The experiments carried out and findings led to accomplish this objective which allowed knowing the real impact of plant bioregulators on the final product.
Finally, the action of these analog natural hormones proved to be similar to the action by endogenous hormones reported by current literature. In the case of brassinosteroids, they are fundamentally involved in cell division, cell elongation and stress mitigation (Vogler et al., 2014; Wang et al., 2015; Zhu, et al., 2015). In the case of gibberellins, they have a particularly proven effect on cell elongation, cell division and action on stress (Hedden and Thomas, 2012; Gallego-Giraldo et al., 2014; Huang et al., 2014; Ding et al., 2015), and cytokinins, with a main effect on cell division, differentiation, antisenescence and stress on plants (Matsuo et al., 2012; Zalabak et al., 2013; Trivellini et al., 2015; Zhao et al., 2015). This thesis demonstrates the positive effect of bioregulators on pollen germination, length of the pollen tube (growth speed), final fruit set and kernel size and dry weight.

5.2. General conclusions

a) In this research, pollen used from cvs. Non Pareil and Carmel almonds, may be considered a high-quality-pollen, after the viability tests were carried out.


c) Brassinolide, gibberellic acid and kinetin solutions sprayed on almond branches in two flowering phenological stages improve the final fruit set of the cvs. Non Pareil and Carmel.
d) Kernel characteristics like dry weight, length and width were increased after sprayings of brassinolide, gibberellic acid and kinetin in flowering on cvs. Non Pareil and Carmel almonds.

References


