Physiological process involved in the flowering habit of ‘Chilean white strawberry’ (*F. chiloensis* subsp. *chiloensis* f. *chiloensis*)

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2020

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Physiological process involved in the flowering habit of 'Chilean white strawberry' (*F. chiloensis* subsp. *chiloensis* f. *chiloensis*)

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Thesis
to obtain the degree of

Doctor
en Ciencias de la Agricultura

Santiago, Chile, June 2020

Thesis presented as part of the requirements for the degree of Doctor in Ciencias de la Agricultura, approved by the

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Santiago, June 2020

Dedication

A todos los agricultores que cultivan con perseverancia y dedicación la frutilla blanca chilena.

Acknowledgements

This work was supported by the Vicerrectoría de Investigación de la Pontificia Universidad Católica de Chile VRI (doctoral fellowship, 2014), the Comisión Nacional de Investigación y Tecnología CONICYT (doctoral fellowship N°21151592/2015) and the Goodberry Project (European Union's Horizon 2020 research and innovation programme; Grant Agreement N° 679303).

Acknowledgements

En este camino, agradezco la compañía y amor de Claudio, mi compañero de vida quien ha estado a mi lado en cada desafío que me propongo. A nuestros hijos, Luciano y Franco, quienes han nacido y crecido durante este periodo. Sin duda, estos hombres fueron la mayor fuente de energía, amor, locura y desconexión que necesité para llegar al fin de este proceso.

Agradezco especialmente mi madre, Miryam, quien me enseñó desde niña lo importante del desarrollo profesional de una mujer. A mi padre, Patricio, de quien no solo heredé genética, sino que también el amor a nuestra profesión y el rol social que debemos desarrollar a través de ella. A mis hermanas Natalia, Francisca y Camila por el constante apoyo y ánimo.

A Marina, mi profesora guía, quien me ha transmitido sus conocimientos y pasión por la frutilla desde hace más de 10 años. Por darme la libertad, y con ello la confianza, de pensar y desarrollar esta investigacion. La autonomía y temple adquirido son fruto de su dirección.

A Elida y Soledad, colegas, socias pero principalmente amigas, a quienes agradezco sinceramente toda la ayuda que me brindaron, tanto con profesional como emocional. También agradezco a todos los que integran o integraron el equipo de trabajo en la Universidad, ya sea en el laboratorio como en campo.

A todos los profesores integrantes del comité de tesis, que en cada reunión aportaron precisos consejos que mejoraron esta investigación. Adicionalmente, agradezco al profesor José Antonio Alcalde, quien también ha contribuido a mi formación durante este periodo.

A todos, muchas gracias

Javiera

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Chapter 1

General Introduction

The *Fragaria* genus belongs to the *Rosaceae* family and is made up of about 20 species widely distributed in temperate, cold and subtropical climates (Staudt, 1999; Potter *et al*., 2000; Rousseau-Gueutin *et al*., 2009; Liston *et al.*, 2014). These species are grouped according to chromosome number in diploid, tetraploid, hexaploid, octoploid and decaploid species (Staudt, 1999; Potter *et al*., 2000; Rousseau-Gueutin *et al*., 2009).

Fragaria chiloensis (L.) Duch is an octoploid species that grows under different climatic conditions. It is geographically distributed along the American Pacific coast, mainly from Canada to the north (ssp. *lucida* and ssp. *pacifica*), Hawaii (ssp. *sandwicensis*) and Chile and Argentina (ssp. *chiloensis*) (Staudt, 1999; Rousseau-Gueutin *et al*., 2009; Liston *et al*., 2014).

In Chile, there are two botanical forms of the subsp. *chiloensis*: *patagonic*a and *chiloensis* form (Fig.1) (Hancock *et al.,* 1999; Staudt, 1999). The *patagonica* form grows from 35° to 47° S, from sandy coastal areas to native forest ecosystems in the Andes mountain range at 1.900 m.a.s.l. (Staudt, 1999; Gambardella and Sánchez, 2016). This plant has a small, globose and red fruit commonly known as ‘Chilean wild strawberry’ or also called ‘llahuen’, ‘lagueñe’, ‘lahueni’ or ‘lahuene’ by the Mapuches and Huilliches (Staudt, 1999; Gambardella *et al*., 2000). On the other hand, the *chiloensis* form was domesticated and cultivated from the wild form

more than a 1,000 years ago, before the arrival of the Spaniards. This last botanical form, whose fruit is pink to whitish and large, is currently known as 'Chilean white strawberry' and also called ‘quellghén’, ‘kelleghen’ or ‘quellgheñ’ by the inhabitants of southern Chile (Staudt, 1999; Hancock *et al.,* 1999; Gambardella *et al*., 2000).

Fig. 1. *Fragaria chiloensis* subsp. *chiloensis* f. *patagónica* (left) and *Fragaria chiloensis* subsp. *chiloensis* f. *chiloensis* (right).

In addition to its local value, the ‘Chilean white strawberry’ has a great historical and genetic background since it is closely related to the commercial red strawberry (*Fragaria x ananassa* Duch). The Frezier's story is widely known. In 1714 he transported plants of *F. chiloensis* f. *chiloensis* to the gardens of the King of France, which were spontaneously crossed with *Fragaria virginiana*, giving rise to the *F. x ananassa* hybrid (Darrow, 1966).

In Chile, the 'Chilean white strawberry' crop is associated with the Mapuche communities of the Nahuelbuta mountain range (38°05'S 73°14'W). The cultivation system is low-tech, on small rain-fed slopes, without fertilization, and farmers themselves produce their own plants. The crop is established in the winter to ensure soil moisture. In the spring, plants develop vegetatively until flowering, which takes place in late November. The plant normally emits only one inflorescence, which produces 5 fruits as a maximum (2 to 4 t ha^{-1}). In addition, in recent years it has been observed that flowering in the field is erratic, since only some plants bloom as in the described behavior. Low flowering and, thus, low fruit production, is one of the main limitations of the crop (Gambardella *et al*., 2000; Carrasco *et al*., 2013) and the trend is replacing it with commercial strawberry plantations, making the 'Chilean white strawberry' at risk of disappearing.

Morphology of the *Fragaria* plant

Plant morphology is well studied in *F. x ananassa* due to the connexion with the flowering habit (Bosc *et al*., 2012; Kurokura *et al*., 2005; Savini *et al*., 2005). The plant has a foliar and radical part united called "crown", a structure that botanically corresponds to the stem in a rosette state. The nodes are arranged helically in this compressed axis and separated by short internodes. Each node is made up of a trifoliate leaf and an axillary bud (Kokura *et al*., 2005; Savini *et al*., 2005; Hytönen *et al*., 2009; Heide *et al*., 2013).

Crown buds will give rise to different structures depending on their location and environmental conditions. The apical bud is responsible for the formation of new

nodes and for the elongation of the main axis. Buds located just below the apex will give rise preferably to lateral or extension crowns, while buds located in lower positions of the crown will give rise to stolons, structures which are responsible for the vegetative reproduction of the species (Kokura *et al*., 2005; Savini *et al*., 2005; Hytönen *et al*., 2009; Heide *et al*., 2013).

Under induction environmental conditions, the apical meristem differentiates to an inflorescence, stopping the growth of the main axis (Savini *et al*., 2005). Despite this growth determination, the plant continues its development through the emission of extension crowns. These new lateral shoots may give rise to terminal inflorescences and the repetition of this pattern allows for an abundant flowering during the season (Kokura *et al*., 2005; Savini *et al*., 2005; Hytönen *et al*., 2009; Heide *et al*., 2013).

Flowering habits *in Fragaria*

The flowering habits observed in different *Fragaria* species are extreme and varied. This is due in part to the complexity of its genome and ploidy levels, as well as the diversity of environments in which they develop (Taylor, 2000; Stewart and Folta, 2010). Of all the factors affecting flowering, environmental aspects have been studied the most, especially temperature, photoperiod and their interaction (Le Miére *et al*., 1996; Hancock, 1999; Durner, 2015).

Darrow and Waldo's work was one of the first to study the effect of temperature and photoperiod in *Fragaria.* They indicated the first classification regarding the

photoperiod response: ‘short day’ (SD) plants are those that form the flower when the temperature and photoperiod begin to decrease in autumn (between 8 to 12 photoperiod hours) (Darrow and Waldo, 1934).

In 1962, the work of Ito and Saito provided new knowledge. They verified that short-day plants are facultative for the photoperiod, therefore it may or not have an influence on flower induction, depending on the temperature. This flowering habit is characterized by a seasonal spring bloom, for 1 to 6 months, depending on the genotype and environmental conditions. This appears to be the natural flowering habit in most *Fragaria* species, including *F. chiloensis* f. *chiloensis* (Staudt, 1999; Serçe and Hancock, 2005; Stewart and Folta, 2010; Hummer *et al*., 2016).

Later, Bringhurst y Voth in 1980 identified the ‘day neutral’ (DN) behavior in *F. virginiana* accessions. This characteristic determines a floral induction regardless of day-length, therefore, plants can bloom continuously if the temperatures are moderate (Ahamadi *et al*., 1990).

The ‘long day’ or remontant genotypes differentiate their buds into inflorescence under a photoperiod exceeding 14 hours. However, stolons emission is practically null since both structures, inflorescences and stolons, are differentiated under the same conditions. Because flower differentiation prevails, these genotypes do not propagate vegetatively, therefore they are not a commercial alternative (Verdier, 1987).

Despite the fact that the classification previously mentioned remains in force, it has been determined that it has diffuse limits, with a response gradient ranging from

strongly SD (obliged), facultative SD, infra SD, weakly DN and strongly DN plants (Verdier, 1987). On the other hand, the terms Junebearing (JB) and Everbearing (EB) have added complexity to the definitions since both terms refer not only to the photoperiod but also to the temperature interaction. JB is generally used as SD, while EN are also referred to as 'perpetual', 'rebloomer or remontant', "cyclic flowering", "double-cropping", "multiple cropping", even DN (Carew and Battey, 2005; Serçe and Hancock, 2005).

Environmental control of flowering in short day genotypes of *Fragaria*

The plant development in *Fragaria* is closely linked to the temperature and photoperiod, which will define the type of structures to produce, the moment and the magnitude (Mouhu *et al*., 2013).

During the long and warm days of spring and summer, plants grow vegetatively, developing abundant leaves and stolons. With the arrival of autumn, temperatures and day-length decrease along with plant growth, allowing for floral induction, branching and dormancy simultaneously. In the spring, growth resumes and flowering occurs (Fig. 2).

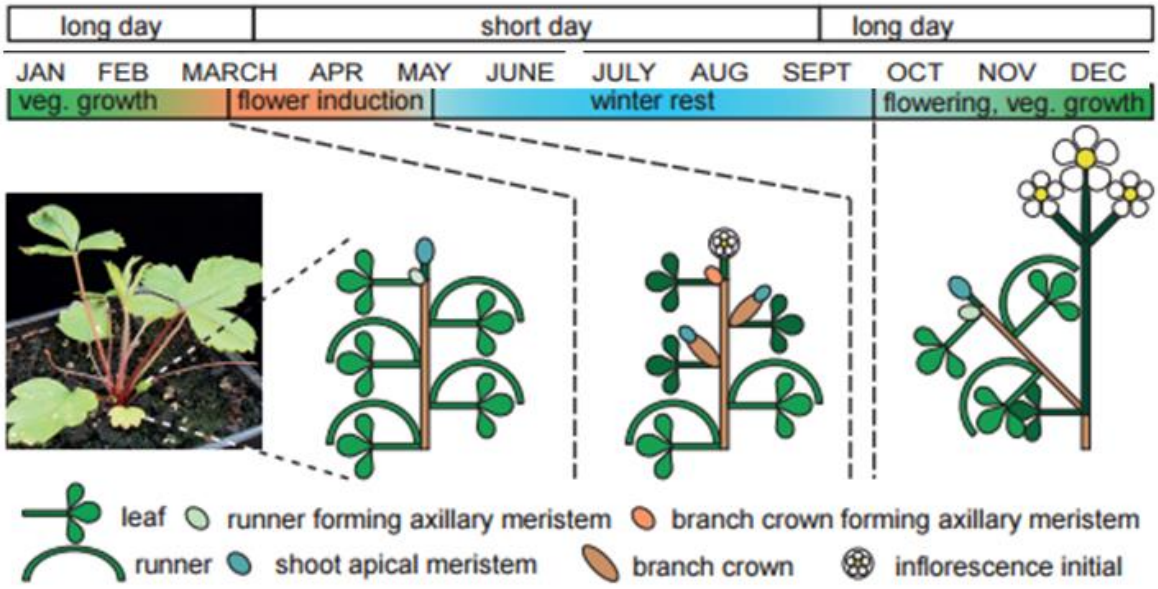

Fig. 2. Strawberry development model (Mouhu *et al*., 2013)

In floral induction, the environmental signal is captured by the leaves and transferred to the meristem, allowing for the transition from a vegetative to a reproductive state (Durner, 2015). For short-day genotypes, this is produced at intermediate temperatures (12 to 22°C) and under short-day conditions, which occur naturally in the autumn. At low temperatures (<10°C), induction occurs under any photoperiod while at warm temperatures (>25°C) the process is inhibited (Taylor *et al*., 1997; Heide *et al.*, 2013). Inductive conditions must be present for a specific period, which has been estimated to be between 3 to 5 weeks (Battey *et al.*, 1998; Sønsteby and Heide, 2006). After this exposition, floral initiation and differentiation will be irreversible (Durner, 2015). Despite this general description, each genotype has particular induction and differentiation conditions, requiring a case-by-case study.

During the autumn, and in parallel to flower induction, plants of *Fragaria* go into dormancy, an evolutionary mechanism that has allowed the species to adapt to temperate and cold climates (Carew and Battey, 2005; Atkinson *et al.,* 2013; Heide *et al*., 2013). In particular, these species go into a state of semi-dormancy, in which the plants apparently stop their growth completely while metabolism is maintained at low rates (Chouard, 1960; Heide *et al*., 2013). To overcome this physiological state, the plant must accumulate a certain amount of chilling hours depending on each genotype. Lack of winter cold will negatively affect the flowering, which will be slow, late and scarce (Koosin *et al*., 2001; Lieten, 2006), all characteristics observed in Chilean white strawberry fields.

In addition to the differentiation of the apical bud to inflorescence, short days also promote bud differentiation into lateral crowns, allowing plant branching (Savini *et al*., 2005; Hytönen *et al*., 2009; Heide *et al*., 2013). Bud differentiation occurs basipetally, therefore, in the first instance, axillary buds are insensitive to the environmental signals that trigger their differentiation, so signal perception varies along the axis (Niwa *et al*., 2013). This supposes an apical control over the growth of lateral shoots. In *F. x ananassa*, research shows that gibberellin hormone is involved in axillary bud differentiation, acting as a signal in the plant response to the photoperiod. A decrease in its content triggers differentiation to lateral crowns, while its increase favors the emission of stolons, both being opposite processes (Hytönen *et al*., 2008; Hytönen *et al*., 2009; Heide *et al*., 2013). However, there are other hormonal and non-hormonal processes that influence bud development and

plant architecture (Gomez-Roldan *et al.*, 2008; Mason *et al.*, 2014; Rameau *et al.*, 2015).

The flowering habit depends on different physiological processes that have been extensively studied in *F. x ananassa*; however *F. chiloensis* f. *chiloensis* does not have the same behavior and the most favorable environmental factors that improv flowering have not been determined.

Hypothesis

Despite to the close genetic relationship between *F. chiloensis* and *F. x ananassa*, the flowering habit observed in the 'Chilean white strawberry' does not respond to the model described for the commercial red strawberry.

Objectives

General objective

To study the main processes that affect the flowering in *F. chiloensis* subsp. *chiloensis* f. *chiloensis*.

Specific objectives

(i) To characterize the plant morphology and flowering habit of *F. chiloensis* subsp. *chiloensis* f. *chiloensis.*

(ii) To determine the environmental conditions that favor the floral induction in *F. chiloensis* subsp. *chiloensis* f. *chiloensis.*

(iii) To determine the chilling requirements for dormancy release in *F. chiloensis* subsp. *chiloensis* f. *chiloensis.*

(iv) To determine the factors that affect the branching habit in F. *chiloensis* subsp. *chiloensis* f. *chiloensis*

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Chapter 2

Induction and floral differentiation in white strawberry (*Fragaria chiloensis* subsp. *chiloensis* f. *chiloensis*)

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This chapter was published in Acta Horticulturae 1156, 433-438 (2017)

Induction and floral differentiation in white strawberry (*Fragaria chiloensis* subsp. *chiloensis* f. *chiloensis*)

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ABSTRACT

The white strawberry (Fragaria chiloensis) has great potential as an alternative product with excellent organoleptic quality. However, this species bears only one inflorescence per year, so productivity is very low (4-6 t·ha^{1}). In red strawberry (Fragaria x ananassa), the morphology, plant architecture model and flower physiology are well known, while in the case of the white strawberry these topics have been little studied and apparently it would have a different behavior. Based on this background, we carried out a phenotypic characterization of Chilean white strawberry (F. chiloensis subsp. chiloensis f. chiloensis) and Chilean wild form (F. chiloensis subsp. chiloensis f. patagonica). To evaluate induction and floral differentiation, we performed a meristematic analysis during the winter rest (June) and early spring (September). Finally, we observed the flowering under greenhouse conditions (November). In June, both accessions had differentiated only the apical meristem of the main shoot. In early September, the Chilean white strawberry maintained its initial condition, whereas the wild form showed more shoots, which were developed from axillary buds. These axillary shoots also were flower differentiated in the terminal meristem. The phenotype in greenhouse was congruent with these observations. The Chilean white strawberry only developed one shoot (crown) per plant with one inflorescence during the season, while wild strawberry developed 10-15 secondary and tertiary shoots per plant, each with its own terminal inflorescence. Results indicated that Chilean white strawberry flower differentiated only the apical meristem in inductive condition. Therefore, likely an apical dominance effect and heavy bud dormancy may limit the formation of the lateral shoots, finally determining a very low production potential, while in the Chilean wild form the apical dominance is lower and the branching capacity is higher.

Keywords: productivity, meristem, flower physiology, apical dominance.

INTRODUCTION

In Chile, *F. chiloensis* (L.) Duch grows in two botanical forms (Hancock et al., 1999; Staudt, 1999). The wild form, *F. chiloensis* ssp. *chiloensis* f. *patagonica,* grows from the mountains to the beach environments. The cultivated form, *F. chiloensis* ssp. *chiloensis* f. *chiloensis*, is known as Chilean white strawberry, producing a big and white fruit, with great flavor and fragrance. In this last species, the crop potential is low (4 to 6 $t \cdot ha^{-1}$) because the plants bloom only once in the season with a harvest interval between 3 to 4 weeks in November-December. This is the main limitation of this crop in Chile because it prevents the commercial development and makes it susceptible to being abandoned (Lopez-Aranda *et al.*, 1997; Gambardella *et al.*, 2000; Lavín and Maureira, 2000; Carrasco *et al.,* 2013).

This flowering habit contrasts with *Fragaria x ananassa* (Weston) Duchesne, the red strawberry. In this species, a model that relates the morphology and development of different structures with the plant cycle has been described (Kurokura *et al.,* 2005; Savini *et al.,* 2005; Bosc *et al.,* 2012; Mouhu *et al*., 2013). In this model, the buds disposed along to the main axis and the apex can develop into different structures according to their position and their sensitivity to environmental and growing factors (Kurokura a *et al*., 2005; Savini *et al.*, 2005; Hytönen *et al.,* 2009; Heide *et al.,* 2013). Under induction conditions, the terminal bud originates an inflorescence and the bud immediately below takes the new terminal position and continues the plant growth. If the conditions are favorable, this new terminal bud can again generate a shoot with terminal inflorescence in a reiteration pattern. Consequently, it is possible to obtain different flowering cycles from the same plant with a productivity between 40 and 80 $t \cdot ha^{-1}$.

Under specific photoperiod and temperature conditions, the induction and differentiation process occurs and the meristem changes in shape. Using a stereomicroscope, a detailed analysis of meristem fate can be carried out to describe the architecture and morphology of the strawberry plant. The bud analysis technique is therefore an important tool to investigate the induction and floral differentiation process and how specific conditions affect flowering of a certain genotype (Savini *et al.,* 2005; Durner, 2015).

The flowering physiology and the environmental control of flower induction in the Chilean white strawberry has been scarcely investigated (Darrow and Waldo, 1934; Sønsteby and Heide, 2009). Since the low crop potential may be attributed to different origins (low flower induction, low branching ability, low flower differentiation, low flower fertility), knowing the plant architecture and the effect of environmental factors on structures development is necessary to manage flowering towards greater fruit production. Based on this background, the objective of this study was to describe the plant architecture of the two botanical forms of *F. chiloensis* subsp. *chiloensis* at meristematic level and phenotypic expression under natural growing conditions in Chile, in order to characterize their flower differentiation pattern.

MATERIALS AND METHODS

In June 2014, 30 plants of each of the two botanical forms were collected in the locality of Purén (38° 01´ S; 184 m altitude; 12.5°C mean annual temperature; 1149 mm mean annual precipitation) and were stored in a cold chamber at 2 °C. In July, 20 of these plants were planted in pots filled with peat and perlite substrate (2:1 ratio) under greenhouse conditions in Santiago (10-15°C mean temperature); the other plants were maintained in the cold chamber. In early September, 10 cold stored plants and 10 growing plants were sampled to perform meristematic analysis, in order to evaluate the floral differentiation state in winter rest and early spring under natural conditions. Plants were dissected and the following parameters were recorded: number of nodes in the main crown, number and position of stolons, number and position of flower buds, number and position of lateral shoots and number of flowers per inflorescence. Plant diameters were measured at the base of the crown. All the buds were excised and examined under a stereomicroscope (63x magnification). The developmental stage at apical meristem was recorded according to the numerical scale proposed by Jahn and Dana (1970) and modified by Savini (2003) and Savini *et al.* (2005).

In late December, 10 plants of each accession maintained in the greenhouse were described phenotypically when the flowering occurred. The observed morphological parameters (growth habit, foliage density, plant vigor; colour, brightness and pubescence of the leaves and stolons; flowers per plant and per inflorescence, petal color) were selected according to the UPOV guideline of this species. Additionally, phenological and productive data (beginning and end of flowering, beginning and end fruiting, fruits per plant and fruit weight) were recorded.

RESULTS AND DISCUSSION

In June, the cultivated white strawberry plants had an average of 13.5 nodes on the main axis and the size at basal diameter was 8.4 mm. Most buds in each node were in vegetative state (11.4). Plants have 1.3 stolons on average. Only 4 of 10 plants had differentiated terminal bud into floral structure, and none showed differentiated side buds. Differentiated plants had in average a terminal inflorescence with 5 flowers and the differentiation stage in primary flower was 4.25. This characterization corresponded to the natural state of the plants in winter rest and indicated that floral initiation happened.

After two months of growth, the Chilean white strawberry maintained a similar state in plant architecture, with the same number of nodes on the main axis and number of stolon. In this moment, 7 of 10 plants had differentiated the apex into inflorescence, which had a differentiation stage of 5.1. Only 4 out of 10 plants developed 1 lateral shoot. Figure 1 shows the meristematic characterization in cultivated form.

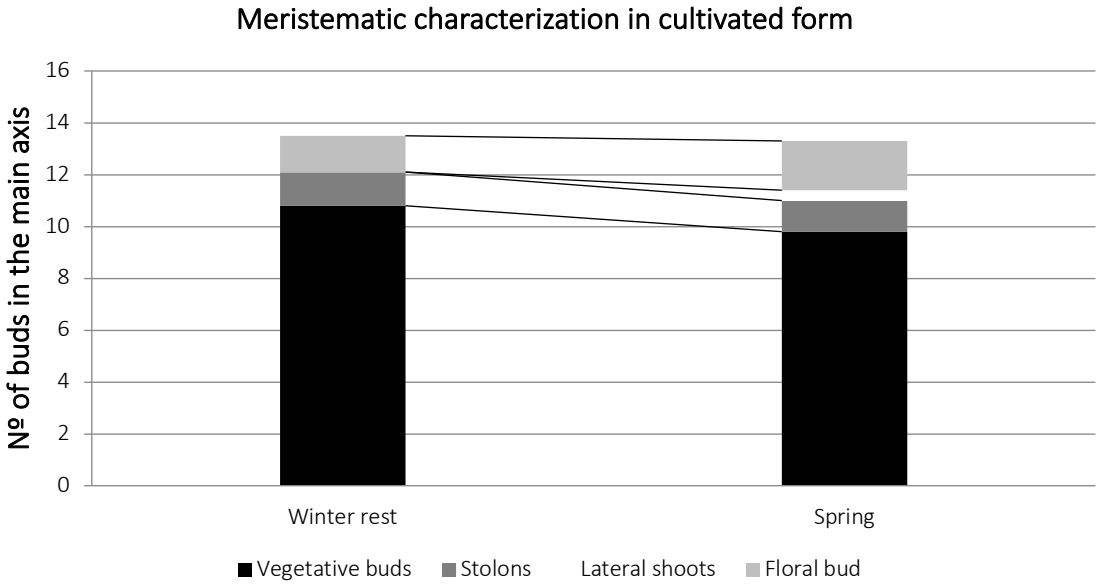

Figure 1. Meristematic characterization Chilean white strawberry. Values are an average of 10 plants.

At flowering plants showed a medium to high vigor, bright green leaves, low brightness and high pubescence in all vegetative structures. They showed low stolon emission (0.9 per plant) and there was no growth of secondary crowns. Only 4 plants bloomed in mid-October and developed solely 1 inflorescence with 5 hermaphrodite flowers as an average, medium to big size and white petals (Figure 2). On average, this plants presented 3.5 fruits of 12.9 grams of white-pink color.

Figure 2. Phenotypic characterization in Chilean white strawberry

Figure 3 shows the meristematic characterization of *F. chiloensis* in its wild form. In June, the size of the plants at basal diameter was 6.5 mm and their main crown had 9.2 nodes as an average. Buds of these nodes were in vegetative state (6) and as stolon (2.2). In this case, 7 out of 10 plants had differentiated the apical

meristem into flower bud. Secondary crowns were not detected. Plant with terminal bud differentiated reached an average stage 5, with 4.4 flowers per inflorescence.

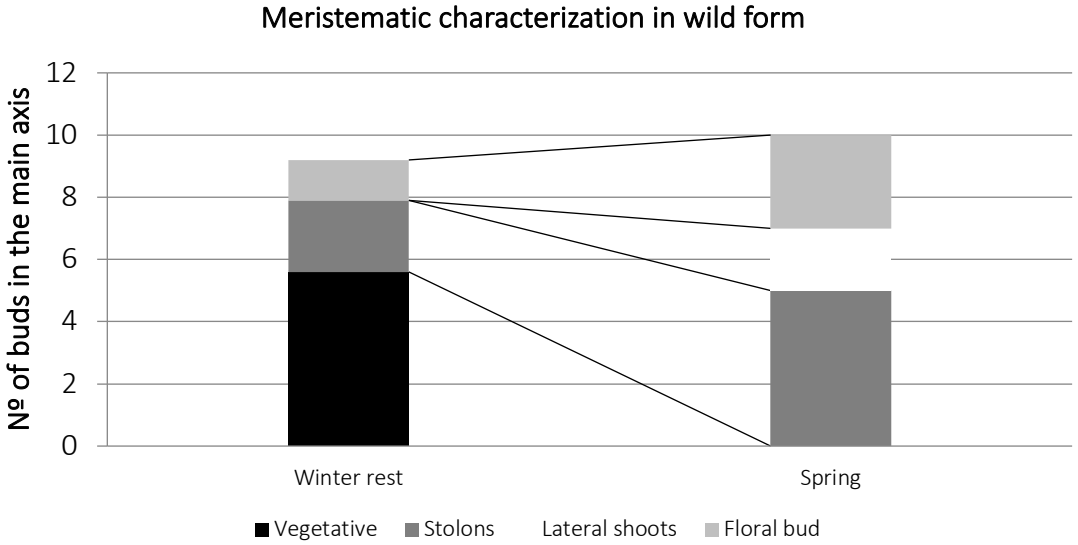

Figure 3.Meristematic characterization in Chilean wild form. Values are an average of 10 plants.

Results of the meristematic analysis after growing under greenhouse conditions are observed in the right side of Figure 3. As in the Chilean white strawberry, plants maintained the number of nodes in the main axis (9.8) since the growth was arrested in all the plants with the differentiation of terminal inflorescence (where 7.5 flowers were formed). In this genotype, the differentiation into plant structures was different. 8 out of 10 plants developed secondary shoots (in average 2 per plant), some of which started to differentiate their own terminal inflorescence. The differentiation stage of terminal inflorescence increased up to 5-6.

In the phenotypic characterization, the wild form demonstrates their vegetative attitude with a higher number of stolons (5.1), lateral shoots (9) and inflorescences per plant (4) characterized wild form (Figure 4). Flowering began in mid-September, one month before the Chilean white strawberry.

Figure 4.Phenotypic characterization in wild form.

In June, both forms only differentiated the terminal bud. In September, the Chilean white strawberry maintained their initial condition where the apical bud was the only inflorescence developed in the season and the extension crown did not develop, whereas Chilean wild strawberry had a longer differentiation period, forming 2-4 lateral shoots and their inflorescences, in addition to the apical bud of the main axis. Further, each of the side shoots generated other 2-3 lateral shoots with their own inflorescences. According to the model reported for red strawberry (Kurokura *et al.,* 2005; Savini *et al.,* 2005; Bosc *et al.,* 2012), the Chilean wild strawberry showed plant structure and developmental model similar to those described in *F. x ananassa*.

In accordance with other studies (Taylor *et al.,* 1997; Savini *et al.,* 2005; Bosc *et al.,* 2012), this study confirmed that there is a close relationship between meristematic characterization and the final phenotype observed in the greenhouse, for both forms of *F. chiloensis* subsp. *chiloensis.*

CONCLUSIONS

The wild form developed smaller plants compared to the cultivated form but showed a higher capacity to develop side inflorescences, useful to extend the fruiting season. While the inductive stimulus was present, differentiation along the main axis was not the same in both accessions, suggesting that in the Chilean white strawberry terminal inflorescence (apical meristem) would control the differentiation in lateral buds located below in the main axis. These results shows that under the same environmental conditions, botanical forms of *F. chiloensis* differ in the physiological response of flowering, suggesting a strong genotypic control in the developmental model.

ACKNOWLEDGEMENTS

The authors want to thank to CONICYT for the Doctoral Fellowship.

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Chapter 3

Floral induction and dormancy behaviour in 'Chilean white strawberry' (*Fragaria chiloensis* (L.) Mill. subsp. *chiloensis* f. *chiloensis*)

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This chapter was accepted in *Scientia Horticulturae*

Floral induction and dormancy behavior in 'Chilean white strawberry'

(*Fragaria chiloensis* (L.) Mill. subsp. *chiloensis* f. *chiloensis*)

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Abstract

The flowering process in *Fragaria x ananassa* has been widely studied, while the favourable environmental conditions for *Fragaria chiloensis* subsp. *chiloensis* f. *chiloensis,* the ''Chilean white strawberry', are not well understood. With the objective to study the floral induction and dormancy process in this species, several experiments were conducted from 2016 to 2019 using the ecotype 'Contulmo' for 'Chilean white strawberry' and the cv. 'Camarosa' for *F. x ananassa*, the latter as a reference genotype. Growth parameters and the differentiation state of apex were evaluated at two locations in Chile (33°38' S and 38°45' S) as well as in climate-controlled growth chamber (9°C and 8-hour photoperiod). Additionally, the chilling requirement for dormancy release after different cold storage periods (2°C in darkness) was determined in this species. This requirement was field-verified (38°45' S) over two seasons. The 'Chilean white strawberry' showed

continuous and reduced growth, without changes under different temperature and photoperiod conditions, with values significantly different from those of ‘Camarosa’. In both locations, the floral and dormancy induction started late (June) and occurred only in a part of the plants (20-30%) and all of them yielded a single inflorescence. In addition, it was determined that the ‘Chilean white strawberry’ require approximately 900-1,000 chilling units for dormancy release. Our results indicated that flowering is favoured only with low temperatures, lower than those necessary for many short-day genotypes. This characteristic, together with the late flowering, suggests that there is a vernalization requirement for this species. Temperature increase is probably the main factor involved in the current floral behaviour of ‘Chilean white strawberry’, however this can decreasing even more in a climate change context.

Keywords: *Fragaria*; chilling requirement; flowering; climate change; vernalization

Highlights

- Despite its historical and genetic relevance, there is little knowledge about the physiological processes related to flowering in *F. chiloensis* subsp. *chiloensis* f. *chiloensis,* the 'Chilean white strawberry'.
- This species has a completely different floral behavior compared to others seasonal flowering genotypes of *F. x ananassa*.
- Floral induction is favoured only at low temperatures (lower than 9ºC) and need approximately 900-1,000 chilling units for dormancy release.

- To prevent its disappearance it is necessary to improve the flowering, so possible vernalization requirements must be studied.

1. Introduction

The origin of the cultivated red strawberry, *Fragaria* × *ananassa* Duchesne ex Rozier, is linked to the native Chilean species, *Fragaria chiloensis* (L.) Mill., one of the cultivated red strawberry's parents (Darow, 1966).

Staudt (1999) described two botanical forms of *F. chiloensis* subsp. *chiloensis*. The *patagonica* form, which has small red fruit and grows wild over a wide range of ecosystems in southern Chile and Argentina. The second is the *chiloensis* form that corresponds to the cultivated **'Chilean white strawberry'**, which has large, soft pinkish-white fruit and a pleasant aroma and flavour.

The cultivation of the 'Chilean white strawberry' is strongly linked to the Mapuche that inhabiting the Nahuelbuta mountain area (38°02'S 73°14'W), since it was domesticated from the wild form before Spanish arrival (Finn *et al.*, 2013; Staudt, 1999). Many centuries ago, the Andean aborigines already used the white strawberry fruit as a food and medicine (Morales-Quintana and Ramos, 2018). Now, in the last decades, the potential effects on human health, thanks to antioxidant and anti-inflammatory action and bioactive compounds (flavonoids, anthocyanins and phenolic acids), in addition to nutritional content (sugar, minerals, vitamin C) have been proven in strawberry fruit (Giampieri *et al*., 2016).

Despite its genetic, cultural and historical importance, the cultivated area has gradually decreased because it flowers very little, with few crowns, inflorescences and flowers, and consequently it has very low yield (between 3 and 6 t ha $^{-1}$) (Carrasco *et al*., 2013; Gambardella *et al.*, 2017; Grez *et al*., 2017). The tendency to replace old cultivars whit new more productive (Mezzetti *et al*., 2018), has been observed also in the "Chilean white strawberry" crop with commercial red strawberry, leaving this crop in a highly vulnerable situation.

For seasonal flowering genotypes of *F. x ananassa*, the plant growth decrease with the arrival of autumn. Under moderate temperatures (12 to 20°C) together short days (< 14 hours), an inflorescence is formed in the terminal position in the apex. Additionally, an independent floral induction pathway has been verified in a large part of these genotypes, and this induction occurs at low temperatures (< 10°C) regardless of the photoperiod conditions (Taylor *et al.,* 1997; Heide *et al*., 2013). The study conducted in *F. chiloensis* subsp. *chiloensis* f. *chiloensis* by Serçe and Hancock (2005) indicated that the floral induction occurred in the apex under short days (< 11 hours) at 18°C. Under natural growing conditions in Chile, the floral induction occurred also in the apex, but only in 40% of the analysed plants (Grez *et al*., 2017). In this report, the moment and condition for floral induction are unknown. The scarce available information does not allow us to identify the most favourable conditions for the formation of apical inflorescences.

Parallel to floral induction the buds enters a state of dormancy, an evolutionary mechanism that has helped the species adapt to temperate and cold climates

(Carew and Battey, 2005; Sønsteby and Heide, 2006; Atkinson *et al*., 2013). Unlike other *Rosaceae*, these species have a semi-dormant state (Atkinson *et al*., 2013; Heide *et al*., 2013; Kurokura *et al*., 2013; Stewart and Folta, 2010), maintaining a minimum amount of foliage during the winter. The entry of dormancy is verified by a decreasing production of leaves during the autumn, being smaller and with shorter petioles. Finally, vegetative growth apparently stops completely, although the apex can continue its floral differentiation (Chouard, 1960; Heide *et al*., 2013; Konsin *et al*., 2001; Lang, 1965; Sønsteby and Heide, 2006). To overcome this state, the buds must accumulate a certain amount of chilling hours that varies according to the genotype (end of endodormancy). Subsequently, with higher temperatures and increasing day lengths, the buds sprout and the plant returns to vegetative growth (end of ecodormancy) (Atkinson *et al*., 2013; Chouard, 1960; Lang, 1965). Due to its semi-dormancy, this plant has the ability to resume its vegetative activity under higher temperatures and day lengths, despite not having completed its chilling requirements (Heide *et al*., 2013). However, when this occurs, the vegetative growth, and consequently the flowering is also affected (Atkinson *et al*., 2013; Koosin *et al*., 2001; Lieten, 2006; Sønsteby and Heide, 2006).

Floral induction and dormancy are well-studied processes in *F. x ananassa*, but the floral behaviour of *F. chiloensis f. chiloensis*, one of its parents, the 'Chilean white strawberry' has not been fully studied. On the other hand, in a climate change context with temperature increases, it is expected that both processes will be negatively affected in regions with a temperate Mediterranean climate, which will

increase productive and economic problems in this species (Atkinson *et al.*, 2013; Uleberg *et al*., 2016). The objective of this study was to describe the floral induction and dormancy processes in the 'cultivated white strawberry' under natural and controlled conditions as well as to determine the chilling requirements for bud break.

2. Materials and Methods

2.1. Plant material

Between 2016 and 2019, three experiments were performed, using the 'Contulmo' ecotype of 'Chilean white strawberry', collected from productive fields in the Nahuelbuta area (38°05'S 73°14'W). In all the experiments, the cv. 'Camarosa' of *F. x ananassa* were used as a reference.

2.2. Floral induction and onset of dormancy under natural environmental conditions.

This study was performed in two different environments, the Central Coast (Santo Domingo, 33°38'S 71°36'W) and the Southern Zone (Temuco, 38°38'S 72°46'W) of Chile. In both locations, frigo plants of two genotypes were field established using the traditional cultivation system (plants each 30 cm in double rows on ridges spaced at 1.2 m, with plastic mulch and drip irrigation). The planting was performed on December 14, 2016 in Temuco and on December 28 in Santo Domingo. The fertilization and irrigation were adjusted at each location to maintain similar nutritional and water conditions. At each location, the experimental design was a

randomized complete block design, with three replicates of 50 plants for each genotype.

Starting on February 15, 2017, samples were taken directly from the field every 10 days, with two plants per repetition. To evaluate the vegetative growth, the petiole length (cm) and the foliar area (cm^2) of the central and left leaflet of the last fully expanded leaf were recorded on each sampling date. The leaf area was determined using ImageJ (ImageJ, US National Institutes of Health, Bethesda, Maryland, USA). The apical bud of the sampled plants was observed under a stereoscopic magnifying glass (63x magnification), recording the number of leaf primordia and the state of floral differentiation of the apex, the latter as an indicator of floral induction on a scale of 0 to 8 (0 = vegetative stage; 1 to 8 = reproductive stages) (Van Delm *et al*., 2009). Finally, during the following spring, the flowering date, the number of plants in flowering, the number of inflorescences per plant and the fruit yield (g $plant^{-1}$) until December 2017 were recorded.

The Fig. 1 show the temperature and photoperiod conditions at both locations throughout the evaluation period.

2.3. Floral induction and onset of dormancy under controlled conditions.

Similar to the experiment described above, both processes were analysed in a growth chamber. For greater homogeneity during the experiment, plants of both genotypes were in vitro propagated and rooted in 200 ml seedling trays using a 2:1 mixture (peat and coconut fibre) with 1 g l^{-1} of slow-release fertilizer (Basacote

6M®). During acclimatization, the plants were maintained in a growth chamber with 16 hours of light and temperatures of 24 ± 1°C. After two months (March 15), the plants were transferred to a growth chamber with a short-day photoperiod (8 hours) and a low temperature (9 ± 1°C). Four treatments were considered, 0, 4, 8 and 12 weeks, corresponding to the period under these conditions. The experimental design for each genotype was a randomized complete block, with three replicates of five plants for each treatment.

At the end of each treatment, the petiole length of the last fully expanded leaf (cm) was recorded. The apex was extirpated and analysed under a magnifying glass (63x magnification) to record the number of leaf primordia and the state of floral differentiation according to the scale indicated above.

2.4. Determination of chilling requirements for dormancy release.

In this experiment, the chilling requirements of the 'Chilean white strawberry' were determined. The cv. 'Camarosa' was included, and Bigey (2002) previously determined its requirements (< 700 hours). Twenty-five plants from both genotypes were propagated in a commercial nursery in 1 l containers using peat and coconut fibre substrate (2:1) and 1 g l^{-1} of slow-release fertilizer (Basacote 6M®). These plants remained in the field until May 31, when theoretically they were in complete dormancy (Sønsteby and Heide, 2006). On June 1, 20 plants from each genotype were stored in a cold chamber at 2°C in the dark. Every 10 days and on four times, five 'Chilean white strawberry' and five 'Camarosa' plants were taken and transferred to a greenhouse with a long photoperiod (16 hours) and growing

temperatures (22 ± 2°C). Five plants of each genotype were left directly in the greenhouse as a control, without cold treatment. To determine the chilling received during each treatments, the following model proposed by Lieten (2006) was used: chilling unit (CU) = 0.9827 + 0.024699*T – 0.00888*T^2 (R^2 = 0.88). Under this scheme, the plants received 195 CUs (cold in field between May 1 and 31, without artificial cold), 434 CUs (cold in field + 10 days in artificial cold), 673 CUs (cold in field + 20 days in artificial cold), 913 CUs (cold in field + 30 days in artificial cold) and 1152 CU (cold in field + 40 days in artificial cold). For each genotype, the experimental design was completely randomized, with five repetitions of one plant per treatment.

During the greenhouse period, every 10 days for 2 months, the number of leaves and plant height were recorded, the latter from the insertion of the leaf in the crown to the upper edge of the tallest leaf (cm). At the end of this period, the petiole length of the last fully expanded leaf (cm) and the length of the flower truss (cm) were recorded. For each treatment, the percentage of plants that flowered was recorded.

Additionally, during the 2017 and 2018 seasons, the dormancy release was evaluated in ‘Chilean white strawberry’ and cv. ‘Camarosa’ in the Temuco field, experiment previously described. Dates of bud break and flowering were recorded as Julian days until 50% of the plants from each replication had their first fully expanded leaf and the first open flower, respectively.

2.5. Data analysis

Data of floral induction and onset of dormancy under natural environmental conditions were analysed by ANOVA, being the genotype (G) and locality (L) the factors. For the other two experiments, each genotype was analysed independently by ANOVA and the means were separated by Tukey's test (5% significance level). In non-normal data cases, the nonparametric Kruskal-Wallis test was performed, using a 5% significance level. STATISTIX 8 (Thomas and Maurice, 2009) and GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) software were used for all the analyses.

3. Results

3.1 Floral induction and onset of dormancy

Due to floral induction and dormancy are both verified with growth arrest, related parameters were evaluated. Fig. 2 shows the results of the petiole length and the leaf area. As observed in both graphs, the response was markedly different between genotypes ($P \leq 0.01$) on all evaluation dates for both parameters. 'Camarosa' showed an increase in growth until end of the summer, followed by a decrease from March 10 in Santo Domingo and March 20 in Temuco. In Temuco, the increase and decrease variations in both parameters were in a greater magnitude than in Santo Domingo. In 'Chilean white strawberry', however, the lengths of the petiole and the leaf area were low throughout the season, similar in both locations and stable compared to cv. 'Camarosa' (Fig. 2).

From March 20 on, a significant effect was also verified for the locality (L) and the interaction with the genotype (G x L), the significance values of which varied between $0.001 \leq P \leq 0.05$ until the end of the evaluations.

The results of the apex analysis are shown in Fig. 3. The production of new leaves was similar between genotypes (G) and locations (L) until March 30, all with an average of two leaves on formation. From this date on, only the genotype (G) had a highly significant effect ($P \leq 0.001$). In cv. 'Camarosa', there was a decrease in the number of leaf primordia at both locations, which stopped completely at the beginning of May. The 'Chilean white strawberry' plants continued to produce leaves.

Regarding floral differentiation, the results of the factorial analysis indicated a significant interaction between the locality and the genotype ($P \leq 0.01$) on March 30, the date at which floral differentiation had already occurred in cv. 'Camarosa' (Fig. 3). For this genotype, the differentiation of the apical meristem occurred in all plants evaluated in each location. From this date on, only the genotype (G) effect was significant ($P \leq 0.001$). At the beginning of May, all plants of cv. 'Camarosa' were in the maximum state of floral differentiation according to scale used. The temperatures present in May in both locations allowed the flowering of most of the differentiated plants of cv. 'Camarosa', inflorescences that were removed.

Since in May 9 the 'Chilean white strawberry' plants did not have their apex in reproductive state (Fig. 3), the evaluations were extended until end of June only for

this genotype. As observed in Fig. 4, the ‘Chilean white strawberry’ responds to shorter days and lower temperatures for the evaluated growth parameters, but in less intensity and after than cv. ‘Camarosa’. The leaf area was the most sensitive parameter to environmental changes since the reduction in leaf size occurs from March 10 onward in both locations, with variations between 30 and 15 cm^2. Subsequently the petiole length and the production of new leaves decreased from May 23 onward. Finally, one month later (June 27), the first signs of floral differentiation were verified, occurring in only 30% of the analysed plants. At the end of the evaluations, there were no significant differences between the locations for the analysed parameters.

During the following spring, flowering occurred in both genotypes. In cv. ‘Camarosa’, the new inflorescences were originated from the lateral crowns since the inflorescence of the apex of the main crown had already developed. Flowering occurred first in Santo Domingo (August 12) and then in Temuco (August 26). At both locations, flowering was continuous in all plants until December, as well as fruiting (Table 1). In the ‘Chilean white strawberry’, the flowering started late in Temuco (October 13). Similar to the floral differentiation results, flowering only occurred in 30% of the plants of each repetition. In Santo Domingo, flowering began a little before (September 29), but only in 16% of the evaluated plants (Fig. 4). At both locations, all the flowering plants produced a single inflorescence, without new flowering periods, which resulted in low yields (Table 1).

As in the field study, the floral growth and differentiation under controlled conditions showed differences between both genotypes (Fig. 5). For cv. ‘Camarosa’, 4 weeks at 9 ± 1°C and a short photoperiod of 8 hours were enough to generate a significant decrease in the petiole length of the last expanded leaf ($P < 0.05$), along with a complete arrest of the production of new leaves ($P < 0.0001$). Simultaneously, floral differentiation occurred in all the evaluated plants, which reached stage 7 of the scale at the end of the first treatment. In the case of ‘Chilean white strawberry’, the length of the petiole showed a reduction, although there were no significant differences between treatments until 12 weeks (Fig. 5). The temperature and photoperiod conditions did not modify the vegetative state of the apex in ‘Chilean white strawberry’, which continued to produce leaves (Fig. 5).

3.2 Chilling requirements in cultivated white strawberry

To determine the chilling requirements for the dormancy release in ‘Chilean white strawberry’, the vegetative response under forced growth conditions after different cold treatments were considered. Fig. 6 shows the plant heights and numbers of leaves for each treatments. For the plant height, after 30 days in the greenhouse, cold treatments produced plants of greater size. Plants of control treatment maintained their heights until the end of the evaluation. After 60 days of forced in greenhouse, it was observed that the growth was saturated with 913 CU since the treatments of 913 and 1152 CU were similar to each other but significantly different from the rest ($P <0.05$). The accumulation of artificial chilling had a positive effect on the production of new leaves, although in this case, changes can be observed

from the beginning of greenhouse evaluations (Fig. 6). Plants that received 913 CU produced more leaves, although without significant differences to other treatments.

At 60 days of growth in the greenhouse, a final evaluation on the vegetative state of the plants was performed by treatment (Table 2). The petiole length of the last expanded leaf was significantly affected by the chilling treatments. Plants without artificial chilling had leaves with short petioles, condition maintained throughout the growth period. The 673, 913 and 1152 CU treatments presented plants with greater petioles length, significantly different from control treatment ($P < 0.01$, $P < 0.001$ and $P < 0.05$, respectively). Plants that received 913 CU produced longer flower trusses, although without significant differences from the rest of treatments.

Similar to the other results presented in this study, flowering in 'Chilean white strawberry' was partial in all treatments. In the control treatment, only 20% of the plants flowered, while in the rest of the treatments it was 40%.

Fig. 7 shows the visual appearance of the 'Chilean white strawberry' plants for each treatment, after their greenhouse evaluation. Plants without artificial chilling treatment were dwarfed and had few leaves, while plants with additional chilling in the cold chamber had greater growth. Although visually the plants with 434, 673, 913 and 1152 CU were visually similar, it was possible to detect differences for some parameters, being the 913 CU treatment the best vegetative response (Fig. 6, Table 2).

In cv. ‘Camarosa’, the 673 CU treatment produced the best response in terms of vegetative growth, evaluated as plant height, number of leaves, petiole length and length of the flower truss. In this genotype, only the control treatment had incomplete flowering at 80%, while in the rest of the treatments, 100% of the plants flowered.

Additionally, the dormancy release of cv. ‘Camarosa’ and ‘Chilean white strawberry’ was verified in plants established in the Temuco field. In cv. ‘Camarosa’, the bud break in the spring occurred on August 15 and 17 (2017 and 2018, respectively). Then, flowering began only a few days later, on August 27 and 23 (2017 and 2018, respectively). In this genotype, all the plants bloomed during both seasons (Table 3). In the case of ‘Chilean white strawberry’, the bud break occurred after than cv. ‘Camarosa’, on September 1, 2017 and August 28, 2018. However, flowering was very late and in low proportion during both seasons. Flowering occurred on October 15 and in 30% of the plants in 2017, while in 2018, flowering began on October 24 in 40% of the plants.

4. Discussion

In the field experiment, floral differentiation in cv. ‘Camarosa’ started at the end of March, which indicates that flower induction took place during the previous 3 to 5 weeks (Sønsteby *et al.*, 2006). During this period, the mean temperatures varied between 20 and 12°C with photoperiods of 13.5 to 12.5 hours in Santo Domingo; and 15 and 12°C with photoperiods of 14 to 12.5 hours in Temuco (Fig. 1). In the

experiment conducted under controlled conditions, it was verified that this genotype was also able to induce and differentiate under low temperatures (9 ± 1 ° C) and short photoperiod (8 hours) in a period ≤ at 4 weeks (Fig. 5). The results of both experiments coincide with the reports for seasonal flowering genotypes, in which floral induction can occur in a wide range of temperatures, which play their role above the photoperiod (Heide *et al*., 2013; Ito and Saito, 1962; Massetani *et al*., 2011; Taylor *et al*., 1997).

The process of floral induction in *F. chiloensis* is less known, even more so in *F. chiloensis f. chiloensis.* There is a consensus that this is a spring seasonal flowering genotype (Hummer *et al*., 2016; Serçe and Hancock, 2005; Staudt, 1999), indicated as scarce in literature reported (Grez *et al*., 2017; Hummer *et al*., 2016; Serçe and Hancock, 2005). The results of the field experiment indicated that unlike the cv. 'Camarosa', the floral induction in 'Chilean white strawberry' does not occur in autumn under decreasing temperatures and photoperiods conditions. The temperatures present one month before floral initiation (during June) were the lowest during the season, with mean and minimum temperatures between 13 to 1°C (Santo Domingo) and 12 to -3°C (Temuco), along with a photoperiod near to 11 hours (Fig. 1). Sønsteby and Heide (2009) reported that the floral induction in 'Chile' population, an ecotype of the wild botanical form of *F. chiloensis* subsp. *chiloensis* from Chile (45º S), occurred at a considerably lower temperature than other two *F. chiloensis* populations from the Northern Hemisphere. The results of this study, together with the literature reports, suggest that the floral induction in Chilean populations of *F. chiloensis* (wild and cultivated forms) occurs with cold

temperatures, lower than other *Fragaria* species, even genotypes of the same species that inhabit higher latitudes. This is consistent with the findings of Heide and Sønsteby (2007), who indicated that high-latitude genotypes do not necessarily require lower temperatures than low-latitude genotypes for floral induction.

For *F. chiloensis* f. *patagonica*, a temperature of 9°C and a photoperiod of 8 hours for 5 weeks were the most favourable inductive conditions among those evaluated (Sønsteby and Heide, 2009). Our results indicated that these same conditions, present for up to 12 weeks, did not produce the induction stimulus in *F. chiloensis* f. *chiloensis* (Fig. 5). It is possible that the plant size was not adequate. However, a similar result to obtained in this study was observed in 'Alta' population of *F. vesca*, in which the floral induction also didn't occur at 9°C with short days of 8 hours (Heide and Sønsteby, 2007). However, when these plants were exposed to 2°C for 5 to 15 weeks, flowering did occur. The authors concluded that this genotype of *F. vesca* would require vernalization (although subject to verification), not common process in the genus but required in other seasonal flowering *Rosaceae* (Carew and Battey, 2005; Kurokura *et al.*, 2013). The vernalization in *Fragaria* species is defined as the acquisition or acceleration of floral induction due to cold exposure (1 to 4° C) for approx. 5 weeks (Chouard, 1960). These conditions occur in cold climate areas, at either high latitudes or altitudes. The vernalization hypothesis for 'Chilean white strawberry' could explain the abundant flowering reported for this genotype by Staudt (1999) in Ambato, Ecuador. Despite being a low latitude location, there is a great thermal oscillation throughout the year (0-30°C) due to the

altitude (2,500 m. a. s. l.). A similar response was observed when low temperatures, applied at night simulating thermal oscillation, favor floral induction in *Rubus* genotypes that require vernalization (Contreras *et al*., 2019). In addition, it should be noted that the domestication and cultivation of the 'Chilean white strawberry' occurred at heights of the Nahuelbuta mountain in Chile, whose farmers indicate that cold winters with snowfall are synonymous with good flowering (farmers, personal communication). Although it was not possible to determine the inductive conditions for *F. chiloensis* f. *chiloensis* in this study, the results obtained suggests the possibility that the pathway for floral induction in 'Chilean white strawberry' is the vernalization, which should be verified in new studies.

The environmental requirements for floral induction of a certain genotype are related to its ability to adapt to different conditions (Uleberg *et al*., 2016). In this study, it was determined that cv. 'Camarosa' presents a wide range of conditions that allow the floral induction, so the flowering period is wide. This condition could explain the great adaptability that this genotype has in response to a great diversity of environments (Shaw and Larson, 2008), and therefore its great productivity, both breeding aims (Mezzetti *et al.,* 2018). On the other hand, domesticated species generally have a better adaptability to the specific environmental conditions in which they were cultivated (Uleberg *et al*., 2016). However, the 'Chilean white strawberry' does not show good floral behaviour in its domestication area, near to Temuco location (Fig. 4). Since the inductive conditions for this genotype were presented in a narrow window in mid-winter, they were not sufficiently effective in

all the plants, differentiating a low proportion of them (Fig. 4). However, a few decades ago, flowering in 'Chilean white strawberry' was uniform in the fields (farmers, personal communication), so it is possible that the increase in autumn and winter temperatures, as effect of climate change, further limits the inductive period. In this context, the low temperature requirement for floral induction leaves 'Chilean white strawberry' in a high vulnerability condition in its place of origin.

As established for temperate and cold climates, onset of dormancy and floral induction were closely related in both evaluated genotypes. The beginning of dormancy in cv. 'Camarosa' was at the end of March both on the central coast and in southern Chile. This process takes approximately one month to reach its deepest state, which was verified both in the field and in the cold chamber, where the apex completely ceased to grow (Fig. 3, Fig. 5). The flowering in May in the field experiment is, therefore produced during the semi-dormant state, which can occur without completing their chilling requirements (Atkinson *et al*., 2013; Heide *et al*., 2013; Kurokura *et al.*, 2013; Stewart and Folta, 2010).

In 'Chilean white strawberry', the dormancy in the field trial started late at the end of May in both locations, which was verified by a decrease in the petiole length of the last expanded leaf, together with the reduction in the vegetative activity of the apex (Fig. 4). Despite of differences in temperatures and photoperiods between Santo Domingo and Temuco (Fig. 1), this plant had a similar behaviour at both locations (Fig. 2, Fig. 3, Fig. 4), indicating a low sensitivity under environmental conditions compared to short-day genotypes of *F. x ananassa*. By the end of June,

the vegetative activity in the apex had not completely stopped in any of the field experiments (Fig. 4); however, the 30% of induced and differentiated plants, did cease their vegetative activity, indicating that only this proportion of plants entry into dormancy state. On the other hand, conditions of 9°C and short photoperiods (8 hours) for 12 weeks did not induce dormancy in 'Chilean white strawberry' (Fig. 5). These results are similar to those described by Sønsteby and Heide (2006) in cv. 'Elsanta', in which exposure to 6°C for 10 weeks restricted visually the growth, but was easily reversed, preventing dormancy.

For dormancy release, this genotype would need near to 900-1,000 CUs, according to the Lieten model (2006), amount with which the vegetative growth was maximum (Fig. 6, Table 3). Sønsteby and Heide (2006) indicates that buds respond to cumulative chilling at all stages of dormancy, so theoretically, the values of chilling accumulation should be considered from the beginning of entry into dormancy, this is at the end of May in 'Chilean white strawberry' plants (Fig. 4). Based on the climatic records (Agromet, 2019), the cumulative chilling units from this moment until bud break correspond to 918 CU (2017) and 1044 CU (2018). However, these values include the period of ecodormancy, so the CUs that are truly effective for dormancy release would be lower and relatively close to those obtained in the chilling experiment. The results in cv. 'Camarosa' validate those obtained in 'Chilean white strawberry', since the chilling requirement was 673 CU, value similar to 700 chilling hours previously reported by Bigey (2002).

Several authors indicate that excessive chilling increases vegetative growth to the detriment of flowering and therefore yield in strawberry, concluding that the chilling requirement for fruit production is lower than that for vegetative growth (Koosin *et al*., 2001; Lieten, 2009; Lieten, 2006). However, this was not consistent with our results, since greater than 900 CUs did not produce a greater vegetative growth in 'Chilean white strawberry' (Fig. 6, Table 3) as well as an additional negative effect on flowering. The flowering was low and the same in all cold treatments (40%), similar to floral induction and differentiation in the field. This would indicate that the low flowering in 'Chilean white strawberry' is not due to a lack or excess of accumulated cold for dormancy release.

Another distinguishing aspect of this species is its late flowering. The flowering started in October, 45-55 days after bud break, unlike cv. 'Camarosa', which bloomed 7-10 days after bud break (Table 5). It is possible that the 'Chilean white strawberry' has a higher thermal time requirement for flowering. However, it should be noted that vernalization has been considered as a mechanism that prevents the premature flowering in the spring (Lang, 1965) In addition, the late flowering was also one of the characteristics observed in 'Alta' population of *F. vesca* with a possible vernalization requirements (Heide and Sønsteby, 2007).

5. Conclusions

The results of this study confirm that *F. chiloensis* subsp. *chiloensis* f. *chiloensis* have a completely different response in relation to the floral induction and

dormancy processes, compared to other short-day genotypes of *F. x ananassa*. In general, the 'Chilean white strawberry' has a low environmental response for all the analysed processes, evidenced by a low growth and low variation during the season. Floral induction and onset of dormancy occur late in response to low temperatures. The specific low temperature ranges that the species needs, together with the temperatures increase in recent years, causes that the flower induction/differentiation and dormancy to occur only in a portion of the plants on a farm. In the traditional production area of 'Chilean white strawberry' in Chile, the cumulative chilling during the winter varies between 800 and 1000 CU, which currently satisfies the cold the chilling requirements for the dormancy release of this species. However, in the next few years this situation could change, decreasing flowering levels even more. It should be noted that 'Chilean white strawberry' plant production is usually performed in the same field where the fruit is produced, without the suitable production techniques. For this reason, to determine the optimal conditions for flower induction, verifying the need for vernalization, will allow professionalizing the plant production for this particular species, ensuring at least a unique inflorescence but uniform flowering in the field. However, increasing the number of inflorescences per plant also depends on branching, a process that is currently being investigated.

Author's contributions

Javiera Grez: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Elida Contreras:** Investigation, Writing - Review & Editing. **Soledad Sánchez:** Investigation, Writing - Review & Editing.

José A. Alcalde: Writing - Review & Editing. **Marina Gambardella:** Writing - Review & Editing, Supervision, Project administration and Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

This study was funding by GOODBERRY (European Union's Horizon 2020 research and innovation programme; Grant Agreement N° 679303), and by CONICYT (Doctoral fellowship N°21151592).

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Tables

Table 1. Flowering, number of inflorescences per plant and fruit yield (g plant^{-1}) of 'Chilean white strawberry' and cv. 'Camarosa' evaluated in Santo Domingo (33°38’ S) and Temuco (38°38’ S) until December 31, 2017. Values are mean of 3 repetitions of 18 plants each ± SD.

Genotype	Location	Plants in flowering	Inflorescence plant^{-1}	Berry yield (g plant^{-1})
‘Chilean white strawberry’	S. Domingo	3.3±0.58	0.2±0.03	55.3±2.97
	Temuco	5.7±1.53	0.3±0.08	61.8±2.37
‘Camarosa’	S. Domingo	18.0±0.00	3.7±0.73	583.7±3.39
	Temuco	18.0±0.00	4.1±0.78	622.8±3.25

Table 2. Effect of different CU accumulated on petiole and inflorescence length, evaluated 60 days after forcing in greenhouse.

Treatment	Petiole length (cm)	Inflorescence length (cm)
195 CU (without artificial cold)	5.30 b	0.50 a
434 CU	8.50 ab	1.25 a
673 CU	10.80 a	3.50 a
913 CU	11.80 a	4.25 a
1152 CU	9.50 a	3.50 a

Means followed by the same letter in each cultivar are not significantly different, according to Tukey test for petiole length ($P<0.05$) or Kruskal-Wallis test for inflorescence length ($P<0.05$).

Table 3. Sprouting and flowering in 'Chilean white strawberry' and cv. 'Camarosa' in field (38°38'S 72°46'W). Values are mean of 3 repetition ± SD

Genotype	Sprouting (Julian days)		Flowering (Julian days)	
	2017	2018	2017	2018
'Chilean white strawberry'	240.3 ± 3.51	243.7 ± 6.66	288.3 ± 5.86	297.3 ± 2.08
'Camarosa'	226.7 ± 1.53	229.3 ± 2.55	239.3 ± 3.79	235.3 ± 3.51

Figures

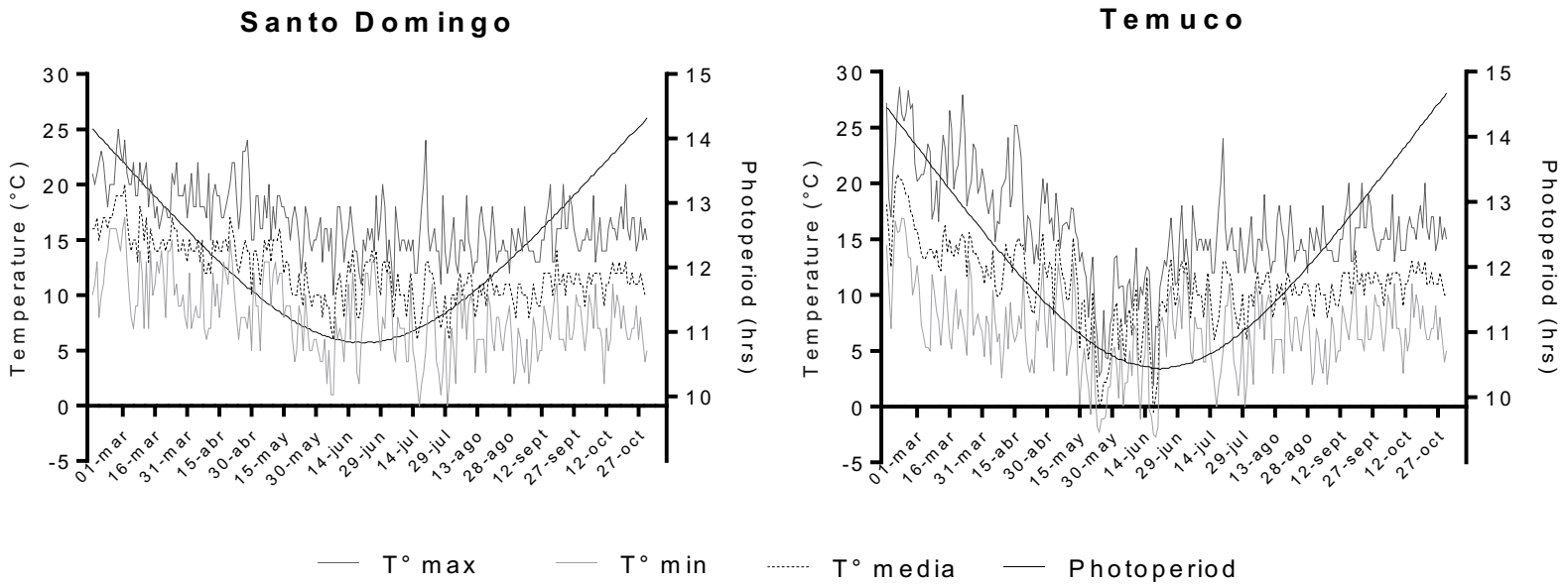

Fig. 1. Mean, minimum, maximum air temperature (°C) and photoperiod (hours between civil twilight) in Santo Domingo and Temuco between February 15 and October 31, 2017.

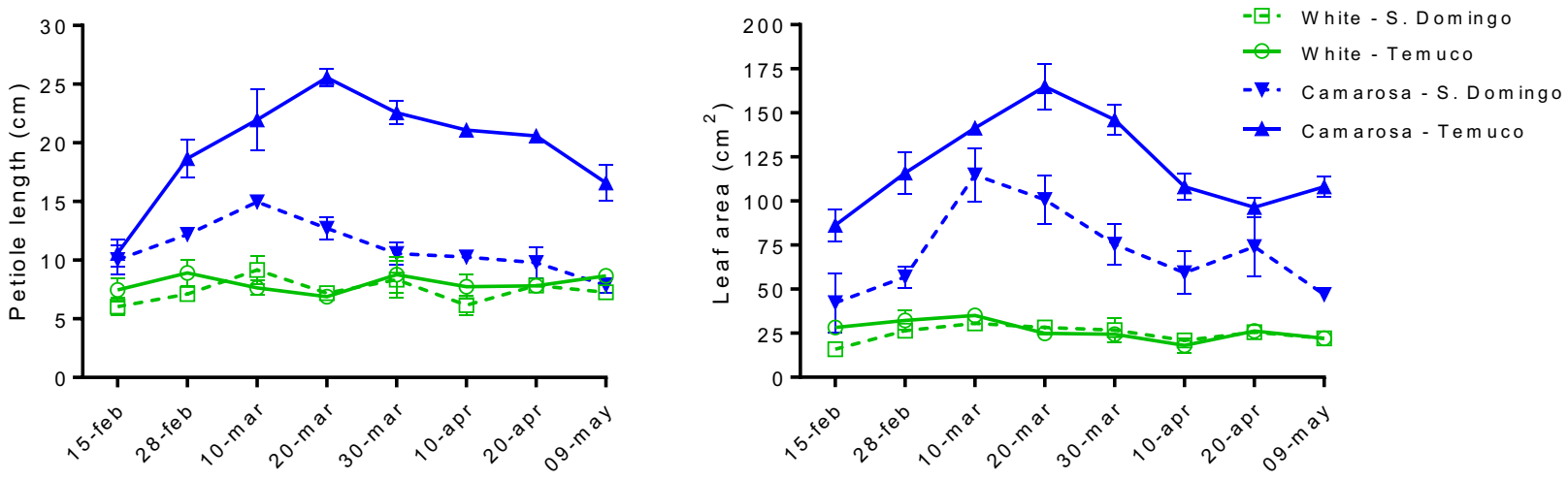

Fig. 2. Petiole length (left) and leaf area (right) of the last expanded leaf in 'Chilean white strawberry' and cv. 'Camarosa' evaluated in two locations, Santo Domingo and Temuco. Values are mean of 6 plants ± SEM.

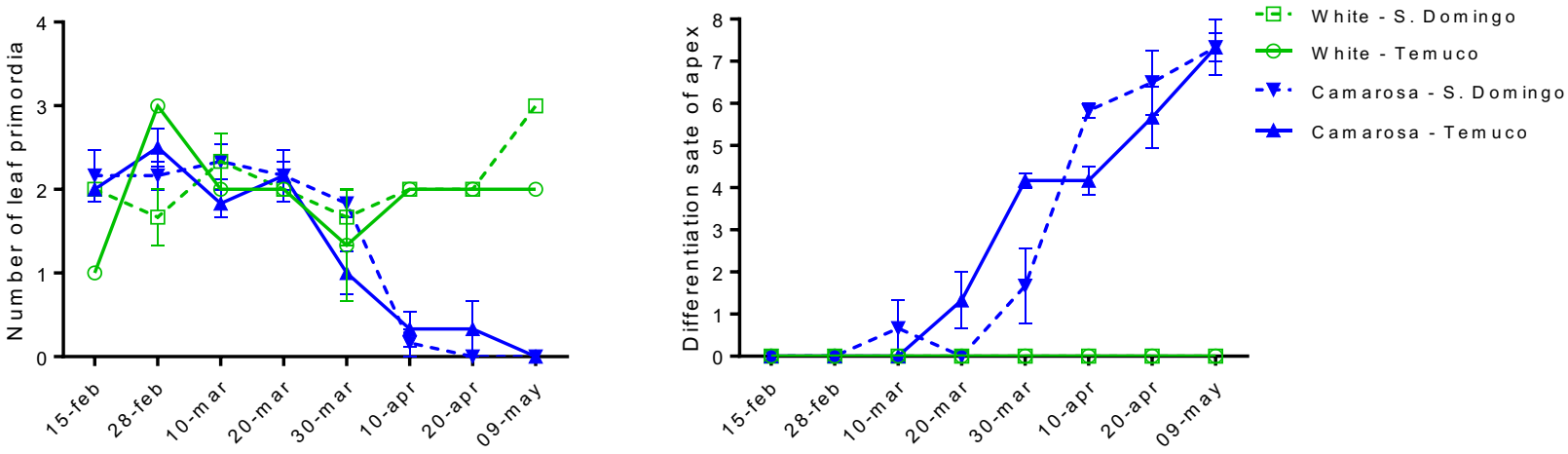

Fig. 3. Number of leaf primordia (left) and state of floral differentiation of the apex (right) in 'Chilean white strawberry' and cv. 'Camarosa' evaluated in two locations, Santo Domingo and Temuco. Values are mean of 6 plants ± SEM.

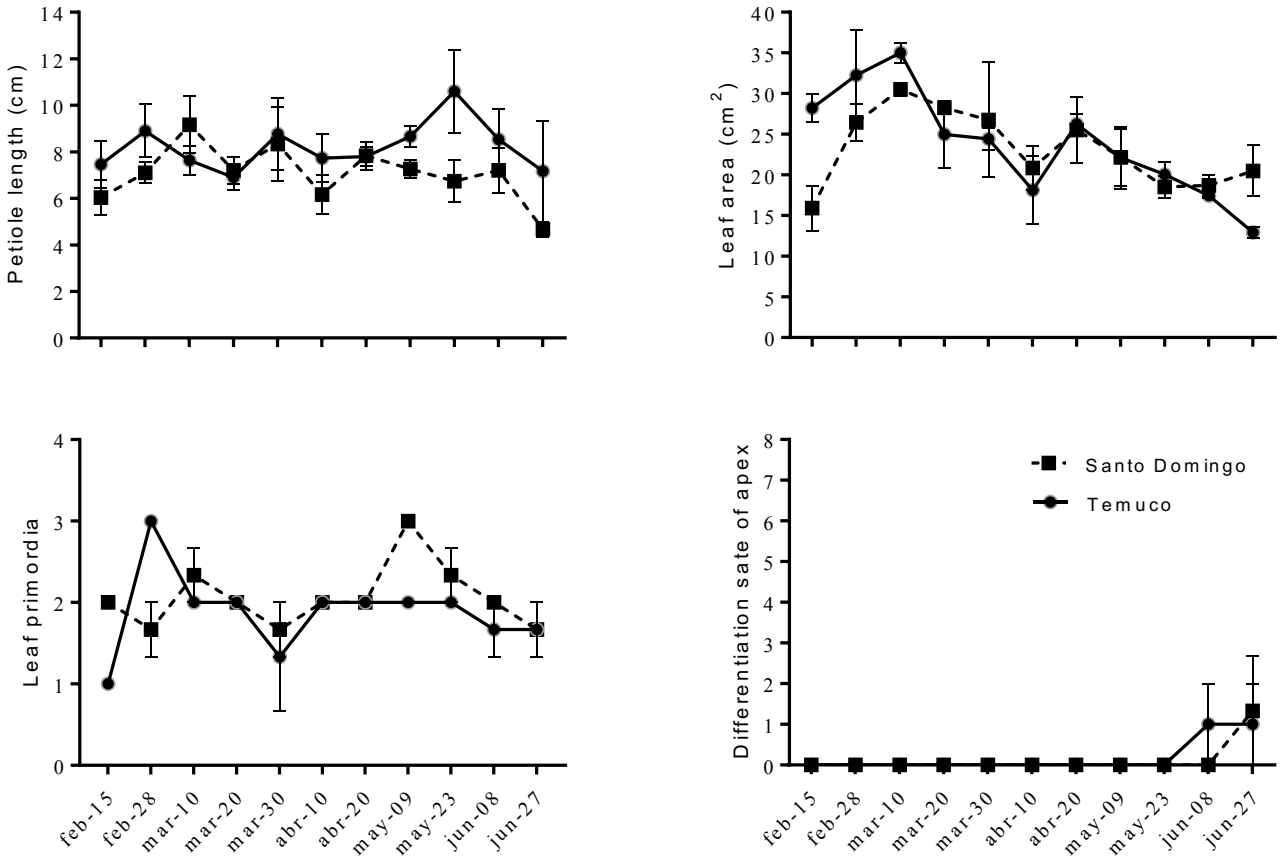

Fig. 4. Petiole length (above left) and leaf area (above right) of the last expanded leaf, number of leaf primordia (down left) and floral apex differentiation (down right) in 'Chilean white strawberry' evaluated in two locations, Santo Domingo and Temuco. Values are the mean of 6 plants ± SEM.

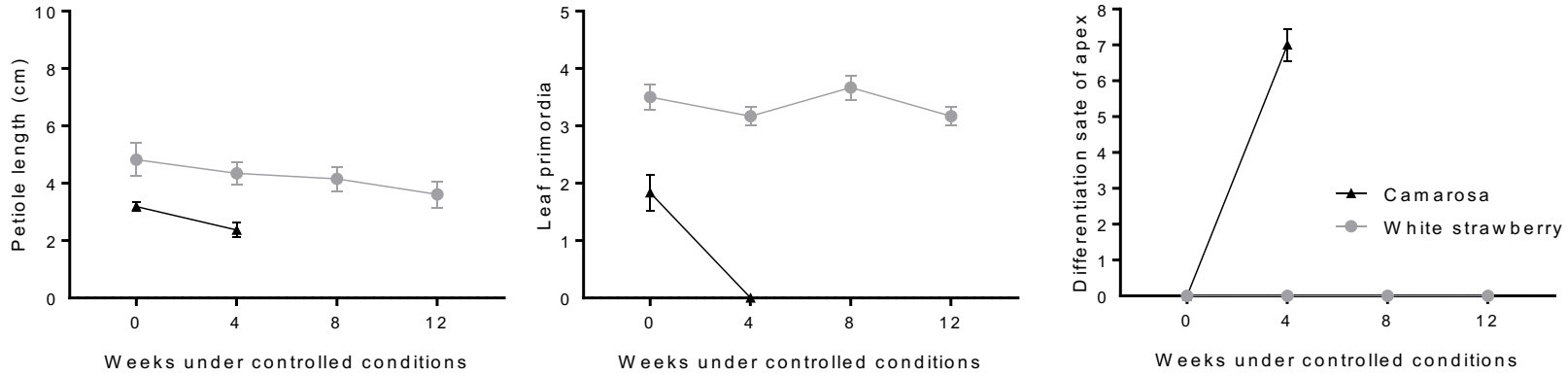

Fig. 5. Petiole length of the last expanded leaf (left), number of leaf primordia (center) and differentiation state of apex (right) in 'Chilean white strawberry' and cv. 'Camarosa' evaluated at 0, 4, 8 and 12 weeks under growth chamber at 9 ° C and 8-hour photoperiod. Values are mean of 9 plants ± SEM.

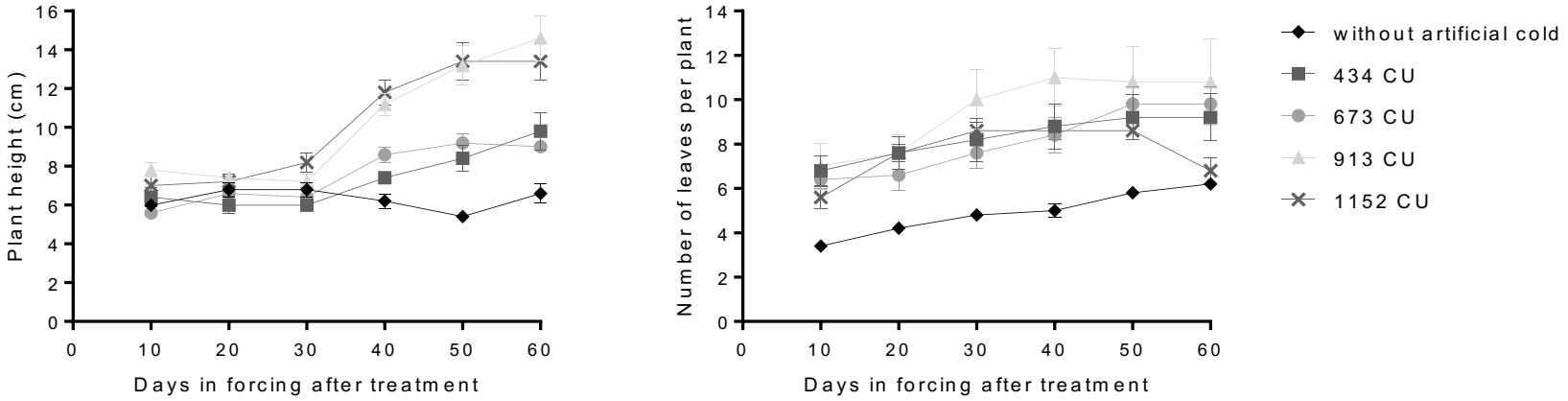

Fig. 6. Plant height (left) and number of leaves per plant (right) for different chilling units (CU) treatments in 'Chilean white strawberry', evaluated under greenhouse conditions for 60 days. Values are means of 5 plants ± SEM.

Fig. 7. Effect of different accumulation of chilling units (CU) in plant performance in ‘Chilean white strawberry.

Chapter 4

Effect of Pro-Ca and decapitation in branching habit in 'Chilean white strawberry' (*Fragaria chiloensis* (L.) Mill. subsp. *chiloensis* f. *chiloensis*)

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Abstract

Unlike most of *F. x ananassa* genotypes, in *F. chiloensis* subsp. *chiloensis* f. *chiloensis,* the 'Chilean white strawberry', the axillary buds remain dormant without developing lateral crowns, limiting strongly their productive potential. In *F. x ananassa*, the formation of new crowns occurs when gibberellin (GA) decreases under short day, associated with a growth decrease. When the GA inhibitor Prohexadione-Calcium is applied, a similar response occurs under non-inductive conditions. On the other hand, it has been indicated that non-branching could be due to apical dominance, which has not been proven. To determine the factors that affect the branching process in 'Chilean white strawberry', several experiments were carried out using Contulmo ecotype of *F. chiloensis* and cv. 'Candonga' of *F. x ananassa*, the latter as a reference. In 'Chilean white strawberry', the application

of 200 mg l^{-1} of Pro-Ca reduced the petiole length and prevented the axillary bud differentiation towards stolons, effect related with a GA3 decreases. Unlike Pro-Ca treatments, the branching in 'Chilean white strawberry' was improved when apex was removal. The decapitation performed in long days allowed a development of 5.8 lateral crowns per plant, similar to cv. 'Candonga' in any treatment. This effect was related with a mayor GA3 decrease in lateral buds and possibly others hormonal relations. These results indicates that the branching process in 'Chilean white strawberry' didn't respond to the *F. x ananassa* model. Despite that the GA would be involved in this trait, there is an apical dominance that prevents the emission of new lateral axes.

Keywords: *Fragaria*, gibberellin, axillary shoot, stolon, bud differentiation, apical dominance.

1. Introduction

The white strawberry (*Fragaria chiloensis* (L.) Mill. subsp. *chiloensis* f. *chiloensis*) is native to Chile and maternal parent of the current red strawberry (*Fragaria x ananassa* Duch.). Due to its distinctive color and flavor, its fruit commands high market prices. In addition, is recognized as a genetic patrimony of world interest. Despite these attributes, its cultivation has been strongly reduced due to its low yield, which varies between 4 to 6 t ha^{-1}, compared to the 50 to 60 t ha^{-1} that normally yielded the cultivated red strawberries.

This difference in crop yield between both species is due to their contrasting flowering habits, which mainly depend on the floral induction and branching processes. Regarding floral induction, it was recently determined that the 'Chilean white strawberry' requires low temperatures (<9ºC), possibly vernalization. They are not fulfilled, therefore, only 30-50% of plants in a field flower, all with only one inflorescence. The increased temperatures could further reduce even more flowering (Grez et al., 2020).

On the other hand, the branching habit varies with each species, depending of the types of buds and structures that each presents (Costes *et al*., 2014). The *Fragaria* species have a main axis or 'crown' with short internodes, which gives it a typical rosette appearance. At the upper end of the crown there is the apex, responsible for the vegetative growth of the axis, and responsible for the formation of new nodes and internodes. The nodes are arranged helically in the crown, and in each one of them a trifoliate leaf and a lateral bud are located. These buds can produce different vegetative structures, or remain dormant depending on their position and environmental conditions. The buds located in basal positions of the crown will preferably give rise to stolons, a modified stem with long internodes that corresponds to the vegetative reproduction system of the species. On the other hand, buds located on the upper part of the crown are normally differentiated from lateral crowns (Staudt, 1999; Savini *et al*., 2005; Heide *et al*., 2013; Costes *et al.,* 2014).

The structures development and their relationship with flowering is well studied in *F. x ananassa*. In optimal conditions for floral induction, the apex changes from

vegetative to reproductive, the terminal inflorescence is formed and the plant stops its growth. At this time, the lateral buds located below the inflorescence are differentiated to lateral crowns, which remain latent until spring. New inflorescences may form at the apexes of these new lateral crowns if conditions are inductive, and so on under a sympodial branching system (Kurokura *et al*., 2005; Savini et al., 2005; Hytönen *et al.,* 2009; Bosc *et al.,* 2012; Heide *et al*., 2013; Costes *et al.*, 2015; Mouhu *et al.,* 2013). The behavior of 'Chilean white strawberry' is completely different from this model and only plants that flowering developed one lateral crown (Gambardella *et al*., 2017; Grez *et al*., 2017).

In *F. x ananassa* it has been determined that the differentiation of axillary buds depends of gibberellin (GA) content, affected mainly by the photoperiod. Increasing day-lengths in spring produce a GA increase in the buds, promoting vegetative growth and stolons differentiation. On the other hand, decreasing day-lengths in autumn produce a GA decrease, the vegetative growth decreases its growth rate until complete cessation, the terminal inflorescence is formed, and the lateral buds are differentiated into lateral shoots (Hytönen *et al*., 2004; Hytönen *et al*., 2008; Hytönen *et al*., 2009). In several cultivars of *F. x ananassa*, it has been observed that the application of a GA inhibitor, Prohexadione-Calcium (Pro-Ca), under long days, produces a similar response to short photoperiods in growth reduction as well in lateral bud differentiation. This has allowed the study of its use, dosage, and application in order to increase potential production, reducing stolon emission and increasing the number of crowns per plant (Black, 2004; Hytönen *et al*., 2008; Hytönen *et al*., 2009). Inside of plant, a low content of bioactive GAs is due to low

activity of GA20-oxidase or GA3-oxidase enzymes (synthesis), and/or the high activity of GA2-oxidase enzyme (catalysis). This allow the formation of DELLA-GAF1 complex, that represses the expression of GA related genes. This generate a growth decrease and favors the cellular differentiation, floral differentiation in the apical buds and new crowns in lateral buds (Hytönen *et al*., 2009; Martins *et al*., 2018). Expression of these GA biosynthesis and signaling genes has been used to confirm the role of this hormone in the branching process of *F. x ananassa* (Hytönen *et al*., 2009).

Apart from the GA role, the lateral shoots emission has been related with an apical dominance, which varies among species and cultivars of *Fragaria* spp. (Sugiyama *et al*., 2004; Hummer *et al.*, 2016). In 'Chilean white strawberry' there are no studies related to the branching, however it has been suggested that this genotype could have an apical dominance effect that limiting the formation of lateral shoots (Grez *et al,* 2017). In relation to this process, it is widely reported that the main gene linked to apical dominance is BRC1/TCP9 transcription factor (Rameau *et al.,* 2015). This gene has been proposed to be the common integrator of the multiple hormonal pathways that regulate branching. Its expression in axillary buds is related to the inhibition of branching and its transcript levels increase in response to strigolactone and giberellin and are reduced by cytokine and sucrose (Gomez-Roldan *et al*., 2008; Dun *et al*., 2012; Enders and Straders, 2015; Rameau *et al*., 2015). The connection with GA and genes of this pathway has not been well studied yet.

In the ‘Chilean white strawberry’ it has been observed that plants that flowering, develop a single inflorescence. In these plants, only one lateral bud is differentiated and becomes a new crown, allowing the continuous plant growth. On the other hand, non-flowering plants do not develop lateral axes. Being the ramification a prerequisite to increase the productive potential, the objective of this work was to study the role of gibberellin as well as the effect of the apex removal in the development of lateral crowns in ‘Chilean white strawberry’.

2. Materials and Methods

2.1. Plant material

The 'Contulmo' ecotype of the ‘Chilean white strawberry’ (*F. chiloensis* subsp. *chiloensis* f. *chiloensis*) was used for all the experiments. This was collected in productive fields in the Nahuelbulta mountain range (38°05’S 73°14’W). In some experiments, the cv. ‘Candonga’ (*F. x ananassa*) was used as reference genotype.

2.2. Pro-ca experiments

To determine the Pro-Ca effect on branching in the ‘Chilean white strawberry’, three experiments were carried out at the Faculty of Agronomy of the Pontificia Universidad Católica de Chile (33°29'S 70°36'W). Both experiments were carried out in white strawberry and in cv. ‘Candonga’. In the first experiment, 45 plants of each genotype, obtained from stolons and rooted in January 2017 in 200 ml plastic containers, were used. The transplant was carried out in September 2017 to 1 l pots with coconut fiber and peat (2:1) and 1 g l^{-1} of slow-release fertilizer

(Basacote®). On November 17, under long photoperiods, the treatments were applied: milli-Q water spray (control treatment) and 200 mg l^{-1} of Pro-Ca (2 g of Regalis® in 1 l of milli-Q water). Each 7 days for 6 weeks, the petiole length of the last expanded leaf was evaluated as a growth parameter as well as the number of crowns per plant. For each genotype, the experimental design was completely randomized, with 2 treatments and 3 replications of 5 plants each.

The second experiment was similar to the previous one but was carried out in late summer, just a few weeks before the natural decrease in the photoperiod, which favors the floral induction in most short-day genotypes. Forty-eight plants of each genotype were used, all obtained from stolons. These were rooted in November 2017 in 200 ml plastic containers. In January 2018, the plants were transplanted into 1 l pots, with coconut fiber and peat (2:1) and 1 g l^{-1} of slow-release fertilizer (Basacote®). Applications were realized in February 14, 2018. The control treatment consisted of spraying milli-Q water, while the second treatment consisted of spraying 200 mg l^{-1} of Pro-ca (2 g of Regalis® in 1 l of milli-Q water). The petiole length of the last expanded leaf and the number of crowns per plant were evaluated every 7 days for 10 weeks. For each genotype, the experimental design was completely randomized, with 2 treatments and 3 replications of 8 plants each.

A third experiment was carried out only on ‘Chilean white strawberry’. The application of Pro-ca was carried out at the same time that the plants were kept under short days and low temperatures, being these conditions inductive for short-day genotypes. Forty-eight plants were vegetatively propagated in January 2018 and rooting in 200 ml plastic containers. From March to August 2018, the plants

were kept in the greenhouse (33°29'S 70°36'W). On August 7, half of the plants were sprayed with 200 mg l^{-1} of Pro-Ca (2 g of Regalis® in 1 l of milli-Q water) and the other half sprayed with milli-Q water. After the applications, all the plants entered a growth chamber with a short photoperiod of 8 hours at 9±1°C, remaining under these conditions for 4 weeks. After this period, the plants were transferred to the greenhouse on September 7, with increasing temperature and photoperiod. Since the growth chamber period, every 7 days during 3 months, the petiole length of the last expanded leaf and the number of crowns per plant were evaluated. The experimental design was randomized complete blocks, with 2 treatments and 3 repetitions of 8 plants each.

2.3. *Decapitation experiments*

To determine the apex effect on the differentiation of lateral buds, a first decapitation experiment was carried out on 'Chilean white strawberry' and cv. 'Candonga'. Thirty plants of the two genotypes were propagated by stolons and rooted in January 2016 in 200 ml plastic containers. In March, the plants were transplanted into 1 l pots, with coconut fiber and peat (2:1) and 1 g l^{-1} of slow-release fertilizer (Basacote®). From March to December 2016, all plants were maintained in a greenhouse in the central zone of Chile (33°38'S 71°36'W). A control treatment was considered in both genotypes with plants without decapitation. The other treatments consisted of decapitation carried out on different dates: April, June and October, the latter only for 'Chilean white strawberry'. The apex excision was performed with a scalpel, extracting the complete apical bud. At the end of the season and after flowering, 5 plants per treatment were dissected

and the following parameters were evaluated: number of secondary, tertiary, total crowns and number of inflorescences per plant. For each genotype, the experimental design was completely randomized, with 3 treatments (for cv. 'Candonga') or 4 (for ‘Chilean white strawberry’), all with 10 repetitions of 1 plant.

In the second experiment, 48 plants of ‘Chilean white strawberry’ were propagated in January 2018. The stolons were rooted in 200 ml plastic containers. From March to August 2018, the plants were maintained in the greenhouse (33° 29'S 70° 36'W). On August 7, half the plants were decapitated. Then, all were transferred to the growth chamber with photoperiod of 8 hours at 9±1°C, remaining in these conditions for 4 weeks. After this period, the plants were returned to the greenhouse with high temperatures and photoperiod. Since August, every 7 days and for 3 months, the length of the petiole of the last expanded leaf and the number of crowns per plant were evaluated. The experimental design was randomized complete blocks, with 2 treatments and 3 repetitions of 8 plants each.

2.4. Hormonal analyses and gene expression

Hormonal and gene expression analysis was performed in ‘Chilean white strawberry’ plants with applications of Pro-Ca and decapitated. These analyses were carried out from the experiment performed in a growth chamber. Plants were sampled before treatments (control plants), taking the apical bud (5-8 mg of fresh weight each) and the 3-4 upper lateral buds located below the apical bud (1-2 mg of fresh weight each, 4-8 mg total fresh weight). Each sample consisted of a pool of 3 plants. After 4 days, sampling was carried out in plants treated with Pro-Ca

(apical and lateral buds) and in decapitated plants (lateral buds). All samples were immediately immersed in liquid nitrogen and then stored at -80° C. Hormone extraction and quantification were carried out by HPLC-MS/MS, a methodology described by Pan *et al*. (2010). For each treatment, two biological and two techniques replicates were taken into account.

The gene expression of GA3-oxidase, RGA (DELLA gene), and TCP9 was analyzed. Sampling was performed at the same treatments and times as described in hormonal analysis. For the RNA extraction, Purelink RNA kit (Ambion®) was used with DNAse treatment. The RNA obtained was purified by precipitation with Lithium Chloride and quantified in a NanoDrop 1000 spectrophotometer (Thermo scientific). For cDNA synthesis, AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies) was used. Both RNA and cDNA were visualized by electrophoresis to test their integrity. One µL of cDNA of each sample was used in the SYBR Green RT-PCR, with 10 µL of Brilliant® II SYBR® Green QPCR Master Mix (Agilent Technologies) and 0.5 µL of each primer in the Mx3000 thermal cycler (Stratagene). The primers used for GA3-ox and RGA were described by Hytönen *et al*. (2009). Primers used for TCP9 were designed by Primers3 program (F: CAAACTGCAATTTCGCTTCA R: CCAAGGGAGAGGGGAAGTAG). Relative quantification was obtained using the FaUBQ gen as a normalizer. Three biological and techniques replicates were used for qPCR.

2.5. *Data analyses*

In all experiments, the data were analyzed for each genotype separately. In case of normal data, ANOVA was performed and the means were separated by Tukey or T-test (5% significance). In the case of non-normal data, non-parametric tests were performed. GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) software was used for all analyzes.

3. Results

3.2 Effect of Pro-Ca in branching in F. chiloensis f. chiloensis

In the first experiment, Pro-Ca was applied in both genotypes under increasing photoperiods. As observed in Fig. 1, the control plants of 'Chilean white strawberry' showed little variation in growth rate throughout the evaluation period, characteristic behavior of this genotype (Grez *et al.,* 2020). However, the application of Pro-Ca produced a decrease in petiole length, significantly different from the control from week 1 until the end of the evaluations ($P < 0.01$).

In cv. 'Candonga' (Fig. 1), plants of all treatments presented a short and slow-growing petiole. However, a significant effect of Pro-Ca was also verified: reduced growth between week 1 and 4 after application ($P < 0.01$).

Despite the effect of Pro-Ca on growth, the number of crowns per plant at the end of the evaluation did not increase in any of the genotypes (Fig. 2).

Considering that short-day genotypes differentiate their lateral buds into stolons under increasing photoperiods, the number of stolons per plant was recorded. The effect of the treatments on the stolons emission in 'Chilean white strawberry' is observed in Fig. 3. Control plants had a mean of 1.3 stolons per plant, a statistically different value to Pro-Ca treatment, with 0.07 stolons per plant ($P < 0.001$). In cv. ‘Candonga’, there were no significant differences between treatments for this parameter (data not shown).

In the second experiment, the application of Pro-Ca was carried out in mid-February. In control plants of ‘Chilean white strawberry’, a low growth response was observed throughout the evaluation period (Fig. 4), similar to the previous experiment. On the other hand, control plants of cv. ‘Candonga’, had the characteristic behavior, with an increase and then a growth decrease in response to the photoperiod (Fig. 4). Despite these differences, the effect of Pro-Ca was similar in both genotypes, where the reduction in petiole length was significant from week 1 and throughout all evaluation period on ‘Chilean white strawberry’ ($P < 0.05$), and until week 8 in cv. ‘Candonga’ ($P < 0.05$) (Fig. 4).

Fig. 5 shows the results for the number of crowns per plant. In ‘Chilean white strawberry’ no significant response to the application of Pro-Ca was observed. All the plants had only one axis. In the cv. ‘Candonga’, the application of Pro-Ca improved branching, although without significant statistical differences with the control treatment.

The results of the third experiment, in which Pro-Ca was applied only to ‘Chilean white strawberry’ plants, growing under controlled conditions of short days, were similar to the previous experiments. Plants treated with Pro-Ca significantly decreased the value of petiole length, differences evidenced in the greenhouse period. The number of crowns per plant did not increase after Pro-Ca applications (Supplementary Fig.).

In this last experiment, the effect of Pro-Ca on the hormonal state was determined in apical and lateral buds in 'Chilean white strawberry'. Fig. 6 shows the results of the gibberellin content according to active form (GA3 and GA4) and tissue (apical and lateral bud). It was observed that the GA3 content in the apex did not change after 4 days from the Pro-Ca application, while in the lateral buds there was a significant decrease in relation to the control treatment ($P<0.05$). In GA4, levels increased significantly in response to Pro-Ca, both at the apex and lateral bud.

Additionally, the content of other hormones are indicated in Table 1. The IAA content increased in lateral buds after the Pro-Ca application, being statistically different from the control treatment ($P<0.001$). Other auxins decreased significantly their content after the application of Pro-Ca, ICA in apical buds and IBA in both tissues. In relation to cytokinins, Pro-Ca produced a significant decrease in *Zea* in both buds ($P<0.01$). Finally, the ABA content increased significantly, especially in lateral buds ($P<0.0001$).

In relation to the gene expression, the alignment with BLAST of the sequences obtained in *F. chiloensis* showed high homology with *F. x ananassa* and *F. vesca*.

FchGA3-oxidase (GenBank accession number MT513720) had 96% identity with *Fragaria x ananassa* gibberellin 3-oxidase GA3ox mRNA (complete cds) and FchRGA (GenBank accession number MT513719) had 98% identity with *Fragaria x ananassa* GAI / RGA-like protein mRNA (partial cds). In FchTCP9 (GenBank accession number MT513718), the sequence obtained with the designed primers showed 100% identity with *Fragaria vesca* PCF transcription factor 9 mRNA (complete cds) in the 140-253bp region.

Fig. 7 shows the results of gene expression in apical and lateral buds of plants treated with Pro-Ca. On day 4, there is an increase in the expression of FchGA3-oxidase in both types of buds. FchRGA expression decreased at the apex, while a slight increase in expression were observed in lateral buds. FchTCP9, integrator and negative regulator of branching, decreased its expression in apices, while in lateral buds the expression increased.

3.2 Effect of decapitation in branching in F. chiloensis f. chiloensis

The results of the decapitation experiment performed in 'Chilean white strawberry' and in cv. 'Candonga' on different times during the season are observed in Table 2.

The 'Chilean white strawberry' emitted between 1 and 2 crowns per plant in those without decapitation. The apex removal in April increased the ramification to 2.8 crowns, although without statistical differences with the control treatment.

Decapitation in June didn't produced changes in branching compared to the control treatment.

In the cv. 'Candonga', the plants without decapitation had between 5 to 9 crowns at the end of the season. Decapitated plants in April and June presented a similar branching, without statistical differences with the control treatment (Table 2).

Since the decapitation treatments in 'Chilean white strawberry' did not have a significant effect on the number of crowns, a final decapitation treatment was performed in October. In this treatment, there was a significant increase in this parameter compared with control treatment ($P<0.01$) and June decapitation ($P<0.01$) (Fig. 8).

At the end of the season, the number of inflorescences in the cv. 'Candonga' was similar to the number of crowns per plant. That is, all the crowns emitted their respective inflorescence. In contrast, in 'Chilean white strawberry', the number of inflorescences was lower than the number of crowns in all treatments (Supplementary Table).

The second decapitation experiment was performed only in 'Chilean white strawberry', before to exposure to short days in the growth chamber. Decapitated plants produced 2 new growth axes as a mean, similar to the decapitation results of April in the previous experiment. In this case, this value was statistically different from the control treatment ($P <0.001$).

As with Pro-Ca, a quantification of the main hormones was performed in response to decapitation. Results are observed in Fig. 9. GA3 content decreased significantly in lateral buds of decapitated plants, being different from the control treatment ($P<0.0001$). In relation to GA4, the levels increased significantly ($P<0.0001$). Cytokinin levels decreased, while IAA and ABA increased significantly ($P<0.05$ and $P<0.0001$, respectively).

4. Discussion

According to several studies, the differentiation of lateral buds into new crowns in *Fragaria* spp. it is naturally promoted for short days in autumn (Hytönen et al., 2004; Hytönen *et al*., 2008; Hytönen *et al*., 2009; Heide *et al.,* 2013; Mouhu *et al*., 2013). Under these conditions, GA biosynthesis is inhibited in plant, slowing growth and promoting tissue differentiation (Hytönen *et al*., 2009; Martins *et al*., 2018). The application of Prohexadione-Calcium on long days produce a similar effect to short days, blocking the action of GA20 3ß-hydroxylase and therefore decreasing the production of biologically actives GAs. In previous research, application of Pro-Ca in long days, affected negatively the growth and stolons emission in cvs. of *F. x ananassa*, but at the same time increased the number of crowns per plant (Black, 2004; Hytönen *et al*., 2008; Hytönen *et al*., 2009). In the present study, these results were achieved in cv. 'Candonga' in the second Pro-Ca experiment, showing a growth reduction (Fig. 4) and an upward trend in the number of lateral crowns (Fig. 5). The low effect of Pro-Ca in cv. 'Candonga' obtained in the first experiment could be attributed to insufficient development of the plant (Fig. 1).

In 'Chilean white strawberry', it was verified that the application of 200 mg l^{-1} of Pro-Ca was effective in all experiments since the plants had a clear response in reducing growth and avoiding the differentiation of buds to stolons (Fig. 1, Fig. 3, Fig. 4). However, unlike to described in other research carried out on *F. x ananassa*, branching did not improve in any experiments (Fig. 2, Fig. 5). In similar studies, even lower doses of Pro-Ca (50 mg l^{-1}) improved branching in cvs. 'Polka' and 'Korona' of *F. x ananassa* (Hytönen *et al*., 2008). Although repeated doses of Pro-Ca have increased the number of crowns in some investigations (Black, 2004), the results obtained in the present study would indicate that the response to branching is due to other factors and not to the dose.

In relation to gibberellin, 13-hydroxylation has been indicated as the main pathway in *Fragaria* species, therefore the quantification of the GA1 and GA3 are of greater interest due to their biological function. In *F. x ananassa* cv. 'Korona', the formation of lateral crowns was correlated with a decrease in GA1 after the application of Pro-Ca (Hytönen *et al*., 2009). Although GA1 was not quantified in this study, it was verified that GA3 levels decreased significantly in lateral buds after the application of Pro-Ca (Fig. 8). This decrease in GA3, together with the slight increase in FchRGA expression observed in lateral buds, would explain the reduction in growth and less stolons differentiation, since both factors contribute to the formation of the DELLA-GAF1 complex that represses the expression of genes in response to GA (Hytönen *et al*., 2009; Martins *et al.,* 2018).

The increase in FchGA3-oxidase expression in axillary buds after Pro-Ca was not expected due to decrease in GA3 content. Similar results were obtained in cv.

Korona where FaGA3-oxidase expression also increased after the application of Pro-Ca despite GA1 levels decreased (Hytönen *et al*., 2009). Since GA4 increased its levels after Pro-Ca application, the higher level expression of FchGA3-oxidase could be related to a feedback with this active form (non-13-hydroxylation pathway). However, a better understanding for this hormone regulation in *F. chiloensis* requires the GA1 quantification.

In contrast to Pro-Ca, the apex removal in short days increased the emission of crowns in white berries between 2 to 3 new axes (experiments 2 and 1, respectively). The low sample size influenced in not obtaining a statistical difference with the control treatment. The highest branching was obtained with the October decapitation in experiment 1 (Table 2, Fig. 8). This response was not expected, since under long days the lateral buds are differentiated to stolons. The greater response with this treatment could be due to the 'Chilean white strawberry' probably have a vernalization requirements to flowering, conditions that occur late in the winter (Grez *et al.,* 2020). This hypothesis, not still tested, would explain that in October (spring) the number of lateral buds differentiated to crowns is greater than in April (autumn), where the induction and differentiation conditions for this genotype do not yet happened. In addition, the emission of these new crowns occurs under temperature and photoperiods that promote the vegetative growth. This would also explain the greater ramification obtained in 'Chilean white strawberry' with the last treatment decapitation. Neri *et al.* (2003) already reported that plants of cv. 'Tochiotome' of *F. x ananassa* were able to develop lateral shoots after apex removal on long days.

The results of this study show that the ‘Chilean white strawberry’ have an apical dominance that prevents branching, since it was improved in response to decapitation in the two experiments carried out (Table 2, Fig. 8), unlike cv. ‘Candonga’ that didn’t respond. Similar conclusions were obtained for Sugiyama *et al.* (2004), where a greater apical dominance was attributed to the cv. ‘Tochiotome’ versus cv. ‘Nyoho’ due to the response to the apex removal.

It should be important noted that, in several crops, domesticated genotypes are less branched than their respective wild relatives. Although some plants modify their degree of branching in response to environmental conditions, in others, apical dominance defines the plant architecture, especially in species where selection has favored fruit size (Kellogs, 1997). This description is coincident with the 'Chilean white strawberry' (*F. chiloensis* subsp. *chiloensis* f. *chiloensis*) and its wild state (*F. chiloensis* subsp. *chiloensis* f. *patagonica*), the latter developing several inflorescences with a greater number of fruits of small size (Fig. 10).

Apical dominance is understood as inhibition of the growth of lateral buds or shoots by a growing apex (Sachs and Thimann, 1967). The apex removal eliminates the main source of auxins, a hormone commonly associated with this process. This could explain the dominance release in 'Chilean white strawberry'. However, 4 days after decapitation, IAA content in axillary bud was increased (Fig. 9). It is possible that the stem compaction in strawberry affects the hormonal relations between the apex and lateral buds due to the proximity that they are. However, to determine the role of auxins in the apical dominance in ‘Chilean white strawberry’, new studies are required, as well as the quantification of strigolactones due to the

importance of this latter hormone in this process (Gómez-Roldán *et al.*, 2008; Dun *et al.,*2012; Rameau *et al.,* 2015).

Branching in decapitated plants may not be regulated by the same processes as those induced in intact plants (Dun *et al.,* 2012). However, the comparison between two types of experiments helps to determine the factors involved. In this way, it is interesting to note that the decapitation also produced a significant reduction of GA3 in lateral buds, greater than that achieved with Pro-Ca (Fig. 3 and Fig. 6). It is possible that, different from *F. x ananassa*, a slight decrease in GA3 is sufficient to avoid differentiation to stolons, but greater decrease is required to promote the bud differentiation to crowns in 'Chilean white strawberry'. This hypothesis would explain that the trade-off between stolons and crowns reported in *F. x ananassa* did not occur in 'Chilean white strawberry'.

The results of this study indicate that the 'Chilean white strawberry' have a different response in the branching in relation to *F. x ananassa*, as reported by other authors. The application of 200 mg l^{-1} of Pro-Ca slightly decreased the levels of GA3 in lateral buds in 'Chilean white strawberry', which would be associated with a growth decreased and non-differentiation buds to stolons. However, the branching was improved with decapitation, which produced an even greater reduction in GA3, as well as other hormonal changes. In this sense, the GA would be involved in the differentiation from buds to crowns in 'Chilean white strawberry', as in *F. x ananassa*, however, the apical dominance presented by the species prevents the emission of new lateral axes.

This research is a first approach to the study of branching in 'Chilean white strawberry' and several questions must be included in new studies. The future findings in this character, together with the determination of the floral induction requirements, can be used to improve the flowering, and therefore the fruit production in this species.

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Tables

Table 1. Hormonal content (ng g^{-1}) in apical and lateral buds in control and Pro-Ca treatment.

Treatment	Bud	IAA	ICA	IBA	Zea	ABA
Control	Apical	12.0±0.42	8.9±0.90	3.2±0.11	11.7±0.28	3.4±0.13
	Lateral	3.0±0.35	3.0±0.38	1.0±0.10	13.3±0.41	3.3±0.26
Pro-Ca	Apical	12.7±1.39	4.6±0.37	2.7±0.20	8.0±0.17	5.1±0.19
	Lateral	4.1±0.19	3.6±0.41	0.6±0.09	10.0±1.16	12.1±0.92

Samples of control treatment were taken before to Pro-Ca application. Samples of Pro-Ca treatment samples were taken 4 days after application. Data were compared in each bud type by T-test with 5% significance. Values are mean of 2 biological and 2 technical replicates ± SEM, each one a pool of 3 plants.

Table 2. Effect of decapitation on the number of crowns per plant in *F. chiloensis* f. *chiloensis* and *F. x ananassa* cv. 'Candonga'.

Decapitation treatment	Number of crowns per plant in *F. chiloensis* f. *chiloensis*	Number of crowns per plant in F. *x ananassa* cv. 'Candonga'
Control	1.2 ± 0.44	6.2 ± 1.63
April	2.8 ± 0.44	7.0 ± 2.00
June	1.0 ± 0.00	6.4 ± 1.14
October	5.8 ± 2.16	w/i

Treatments were compared within each genotype by Kruskal-Wallis test with 5% significance. w/i without information. Values are mean of 5 plants ± SD.

Figures

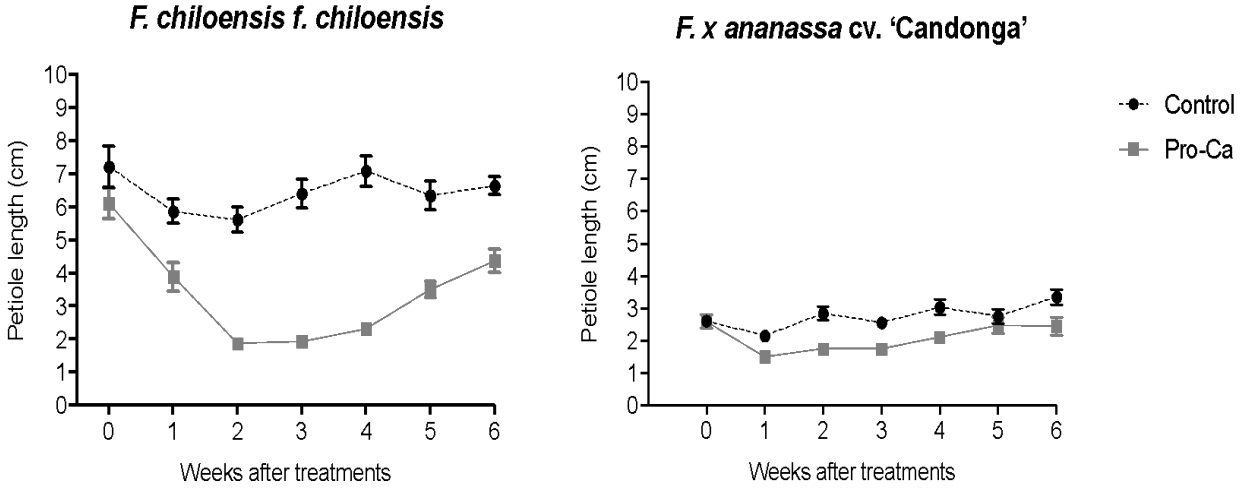


Fig. 1. Effect of Pro-Ca application in November in *F. chiloensis* f. *chiloensis* plants (left) and *F. x ananassa* cv. 'Candonga' (right) in the petiole length of the last expanded leaf. Values are mean of 15 plants ± SEM.

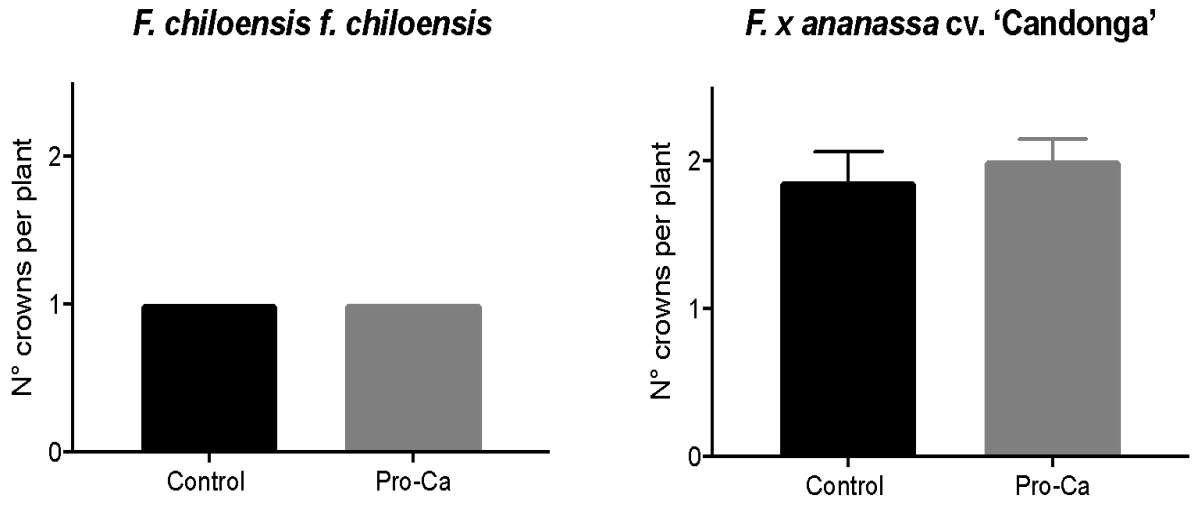


Fig. 2. Effect of Pro-Ca application in November in *F. chiloensis* f. *chiloensis* plants (left) and *F. x ananassa* cv. 'Candonga' (right) in the number of crowns per plant at week 6 after treatment. Values are mean of 15 plants ± SEM.

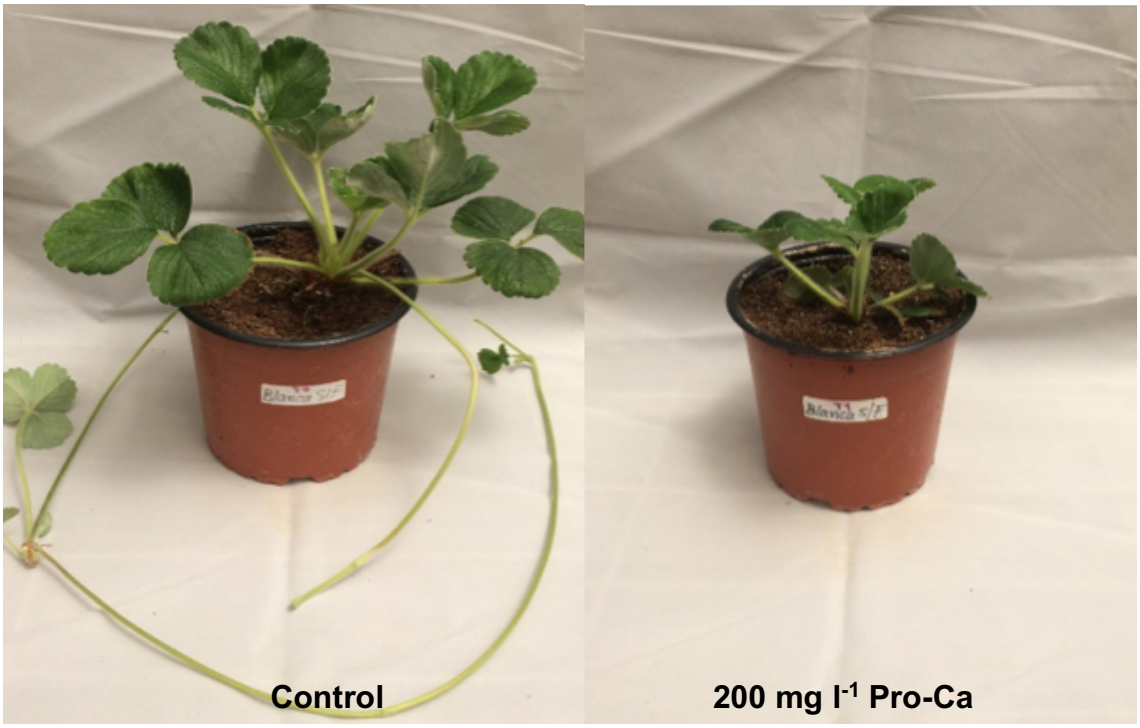


Fig. 3. Appearance of *F. chiloensis* f. *chiloensis* plants of control treatment (left) and applied with 200 mg l^{-1} of Pro-Ca (right).

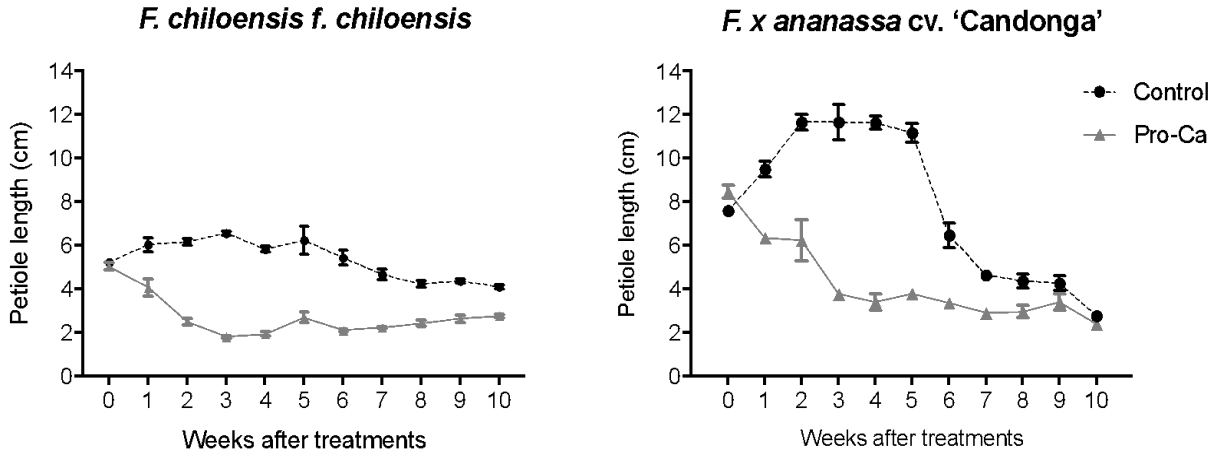


Fig. 4. Effect of Pro-Ca application in February in *F. chiloensis* f. *chiloensis* plants (left) and *F. x ananassa* cv. 'Candonga' (right) in the petiole length of the last expanded leaf. Values are mean of 24 plants ± SEM.

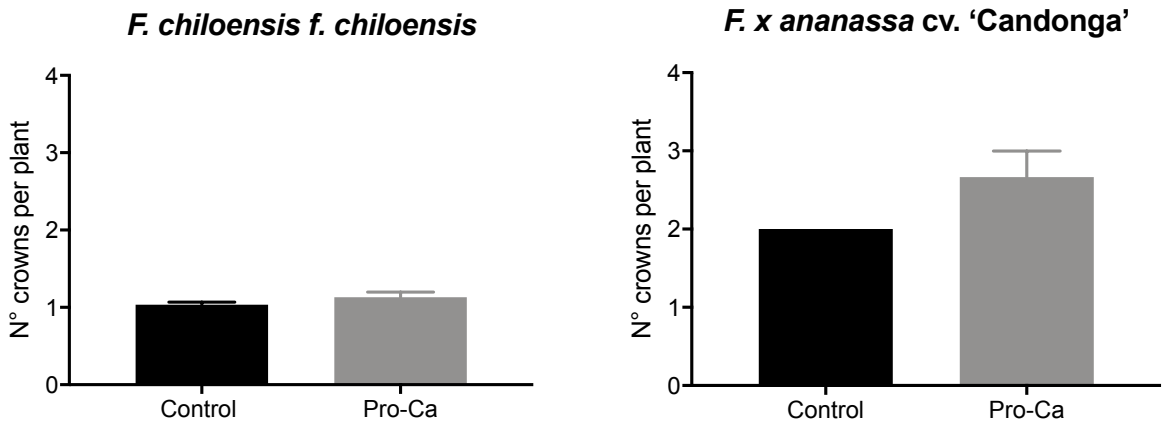


Fig. 5. Effect of Pro-Ca application in February in *F. chiloensis* f. *chiloensis* plants (left) and *F. x ananassa* cv. 'Candonga' (right) in the number of crowns per plant at week 10 after treatment. Values are mean of 24 plants ± SEM.

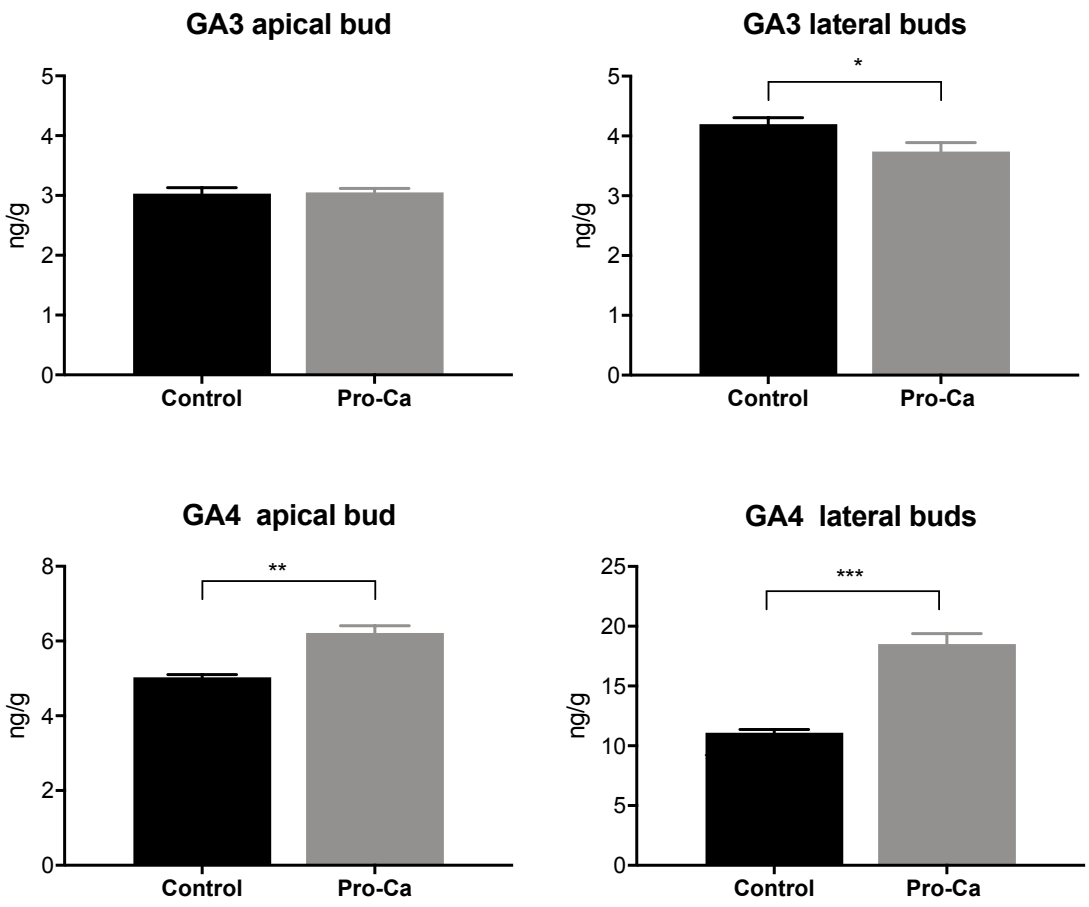


Fig 6. Effect of Pro-Ca on the content of GA3 (above) and GA4 (below) in apical bud (left) and lateral buds (right) in *F. chiloensis f. chiloensis* plants. Samples of control treatment were taken before to Pro-Ca application. Samples of Pro-Ca treatment were taken 4 days after application. Treatments were compared by T-test with 5% significance. Values are mean of 2 biological and 2 technical replicates ± SEM, each one a pool of 3 plants.

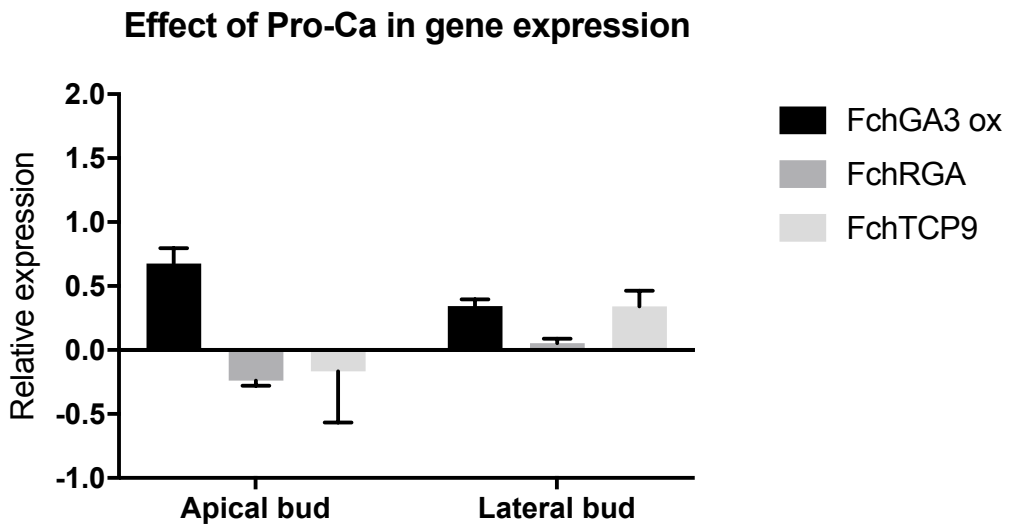


Fig 7. Relative expression of FchGA3ox, FchRGA and FchTCP9 in apical and lateral buds in *F. chiloensis f. chiloensis* after Pro-Ca application. Samples of control treatment were taken before to Pro-Ca application. Samples of Pro-Ca treatment samples were taken 4 days after application. Values are mean of 3 biological and 3 technical replicates ± SEM, each one a pool of 3 plants.

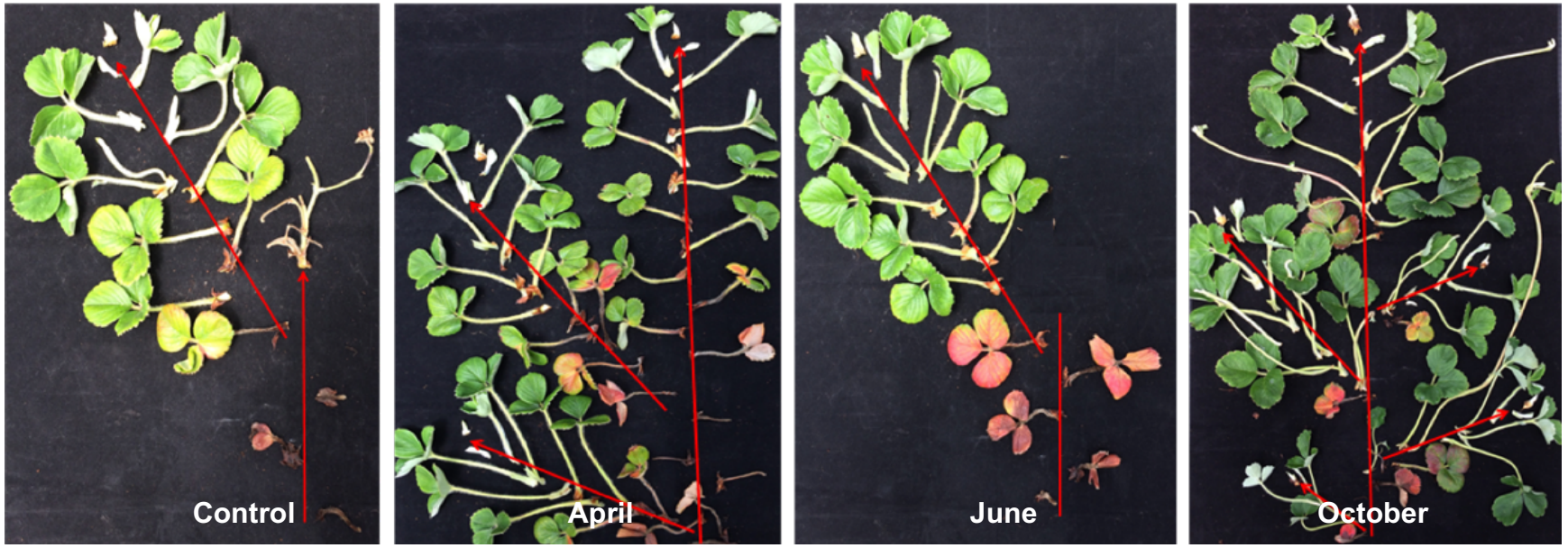
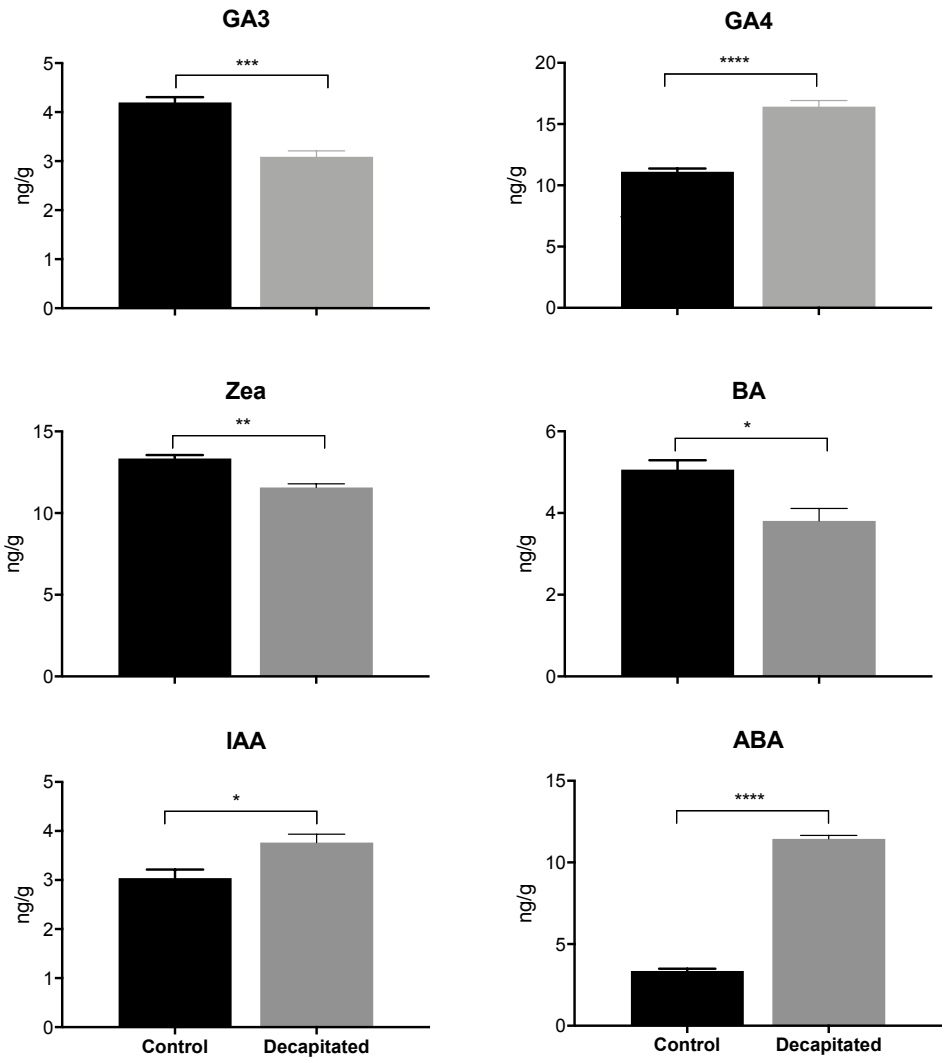


Fig. 8. Effect of decapitation in *F. chiloensis* f. *chiloensis* in the number and type of crowns per plant. From left to right, plants without decapitation, April decapitation, June decapitation and October decapitation.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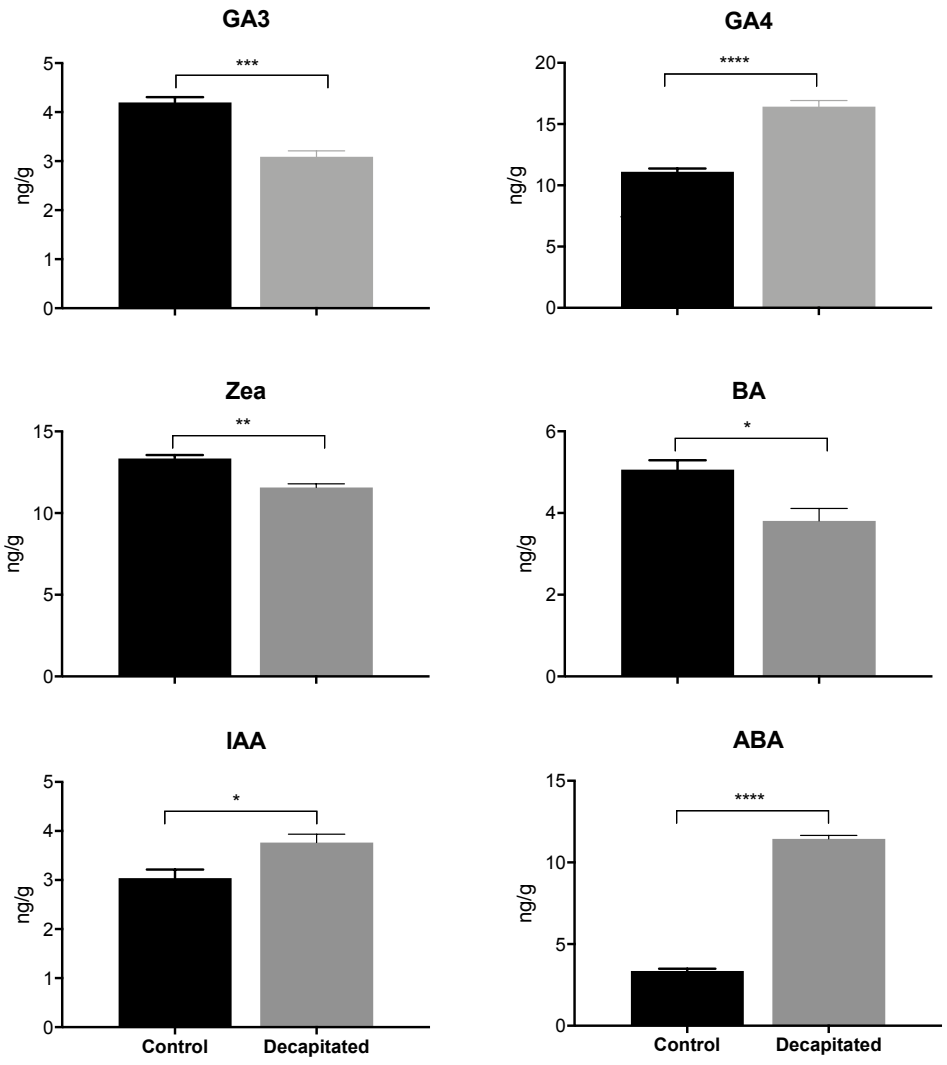


Fig 9. Hormonal profile in *F. chiloensis f. chiloensis*, before and after of decapitation. Samples of decapitation plants were taken 4 day after treatment. Data were compared by T-test with 5% significance. Values are mean of 2 biological and 2 technical replicates ± SEM, each one a pool of 3 plants.

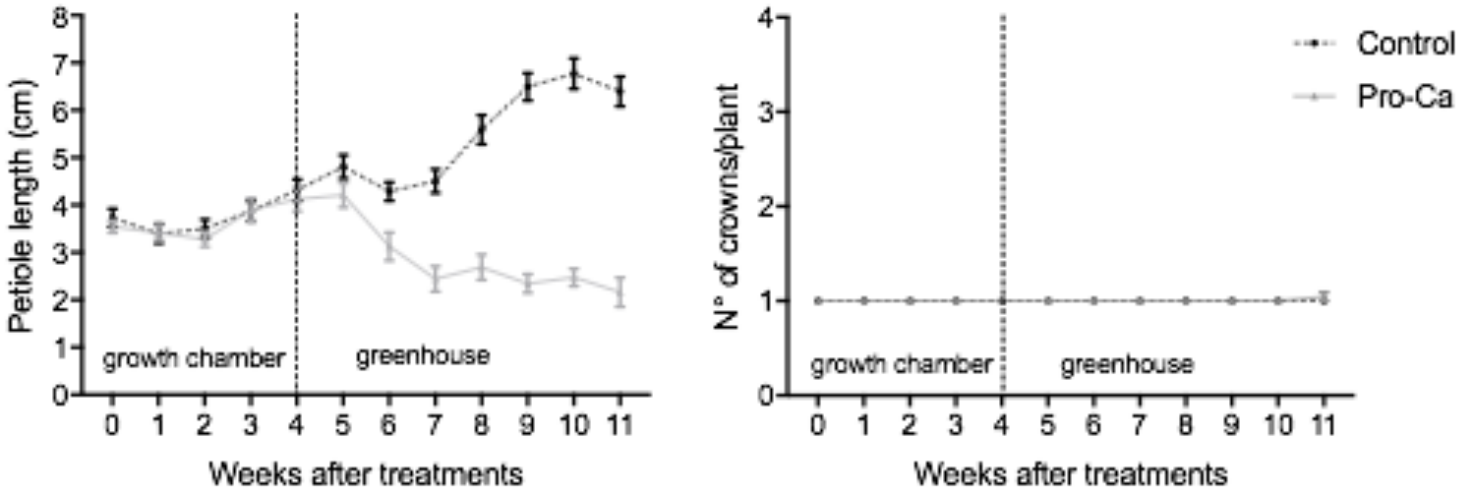
Fig. 10. Natural branching habit, inflorescence and fruit size in *F. chiloensis* subsp. *chiloensis* f. *chiloensis,* 'Chilean white strawberry' (left) and *F. chiloensis* subsp. *chiloensis* f. *patagonica*, wild strawberry (right).

Supplementary material

Supplementary Table 1. Effect of decapitation on the number of crowns and inflorescences per plant in *F. chiloensis* f. *chiloensis* and *F. x ananassa* cv. 'Candonga'.

Treatment	'Chilean white strawberry'		cv. 'Candonga'	
	Number of crown	Number of inflorescences	Number of crown	Number of inflorescences
Control	1.2 ± 0.44	0.3±0.48	6.2 ± 1.63	5.1±1.25
April	2.8 ± 0.44	0.0±0.00	7.0 ± 2.00	5.6±0.8
June	1.0 ± 0.00	0.1±0.32	6.4 ± 1.14	5.2±1.12
October	5.8 ± 2.16	0.0±0.00	w/i	w/i

Values are mean of 5 plants ± SD. w/i without information.



Supplementary Fig. 1. Effect of Pro-Ca application in *F. chiloensis* f. *chiloensis* in the petiole length of the last expanded leaf (left) and crowns per plant (right). Values are mean of 24 plants ± SEM.

Chapter 5

General discussion and final remarks

In the 'Chilean white strawberry' (*F. chiloensis* subsp. *chiloensis* f. *chiloensis*), the productive fruit potential has been indicate as the one of major restrictions for the crop commercial development (Hancock *et al.,* 1999; Retamales *et al*., 2005; Carrasco *et al.,* 2013; Finn *et al.,* 2013). However, there are few studies of flowering in this species. Their comparison with the *F. x ananassa* physiology is the natural way for understanding it.

In this research, a morphological plant description was performed (Grez *et al.,* 2017), which allowed determining the two main processes that affect the flowering; floral induction and branching. In F. x ananassa, both are stimulated by similar conditions but different pathway are involved (Savini *et al.,* 2005; Heide *et al.,* 2013; Mouhu *et al.,* 2015). Under effective environmental conditions in most *Fragaria* genotypes, the differentiation towards inflorescences (floral induction) and lateral crowns (branching) in 'Chilean white strawberry' was significantly lower than in *F. x ananassa*, determining the differences in their flowering habits. Surprisingly, these processes were similarly affected in 'Chilean wild strawberry' and *F. x ananassa*, opening, together with other background, a new hypothesis about the hybrid origin (Gambardella *et al*., 2017).

The flower induction in short day genotypes of *Fragaria* occurs under decreasing temperature and photoperiod in autumn (Taylor *et al.,* 1997; Heide *et al*., 2013). The environmental conditions of this study were effectives in cv. 'Camarosa' but not in 'Chilean white strawberry'. The floral induction could be favored by vernalization, uncommon pathway in *Fragaria* spp. This hypothesis is coincident with the late flowering of this species. A similar behavior was reported by Heide and Sønsteby (2007) in a specific population of *F. vesca*.

The dormancy in *Fragaria* also occurs in autumn under decreasing temperatures and day-length (Koosin *et al*., 2001; Sønsteby and Heide, 2006; Heide *et al*., 2013).The vegetative activity decrease to prevent the damage in the formed flower during the winter, and the sprouting and flowering are conditioned to chilling accumulation (Chouard, 1960; Lang, 1965; Atkinson *et al*., 2013). Therefore, the flowering can be affected if this process is not completed. The 'Chilean white strawberry' enter into dormancy late in the autumn and require near to 900-1,000 chilling hours to dormancy release, amount currently covered in the cultivated area in Chile (Grez *et al*., 2020).

The flowering of 'Chilean white strawberry' in Chile has been historically low, with 1 inflorescence per plant. However, in the last decades, the number of flowering plants has decreased. The climate change could be the cause of this detrimental effect due to low temperatures required to flower induction (Grez *et al*., 2020). In addition, the chilling requirements for dormancy release could be less covered in the next years due to the temperatures increase (Atkinson et al., 2013).

Branching in *F. x ananassa* improves in response to a decrease in GA content, mediated by a decrease in day length. The Prohexadione-Calcium is a GA inhibitor that is being used to improve this trait, and therefore the fruit production (Black, 2004; Hytönen *et al*., 2008, Hytönen *et al*., 2009). The application of Pro-Ca in 'Chilean white strawberry' had no effect in branching, unlike what has been observed in *F. x ananassa* The apical dominance that present this species prevents the emission of new lateral axes since the crowns number was improved after apex removal. This response was associated with a GA3 decrease, but possibly also to the elimination of the source of auxin.

This work is the first in-depth study of floral physiology in the 'Chilean white strawberry'. However, new knowledge are required, specifically in flower induction and branching processes. Their application would allow to adjust the plant propagation and cultivation techniques in the 'Chilean white strawberry' to obtain a better fruit production and avoid the disappearance of this important crop in Chile.

Appendix

Table 1. Botanical description of 'Chilean white strawberry' (*Fragaria chiloensis* (L.) Mill. subsp. *chiloensis* f. *chiloensis*) according UPOV guideline.

Plant	
Growth habit	semi-upright
Density of foliage	medium
Vigor	medium
Stolon	
Anthocyanin coloration	absent
Density of pubescence	medium
Direction of pubescence	upward
Leaf	
Size	medium
Color	ligth green
Color intesity	medium
Brightness	weak
Pubescence	dense
Variegation	absent
Shape of base	obtuse
Margin	serrate

Petiole	
Pubescence	dense
Direction of pubescence	horizontal
Anthocyanin coloration	absent
Flower	
Diameter	large
Size of calyx in relation to corolla	smaller
Sex	hermaprodite
Color	white
Inflorescence	
Numbers of flowers	medium
Position in relation to foliage	beneath
Petal	
Number per flower	7,5
length in relation to width	equal
Color	white
Arrangement	overlapping

Fig. 1. Morphological characteristics of plant, flower, inflorescence and fruit of 'Chilean white strawberry' (*Fragaria chiloensis* (L.) Mill. subsp. *chiloensis* f. *chiloensis*).