Can "Caricia" and "Princesa" apples be considered low-chilling cultivars?

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ABSTRACT. The purpose of this work was to study the response of two apple cultivars bred for low chilling environments to artificial chilling accumulation. Two trials were carried out; in experiment one, excised shoots were randomly taken from "Caricia" and "Princesa", and in experiment two, intact and excised shoots of "Caricia", "Princesa" and "Gala" (control) were collected. After collection, both shoot types were exposed to artificial chilling accumulation (4.0 ± 0.5°C) from 0 to 1200 chill units (CU). Bud break of mixed buds of "Caricia" and "Princesa" was higher than 50% between 0 CU to 1200 CU, irrespective of shoot type. Bud break of "Gala" mixed buds exceeded 50% only in intact shoots after accumulating 900 CU. The mean time to bud break of "Caricia" and "Princesa" diminished with increasing chilling accumulation and stabilized after ∼600 CU, depending on the type of shoot and the year of experimentation. The low-chill apple cultivars tested in this work showed shallow dormancy, but they required moderate cold accumulation (800 – 1150 CU) to fully satisfy their chilling requirements. Thus, although their shallow dormancy makes them suitable for cultivation in chill-deficient environments, they cannot be considered low-chill cultivars.

Keywords: *Malus domestica* Borkh, dormancy, bud break, lack of chilling, heat requirements.

Os cultivares de maça "Caricia" e "Princesa" podem ser considerados de baixo requerimento de frio?

RESUMO. O objetivo deste trabalho foi avaliar a resposta ao acúmulo de refrigeração artificial de dois cultivares de maça de baixo requerimento de frio. Coletaram-se ramos a campo das cultivares "Caricia" e "Princesa", e da cultivar "Gala" (utilizada como controle), com e sem gemas apicais (ramos intactos e decapitados, respectivamente), as quais foram expostas ao acúmulo de refrigeração artificial (4.0 ± 0.5°C) de zero a 1200 unidades de frio (UF). A brotação das gemas em ambos os tipos de ramos foi maior do que 50% em todo o intervalo de acumulação de frio avaliado em "Caricia" e "Princesa". No entanto, em "Gala·" a brotação excedeu 50% somente nos ramos intactos depois de acumular 900 UF. Observou-se que o tempo médio para a brotação das gemas de "Caricia" e "Princesa" estabilizou-se acima das 600 UF, dependendo do tipo de ramo e do ano de experimentação. As gemas dos cultivares avaliados neste trabalho mostraram uma dormência pouco profunda, no entanto precisaram de acumulação moderada de frio (800 – 1150 UF) para satisfazer seus requerimentos ecofisiológicos. Portanto, apesar da dormência pouco profunda permitir que estes cultivares sejam adequadamente cultivados em zonas com invernos amenos, não podem ser considerados de baixo requerimento de frio.

Palavras-chave: *Malus domestica* Borkh, dormência, brotação, inverno ameno, requerimento térmico.

Introduction

Apple production in chill-deficient environments became possible with the release of low-chill apple cultivars (Hauagge & Cummins, 2001). Low-chill apple production has since been extended to the tropical and subtropical regions of Latin America, Africa and Asia (Ashebir et al., 2010; Castro, Cerino, Gariglio, & Radice, 2016; Mohamed, 2008; Njuguna, Wamocho, & Morelock, 2004; Pommer, & Barbosa, 2009). Furthermore, in the Americas, novel cultivars, such as "Caricia" and "Princesa", are becoming important in mild-winter areas either for cultivation or as genetic material for apple breeding programs (Pommer, & Barbosa, 2009).

One of the major challenges of temperate-zone fruit production in warm-winter areas is to overcome the dormancy period (Erez, 2001). Dormancy has been defined as the inability to initiate growth from meristems or other organs and cells, with the capacity to resume growth under

favorable conditions (Rohde, & Bhalerao, 2007). In natural conditions, dormancy release and growth resumption in apple and other temperate deciduous fruit trees is mediated by a quantitative accumulation of chilling (Alburquerque, García-Montiel, Carrillo, & Burgos, 2008; Gariglio, Weber, Castro, & Micheloud, 2012; Hauagge & Cummins, 1991c; Oukabli & Mahhou, 2007; Rahemi & Pakkish, 2009). However, the chilling requirement is highly variable, depending on the genotype (Hauagge & Cummins, 2001), the environmental conditions in autumn (Heide, 2003) and the type of bud (apical versus lateral) (Erez, 2001).

Low-chill apple cultivars cultivated in mild-winter areas frequently show symptoms of a lack of chilling. Such symptoms comprise low bud break, erratic and delayed flowering, low seed set under open pollination and a shift to self-fertility (Castro et al., 2016; Erez, 2001; Mohamed, 2008). Consequently, chemical dormancy-breakers are usually applied in these mild-winter areas to promote the growth and flowering of low-chill apple cultivars (Botelho, & Müller, 2007; Erez, 2001; Mohamed, 2008; Njuguna et al., 2004).

Knowledge of the behavior of the apical and lateral buds under a wide range of chilling accumulation conditions is necessary to predict their performance in warm-winter regions. This knowledge is essential in the global warming context that will affect the chilling availability in warm regions (Luedeling, Girvetz, Semenov, & Brown, 2011).

Hence, the aim of this work was to determine the chilling requirements of two low-chill apple cultivars ("Caricia" and "Princesa"). We hypothesized that the chilling requirements of these low-chill apple buds (cv. "Caricia" and "Princesa") reside within the range of 0 to 600 CU. We expected that within this range of cold accumulation, a stabilization of the mean time to bud break (MTB) and bud break over 50% after adequate forcing would be observed.

Material and methods

The experiments were carried out in the experimental field of the *Facultad de Ciencias Agrarias* of the *Universidad Nacional del Litoral (CECIF)*, Santa Fé, Argentina (31° 26' S; 60° 56' W.; 40 m above sea level) during two years, 2011 and 2014.

Experiment 1

Seven-year-old apple trees (*Malus × domestica* Borkh.) of "Caricia" (IAPAR 77; "Anna" × "Prima") and "Princesa" ("Anna" × "NJ56") grafted onto "M9" rootstocks were used. According to the literature, the chilling requirement of these cultivars ranges between 350 and 450 chill units (CU) (Denardi, Hough, & Camilo, 1988; Hauagge, & Tsuneta, 1999).

In May (end of autumn in the southern hemisphere) of 2011, one-year-old shoots without apical mixed buds were randomly collected from 20 plants of each cultivar. The chilling that had accumulated before the collection date was 10 chilling hours below 7°C. The remaining leaves were removed, and the collected shoots were cut into pieces 15 cm long. Their basal and non-lignified apical portions were discarded. In the resulting excised shoots, the uppermost buds (near the wound) were eliminated. Thus, only the three central mixed buds were left on every excised shoot. Then, the excised shoots were treated with carbendazim [methylbenzimidazol-2-ylcarbamate] (2 mL L^{-1}) for 10 minutes and allowed to dry naturally on absorbent paper.

Five groups of 40 excised shoots per cultivar were placed in plastic bags and exposed to artificial low temperature (4.0 ± 0.5°C) in a cold chamber to simulate five chilling accumulation treatments: 0, 300, 600, 900 and 1200 chilling hours (CH). According to the Utah Model, one hour at 4°C is equivalent to 1 Chilling Unit (Richardson, Seeley, & Walker, 1974). Thus, the chilling accumulation treatments were expressed as chilling units. All shoots were placed in darkness and horizontally within the cold chamber until cold treatment was finished.

After cold treatment, the shoots from each treatment were divided into 8 groups of 5 shoots each. Each of these groups was placed in a 250-cm^3 plastic container, with their basal tip in a sodium hypochlorite solution (1:1000 v/v), and kept for 30 days in a growth chamber at 25 ± 0.5°C, with a 16-hour photoperiod, and 50 μmol m^{-2} s^{-1} light intensity to force bud break. The basal tips of the shoots were cut weekly, and water was replaced daily.

The bud break times of the mixed buds were recorded every other day. Bud break occurrence was defined as the time when the mixed buds reached stage 53 on the pome fruit BBCH scale (Meier, 2001). The bud break percentage (BP) and the mean time to bud break of the mixed buds (MTB) were obtained using the following equations:

$$BP\ [\%] = \sum_{1}^{j} \frac{\sum_{1}^{n} Bi/n}{j} \times 100$$

where j is the number of excised shoots per experimental unit ($j = 5$), B_i is the i-th bud in the j-

th excised shoot broke within the forcing period, and *n* is the number of buds per excised shoot (*n* = 3).

$$\text{MTB [days]}= \sum_{1}^{j} \frac{\sum_{1}^{n} Ti/n}{j}$$

where *j* is the number of excised shoots per experimental unit (*j* = 5) and *Ti* is the time from the beginning of the forcing period at 25 ± 0.5ºC to the occurrence of budbreak of the i-th bud in the j-th excised shoot and *n* is the number of buds per excised shoot (*n* = 3). We considered the MTB to have stabilized when an inflection point was observed above which the reduction in its value was less than one day (e.g.; the MTB value tended to a limiting value).

The experimental model was a 2×5 factorial and completely randomized design (CRD) with two cultivars and five chilling accumulation treatments. The experimental unit was a container with 5 excised shoots. Each experimental unit was repeated eight times (n = 40 containers and 200 excised shoots per cultivar).

The data were analysed with general linear models (GLM) adjusted with the *lme* function of the *nlme* package (Pinheiro, Bates, DebRoy, & Sarkar, 2011) of the R statistical language (R Development Core Team, 2011) using the InfoStat interface (Di Rienzo et al., 2012). The results from the GLM indicated the significance of a cultivar × chilling treatment effect for MTB; hence, regression analysis was performed. To test the effect of each cultivar within a chilling treatment interval, data for the cultivars within the chilling interval were pooled, and cultivar was included as a dummy variable in the regression model. The linear, quadratic and cubic components of chilling treatment were included in this analysis. The selection of variables was made, and the best model, chosen by the backward elimination procedure of InfoStat (Di Rienzo et al., 2012).

Normality and homoscedasticity were tested graphically with a Q-Q plot, and a plot of residuals vs. predictors, respectively).

Experiment 2

Seven-year-old apple tree (*Malus × domestica* Borkh.) "Caricia", "Princesa" and "Gala" apples grafted onto "M9" rootstocks were used for the experiment. The "Gala" cultivar has a high chilling requirement (> 800 CU) according to Hauagge, and Cummins (1991c) and was utilized as a control cultivar.

In May (end of autumn in the southern hemisphere) of 2014, one-year-old shoots with (intact shoots) and without (excised shoots) apical

buds were randomly collected from 20 plants of each variety. No chilling accumulation had been recorded before the collection date. The remaining leaves were removed. All shoots were cut into pieces 15 cm long; the basal and non-lignified apical portions of excised shoots (without apical buds) were discarded. Furthermore, in the resulting excised shoots, the uppermost buds (near the wound) were eliminated. Thus, only the three central mixed buds were left on each excised shoot.

However, in the intact shoots (with apical bud), only the basal portion was removed. Intact shoots were used in addition to excised shoots to compare the effect of chilling accumulation on lateral versus apical mixed buds. Thus, in the intact shoots, only the apical and three central mixed buds were left.

The experimental conditions, sample handling procedures and variables analysed were the same as in Experiment 1.

The experimental model was a 3×2×5 factorial CRD with three cultivars, two types of shoots and five levels of chilling accumulation. The experimental unit was a container with 5 excised shoots. Each experimental unit was repeated eight times (n = 80 containers and 400 shoots per cultivar). The data were analysed using GLMs as in experiment 1. As interactions on MTB and BP were detected, data for each cultivar and shoot type within the chilling interval were pooled. Thus, "cultivar" and "shoot type" effects were included as dummy variables in the regression model. In this analysis, the linear, quadratic and cubic components of chilling treatment and dummy variables were included. Variables were selected, and the best model, chosen by a backward elimination procedure using InfoStat (Di Rienzo et al., 2012).

To compare the season effect on the chilling response of "Caricia" and "Princesa" mixed buds, data of excised shoots of both experiments (2011 and 2014) were analyzed together in a 2×2×5 factorial in a CRD (two seasons, two cultivars and five levels of cold accumulation). The data were analysed using GLM, as previously described. The MTB and BP response was modelled by regression analysis using "cultivar" and "season" as dummy variables. As in the previous regression analysis, variables were selected, and the best model, chosen by a backward elimination procedure using InfoStat (Di Rienzo et al., 2012).

Results and discussion

In experiment 1, the MTB of lateral mixed buds decreased significantly with increasing chilling accumulation, but the response differed between the varieties, with a highly significant interaction between

the factors cultivar and chilling (p = 0.001, r^2 = 0.59). The variation in MTB in response to chilling accumulation was fit to a cubic function on both apple cultivars, a stabilization point at approximately 800 CU was found according to our criterion (Figure 1A). The intercept of both curves was the same (p = 0.10), but the slopes of the models showed significant differences between the varieties (p < 0.01). In fact, "Caricia" excised shoots were the least sensitive to chilling accumulation (Figure 1A). Consequently, the MTB decreased by only seven days in the range of 0 to 1200 CU. In contrast, the MTB of "Princesa" excised shoots decreased by approximately ten days in the same range (Figure 1A).
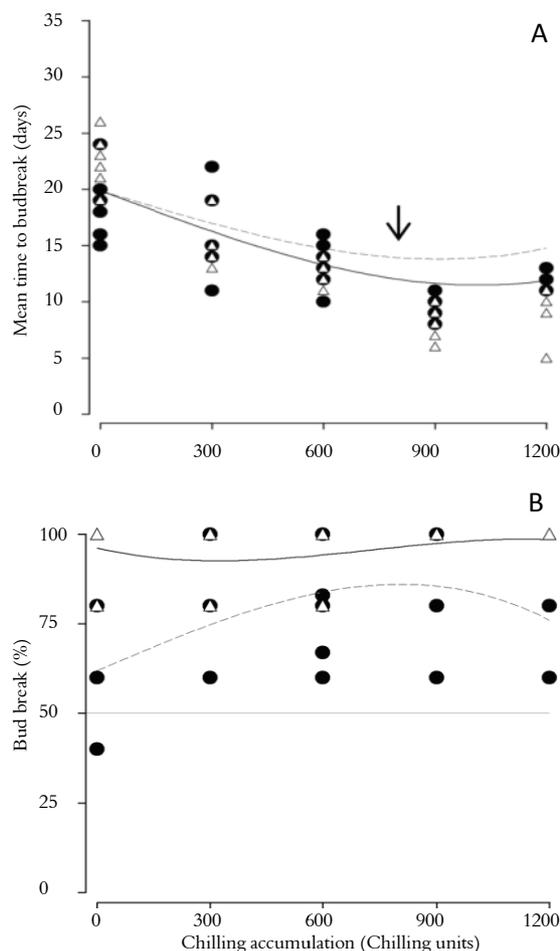

Figure 1. A) Effect of chilling accumulation on mean time to bud break in excised shoots of "Caricia" (dotted line, black circles) and "Princesa" (solid line, white triangles) apples. All curves were adjusted to a cubic regression model against the chilling accumulation (Caricia model: $MTB = 19.27 - 7.33 \star 10^{-3} \star CU - 9.69 \star 10^{-6} \star CU^2 + 9 \star 10^{-9} \star CU^3$. Princesa model: $MTB = 19.97 - 1.07 \star 10^{-2} \star CU - 9.69 \star 10^{-6} \star CU^2 + 9 \star 10^{-9} \star CU^3$). The arrow indicates the beginning of stabilization of the MTB values according to our criterion. B) Effect of cultivar and chilling accumulation on the bud beak percentage of excised shoots of "Caricia" and "Princesa" apples. (Caricia model: $PB = 61.94 + 0.04 \star CU - 2.30 \star 10^{-8} \star CU^3$. Princesa model: $PB = 96.2 - 0.03 \star CU + 5.0 \star 10^{-5} \star CU^2 - 2.30 \star 10^{-8} \star CU^3$). The excised shoots had lateral buds only. Data for experiment 1.

The bud break percentage (BP) of lateral buds was affected both by the chilling accumulation treatments (p = 0.001) and by the cultivar (p = 0.001; r^2 = 0.26). The BP was higher in "Princesa" than "Caricia" (Figure 1B). Additionally, in both cultivars the BP was slightly greater than 900 CU; nevertheless, both cultivars showed a BP higher than 50% over the entire range between 0 to 1200 CU.

The fact that the BP of low-chill apples is quite stable with respect to chilling accumulation is a well-known trait of those genotypes that is crucial to their adaptation to warm-winter areas (Hauagge, & Cummins, 2001). In low-chill genotypes, the rate of bud break appears to be more related to a reduced need to accumulate growing degree days for sprouting than an effect of chilling (Hauagge, & Cummins, 1991a; Putti, Petri, & Mendez, 2003). However, the MTB value of those cultivars is affected both by the chilling accumulation and by the accumulation of growing degree-days. In fact, the rate of bud break of "Anna" apple buds was approximately 90% from zero CU to 1000 CU, but the time needed to reach this rate of bud break diminished linearly from 14 days to 2 days over the same interval of chilling accumulation (Hauagge, & Cummins, 1991b). This evidence indicates that the chilling is not necessary to promote bud break in this cultivar, but affects its speed.

In experiment 2, the MTB for mixed buds was significantly affected by an interaction between cultivar, chilling treatment and shoot type (p = 0.002; r^2 = 0.75). The MTB of "Caricia" intact shoots was greater than the MTB of excised shoots (Figure 2A). The regression analysis showed that for both shoot types, the effects of the linear, quadratic and cubic components of chilling accumulation and the slopes of the curves were significant (p < 0.02; r^2 = 0.72). Hence, the initial MTB of both types of shoots was the same, whereas the MTB response to chilling accumulation was significantly different (p < 0.001). A maximum value was observed at 425 CU accumulation on intact shoots. According to our criteria, a stabilization of MTB up to 1150 CU was observed. In contrast, the MTB of excised shoots was higher at 175 CU and displayed stabilization up to 900 CU accumulation.

As in "Caricia", the MTB of "Princesa" intact shoots was greater than the MTB of excised shoots (Figure 2B). Additionally, the effects of the linear, quadratic and cubic components of chilling accumulation and slopes of the curves differed significantly between the two types of shoots (p < 0.03; r^2 = 0.53). The MTB of intact shoots exhibited a maximum value at 350 CU. A stabilization of MTB up to 1150 CU was observed. In contrast, the MTB of excised shoots reached a maximum value at 0 CU and displayed stabilization up to 1100 CU.
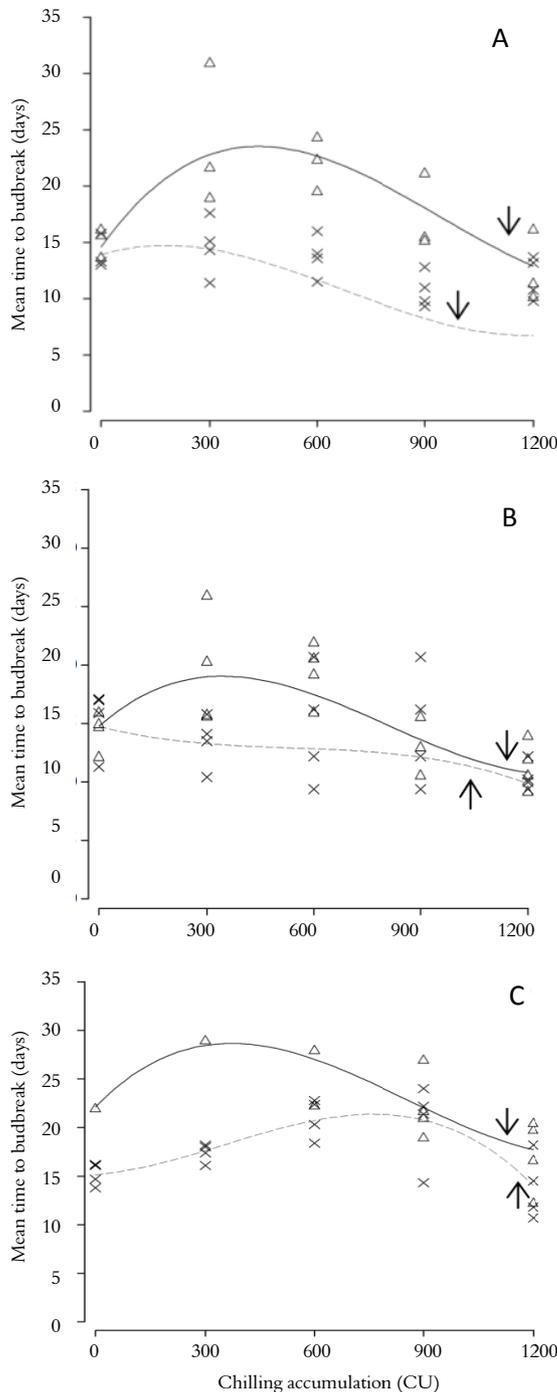
Figure 2. Effect of chilling accumulation on the MTB of intact (solid lines and white triangles) and excised shoots (dotted lines and crosses). The arrows indicate the approximate stabilization points of MTB detected on both types of shoots, according to our criterion. (A) "Caricia" (Intact shoot model: $MTB = 14.58 + 4.56 \star 10^{-2} \star CU - 6.80 \star 10^{-5} \star CU^2 + 2.40 \star 10^{-8} \star CU^3$. Excised shoot model: $MTB = 13.9 + 0.01 \star CU - 3.25 \star 10^{-5} \star CU^2 + 1.6 \star 10^{-8} \star CU^3$). (B) "Princesa" (Intact shoot model: $MTB = 14.76 + 2.75 \star 10^{-2} \star CU - 5.08 \star 10^{-5} \star CU^2 + 2.1 \star 10^{-8} \star CU^3$. Excised shoot model: $MTB = 14.76 - 8.09 \star 10^{-3} \star CU + 1.29 \star 10^{-5} \star CU^2 - 8.0 \star 10^{-9} \star CU^3$). (C) "Gala" (Intact shoot model: $MTB = 22.14 + 3.87 \star 10^{-2} \star CU - 6.65 \star 10^{-5} \star CU^2 + 2.6 \star 10^{-8} \star CU^3$. Excised shoot model: $MTB = 15.15 + 2.98 \star 10^{-3} \star CU + 2.43 \star 10^{-5} \star CU^2 - 2.3 \star 10^{-8} \star CU^3$). The intact shoot had an apical bud, whereas the excised had lateral buds only. Data for experiment 2.

The data showed that lateral buds on excised shoots of "Caricia" and "Princesa" enter into dormancy before apical buds on intact shoots (i.e., the MTB of excised shoots showed a maximum earlier than intact shoots). Furthermore, the dormancy of the lateral buds on excised shoots was shallower as their MTB was smaller than that of apical buds on intact shoots.

As in the tested low-chill cultivars, the MTB of "Gala" intact shoots was greater than the MTB of excised shoots (Figure 2C). In both types of shoots, the linear, quadratic and cubic components of chilling accumulation, as well as the intercepts and slopes of the curves, were significant predictors of the MTB response ($p < 0.05$; $r^2 = 0.76$). The MTB of intact shoots increased up to 375 CU and then decreased continuously, showing apparent stabilization at 1100 CU. In the same way, the MTB of excised shoots rose to a maximum value at 750 CU, and stabilization seems to begin at 1175 CU. In both types of shoots, true stabilization of MTB likely occurs above 1200 CU.

BP varied depending on the shoot type and cultivar and with chilling accumulation ($p < 0.05$; $r^2 = 0.57$) (Figure 3). The quadratic and cubic components of chilling accumulation, as well as the intercepts and slopes of the curves, were significantly different. The bud break percentages of intact and excised shoots of "Caricia" and "Princesa" were higher than "Gala". However, within each cultivar, the BP of intact shoots was smaller than the BP of excised shoots below 900 CU. However, the BP of both shoot types of "Caricia" and "Princesa" was greater than 50% over the entire evaluated range of chilling. In contrast, the BP of excised shoots of "Gala" never exceeded 50%. Furthermore, bud break of intact shoots on this cultivar surpassed 50% only after 900 CU had accumulated. Noting the data of all cultivars, incremental rates of bud break per unit of chilling accumulated in intact shoots were greater than those in excised shoots, indicating that intact shoots' dormancy was deeper and more strongly influenced by chilling accumulation.

The differential response of lateral and apical buds has been related to their different status and dynamics of dormancy in relation to chilling accumulation (Dennis, 2003; Hauagge, & Cummins, 1991b; 1991c). However, apple buds do not behave uniformly in this respect (Dennis, 2003). Our research demonstrates that the dormancy of apical buds of "Caricia", "Princesa" and "Gala" grown in a mild-winter area is more intense than that of lateral buds. Furthermore, the dormancy of apical buds of "Caricia" and "Princesa" apples was less intense than those of medium-chill cultivars such as "Gala". Our results agree with the data reported by Hauagge, and Cummins (1991b); they found that the time required for bud break (which is equivalent to MTB) of apical buds of low-chill apples was lower than that required for medium-chill cultivars. Additionally, they found that excised shoots had shallower dormancy than intact shoots on low and medium-chill cultivars.
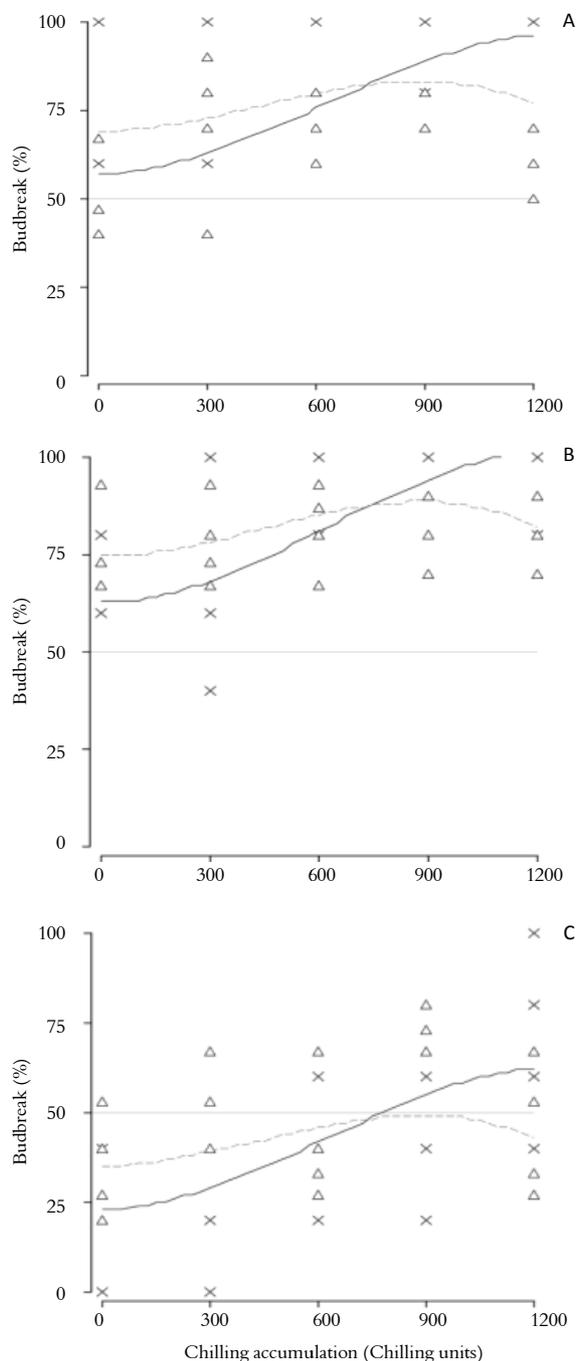
Figure 3. Bud break of mixed buds on intact (solid line and white triangles) and excised shoots (dotted lines and crosses) as an effect of cultivar × chilling accumulation × shoot type interaction. (A) "Caricia" (Intact shoot model: *PB = 0.57+7.5*10^-7*CU^2—4.0*10^-10*CU^3*. Excised shoot model: *PB = 0.57+5.32*10^-7*CU^2—4.0*10^-10*CU^3*). (B) "Princesa" (Intact shoot model: *PB = 0.63+7.5*10^-7*CU^2—4.0*10^-10*CU^3*. Excised shoot model: *PB = 0.63+5.32*10^-7*CU^2—4.0*10^-10*CU^3*). (C) "Gala" (Intact shoot model: *PB = 0.23+7.5*10^-7*CU^2—4.0*10^-10*CU^3*. Excised shoot model: *PB = 0.23+5.32*10^-7*CU^2—4.0*10^-10*CU^3*). The intact shoot had an apical bud, whereas the excised had lateral buds only. Data from experiment 2. Horizontal gray line indicates a threshold of 50%.

If the MTB of lateral buds is analysed between years, an interaction effect of cultivar ×

chilling accumulation × year can be seen (p = 0.01; r^2 = 0.76). The regression analysis on "Caricia" MTB showed that the quadratic and cubic components of chilling accumulation, as well as the intercepts and slopes of the curves, were statistically significant (p < 0.02; r^2 = 0.53) (Figure 4A). The same pattern was observed for "Princesa" MTB (p < 0.001; r^2 = 0.75) (Figure 4E). In both cultivars, the MTB of excised shoots stabilized up to 600 CU, with differences between years (Figure 4).

However, bud break was greater in 2011 than in 2014 and was affected by chilling accumulation in both cultivars (p < 0.05) (Figure 4). The discrepancies observed between the two years of experimentation can be explained by slightly different initial statuses of dormancy of buds on excised shoots at the time of their collection. Although a relationship between MTB and BP with respect to chilling accumulation was evident in experiment 2, the patterns were slightly different than in experiment 1. In experiment 2, the initial dormancy intensity of "Caricia" and "Princesa" was shallower than in experiment 1 (Figure 4). Therefore, the subsequent dynamic of dormancy was different in its response to increases in chilling accumulation; however, in both experiments, stabilization of the MTB up to 600 CU was observed. In contrast, Putti et al. (2003) reported no evident MTB stabilization in "Condessa", "Gala" or "Fuji" excised shoots and a rise in BP with chilling accumulation until 1500 CU, when samples were collected without chilling accumulation.

Dormancy is induced in apples when temperatures fall below 12ºC (Heide, & Prestrud, 2005). Hence, with moderate variations in chilling availability at the moment of sample collection, slight variations in initial bud dormancy are expected. Thus, it was likely that the temperature in the cold chamber was slightly different in its effects on the progression of dormancy in experiments 1 and 2. Different effects of different low-temperature regimes on dormancy progression in buds collected with different initial dormancy intensity have been observed in apricots (Campoy, Ruiz, & Egea, 2011). However, in our experiments, there was a clear response of apical and lateral buds to chilling accumulation. Furthermore, for excised shoots the general MTB response pattern was very stable between years and cultivars (i.e., MTB was stable over 600 CU, with some differences between years and cultivars).
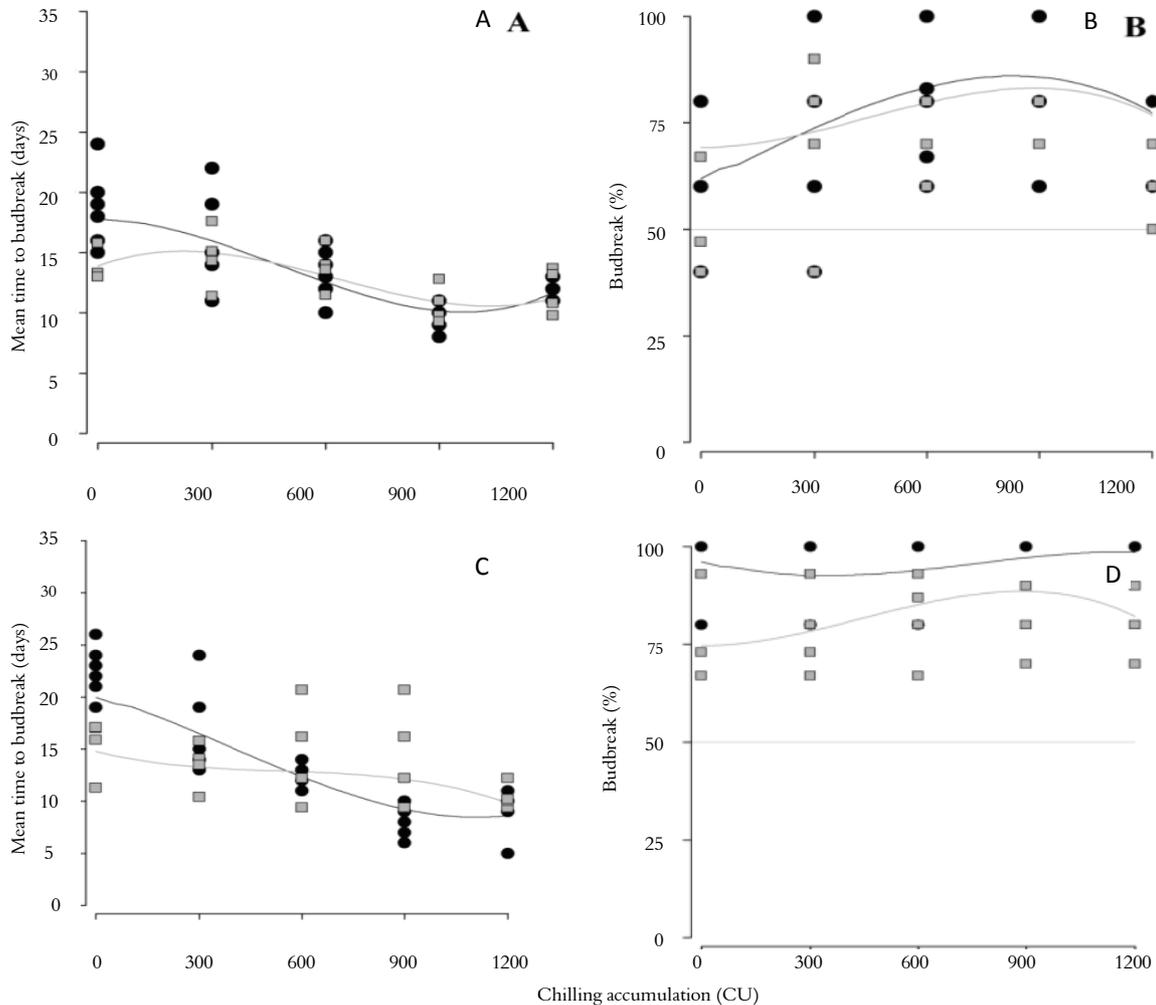
Figure 4. Mean time to bud break (days) and bud break (%) of mixed buds on excised shoots as an effect of chilling accumulation × cultivar × year interaction for cv. "Caricia" (A-B) and "Princesa" (E-F). Black circles and lines correspond to 2011, and the gray squares and lines, to 2014. Horizontal gray lines on figures B and D indicate a threshold of 50%. The equations for MTB and PB and the values of the coefficients are shown in previous figures. The excised shoots had lateral buds only. Data for experiments 1 and 2.

In summary, our results may be interpreted differently depending on how the chilling requirement is considered satisfied in "Caricia" and "Princesa" buds. Dennis (2003), Gardea, Carvajal-Millan, Orozco, Guerrero, & Llamas (2000) and Rahemi, and Pakkish (2009) consider chilling requirements to have been satisfied when the percentage of bud break during the forcing period is over 50%. Under this criterion, it may be assumed that lateral and apical buds of "Caricia" and "Princesa" apples do not require chilling because bud break was always above this threshold value. However, the chilling requirements of the lateral and apical buds of "Gala" would exceed 900 CU (Figures 1 and 3).

In contrast, other researchers consider the chilling requirement to be satisfied only when the MTB response to cold accumulation stabilizes (Balandier, Bonhomme, Rageau, Capitan, & Parisot,

1993). According to Hauagge, and Cummins (1991b), the time required to reach bud break shows less variability and is less subjective than other indices, such as the percentage of bud break. Furthermore, it represents the time necessary to satisfy the heat requirement of the buds. This value is not constant and decreases with increasing chilling accumulation because the bud break process is blocked or at least delayed by dormancy, which increases the activation energy of their biochemical processes (Gardea, Carvajal-Millan, Orozco, Guerrero, & Llamas, 2000). For this reason, when the MTB reaches a stable, low value and does not decrease any further with longer exposure to low temperature, the chilling requirement may be considered satisfied. Hence, the MTB value is the better index for evaluating the depth of bud dormancy (Balandier et al., 1993; Hauagge, &

Cummins, 1991b; Oukabli, & Mahhou, 2007). Following the above discussion, we consider the MTB to have stabilized when an inflection point was observed on the chilling accumulation curve, above which the reduction in its value was less than one day (e.g., the MTB value tended to a limiting value). Using this criterion, we found that the chilling requirement of apical and lateral buds of "Caricia" and "Princesa" was over 600 CU in both experiments, which refutes our research hypothesis. This value represents almost twice the requirement cited for these cultivars (Denardi et al., 1988; Hauagge, & Tsuneta, 1999). Using this same metric, the chilling requirement of apical and lateral buds of "Gala" was over 1200 CU.

It is important to mention that some methodological factors can affect the results. On excised shoots, the cut performed on the shoots can itself promote bud break (wound effect) (Naor, Flaishman, Stern, Moshe, & Erez, 2003). Additionally, the absence of the apical buds, which may dominate lateral buds (Cook, & Jacobs, 1999), can increase the percentage of bud break on the lateral buds of excised shoots. Neither Naor et al. (2003) or Cook, and Jacobs (1999) evaluated the wound effect or the absence of the apical bud on the MTB response or any other variable that expresses the time needed for buds to break (e.g., T50) in their experiments. Furthermore, according to Hauagge and Cummins (1991a), the low-chill apple cultivars never enter into deep endo-dormancy, so low temperatures may not be required to promote bud break in excised shoots. Nevertheless, our data demonstrate that up to 800-1150 CU of chilling accumulation reduces the heat requirement and accelerates the bud break on "Caricia" and "Princesa" apples, with additional chilling having minimal effects.

Despite showing some disadvantages, the methodology used in our work has been suitable for evaluating the chilling requirement or dormancy progression in many fruit species, both under artificial chilling accumulation (Mohamed, 2003; Putti et al., 2003; Rahemi, & Pakkish, 2009) and under natural chilling accumulation (Campoy et al., 2011; Dennis, 2003; Hauagge, & Cummins, 1991c; Mohamed, 2008; Oukabli, & Mahhou, 2007). Moreover, this methodology is one of the most suitable for evaluating chilling requirements because it is possible to control several key factors, such as light, thermal amplitude and temperature (Dennis, 2003).

Despite the above-discussed factors, lateral buds of excised shoots of the low-chill apple cultivars studied in our work showed a significant reduction in dormancy intensity (MTB) with increased chilling accumulation. Consequently, both apical and lateral buds of the tested cultivars showed shallow dormancy but required a moderate chilling accumulation (800–1150 CU) to stabilize their MTB value. The shallow dormancy of "Caricia" and "Princesa" makes them suitable for cultivation in areas with very low chilling availability (Denardi et al., 1988; Hauagge, & Cummins, 2001; Hauagge, & Tsuneta, 1999) despite the occurrence of symptoms of lack of chilling, such us a shift to self-fertility and a wide flowering period (Castro et al., 2016).

Conclusion

The apple cultivars "Caricia" and "Princesa", bred for chill-deficient environments, showed shallow dormancy but required moderate cold accumulation (800–1150 CU) at 4ºC to fully satisfy their chilling requirements. These characteristics make these cultivars suitable for successful cultivation in mild-winter areas, but chill-deficiency symptoms may still be seen in some years without dormancy-breaking treatments. "Caricia" and "Princesa" therefore cannot be considered low-chill cultivars.

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