Pollen meiosis and chilling requirements in sweet cherry

E. Fadón[1,2], M. Herrero [1] and J. Rodrigo[2]

[1] Estación Experimental Aula Dei (CSIC), Zaragoza, Spain; [2] Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Instituto Agroalimentario de Aragón-IA2 (CITA-Universidad de Zaragoza), Zaragoza, Spain.

Abstract

In sweet cherry, as other temperate woody perennials, cycles of growth and dormancy are synchronized with the seasons. Flower primordia survive to low winter temperatures entering a dormant stage, but chilling is also required for a proper blooming. However, the biological mechanisms behind dormancy and chilling requirements are still not fully understood. In other species, pollen meiosis has been reported as one of the first biological events occurring after dormancy, but this process has not been studied in sweet cherry. In this work, pollen development has been characterized under the microscope in the cultivars 'Burlat' and 'Bing' in relation to winter dormancy up to anthesis during five years. The chilling requirements of both cultivars were empirically determined, and the dates of chilling fulfilment were related to meiosis and flowering time. During endodormancy, the anthers presented the sporogenous tissue formed by the pollen mother cells. First changes in pollen mother cells were detected about three weeks after chilling fulfillment as prelude of anther meiosis, which was completed within a week. Then pollen development continued during two or three weeks up to anthesis. Positive correlations were found between the dates of chilling fulfilment, pollen meiosis and full bloom for both cultivars. These results showed the influence of chilling winter temperatures on pollen meiosis time.

Keywords: anther development, dormancy, cold temperatures, Burlat, Bing

INTRODUCTION

In sweet cherry, as other temperate woody perennials, cycles of growth and dormancy are synchronized with the seasons (Kurokura et al., 2013). Flower primordia differentiate in the autumn and, to survive to low winter temperatures, enter a dormant stage (Fadón et al., 2017). During dormancy, growth is arrested and cannot be resumed until pass a certain period under chilling conditions (Perry, 1971; Sedgley and Griffin, 1989). In temperate fruit trees, the term endodormancy refers to dormancy regulated by internal physiological factors, and the term ecodormancy refers to regulation by environmental conditions (Lang et al., 1987). Despite of the importance of this process on cherry production, and the interest of dormancy for the adaptation of sweet cherry to warmer latitudes, the biological mechanisms behind dormancy and chilling requirements are still not fully understood.

Chilling requirements of particular cultivars can be empirically calculated by empirical methods, in which the end of dormancy is estimated when flower buds from cut shoots recovered their capacity to grow under favorable conditions in controlled chambers (Brown and Kotob, 1957). Different models have been developed to quantify chilling temperatures until the date of chilling fulfillment. Among the different models existing (Fishman et al., 1987; Richardson et al., 1974; Weinberger, 1950), the Utah model is the

most used under our conditions. Theoretically, the chilling requirements can be used to predict the capacity of the cultivars to adapt to the different climatic regions of cultivation. However, a drawback of these models is that they do not behave equally well in different climates and latitudes, and there is no agreement on when begin and end to record the temperatures (Dennis, 2003). This is related to the fact that there is a lack of biological markers to determine the dormancy status.

In other *Prunus* species, pollen meiosis has been reported as one of the first biological events occurring after endodormancy (Citadin et al., 2002; Julian et al., 2011, 2014). This led us to explore the process of pollen meiosis in sweet cherry in relation to the breaking of endodormancy. In this work, pollen development has been characterized under the microscope in sweet cherry cultivars 'Burlat' and 'Bing' during winter dormancy up to anthesis over five years, paying special attention when pollen meiosis occurred. The chilling requirements of both cultivars were empirically determined, and the dates of chilling fulfilment were related to meiosis and flowering time.

MATERIAL AND METHODS

Plant material

Two sweet cherry (*Prunus avium* L.) cultivars were selected for this study: 'Bing', the reference cultivar in North America, and 'Burlat', the reference cultivar in Europe (Bargioni, 1996). Three trees per cultivar were used from a cultivar collection located at "Centro de Investigación y Tecnología Agroalimentaria de Aragón" (CITA) in Zaragoza (Spain) at 41º44´30" N, 0º47´00" W and 220 m above sea level. This region is characterized by a Mediterranean- continental dry climate, irregular rainfall and a large temperature range, with cold winters and hot and dry summers.

Determination of chilling and heat requirements

Temperature was recorded daily at a meteorological station located in the experimental orchard at CITA over five years. Winter temperatures varied between years, with a mild winter in 2012, 2014 and 2015, and a cold winter in 2011 and 2013.

To determine the chilling requirements of both cultivars, shoots were weekly sampled from October until the onset of budbreak, in late February (Figure 1 A). Cut shoots were placed in a growth chamber (22 ± 1 ºC) for a week. The breaking of dormancy was established when flower buds increased by at least 30% in fresh weight (Figure 1 B). Chilling requirements was calculated by the sum of chill-units (CU) until the breaking of endodormancy for each cultivar (Richardson et al., 1974). Heat accumulation was measured using the Growing Degree Hours (GDH) model (Richardson et al., 1974).

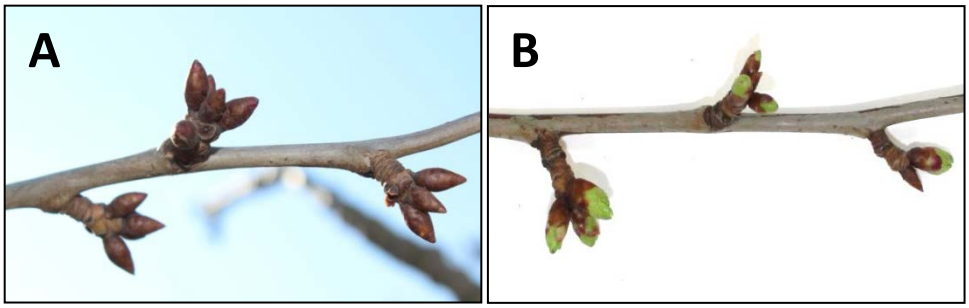

Figure 1: Endo-dormancy determination. (A) Shoots during endodormancy. (B) Bud burst in cut shoots after a week under controlled conditions.

Pollen development characterization

Experiments were performed over five years (2011-2015). Three flower buds per cultivar were randomly collected weekly from mid-winter until flowering time. Flower buds were fixed in ethanol:acetic 3:1 (v/v). To examine male meiosis, anthers were removed from fixed flower buds with the help of a scalpel and mounted by squash with a solution of 0.25 µg/ml of DAPI in 0.05 M TRIS buffer (pH 7.2) to observe nuclei (Williams et al., 1999) followed by adding 0.1% aniline blue in 0.1 N K_3PO_4 to stain callose (Currier, 1957). Microscopic preparations were observed under a UV epifluorescence Leica DM2500 microscope with a 340–380 bandpass and 425 longpass filter. Micrographs were taken with a Leica DFC-310 digital camera for fluorescence microscopy compatible with the Leica Application Suites Version 4.2.0 software (Leica Microsystems, Cambridge, UK).

RESULTS AND DISCUSSION

The breaking of dormancy was established between 21 and 28 December depending on year, when flower buds increased about 60% in fresh weight in 'Bing' and about 40% in 'Burlat'. The calculation of the number of chill-units accumulated until the breaking of endodormancy showed that both cultivars had the same chilling requirements (1082 ± 27 CU). The date of breaking of endodormancy occurred for both cultivars in the last week of December. During the five years of pollen meiosis characterization, chilling fulfillment of cultivars studied occurred in January, presenting an elapse of time of 15-20 days between cold- and mild-winter years. Between 5804 and 6874 GDH for 'Bing' and 6064-6788 GDH for 'Burlat' were required from chilling fulfillment to blooming.

During endodormancy, the anthers showed the sporogenous tissue formed by mononucleated pollen mother cells (Figure 2 A). First changes in pollen mother cells were detected about three weeks after chilling fulfillment as prelude of pollen meiosis, which occurred when callose was layering around the pollen mother cells (Figure 2 B – C). Inside the callose layer chromatin changes associated with meiosis occurred; first division which result in two nuclei cell at telophase I (Figure 2 B) and a subsequent second division until telophase II with four nuclei (Figure 2 C). Cytoplasm division occurred by the formation of inside callose layers until formed the characteristic tetrad (Figure 2 D). Then callose was progressively degraded until microspore released and the formation of the young pollen grains (Figure 2 E). Pollen meiosis was completed within a week, following a conserved pattern (sporogenous tissue, meiosis time, indicated by the pollen mother cells surrounded by callose, tetrad and young pollen grains) common to other model species as *Arabidopsis* (Owen and Makaroff, 1995), tobacco (Koltunow et al., 1990) or tomato (Brukhin et al., 2003). However, pollen development in sweet cherry required several months to complete the process while in annual plants just last few days (Smyth et al., 1990).

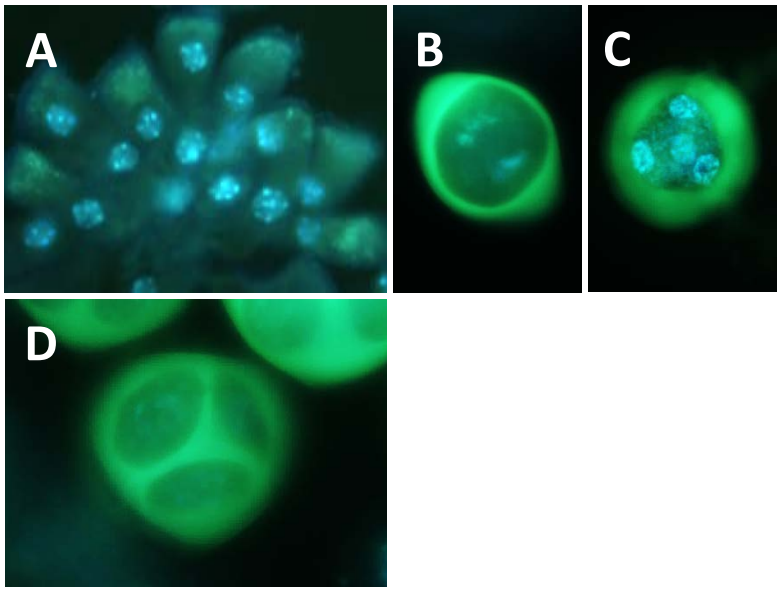

Figure 2: Microsporogenesis in sweet cherry. (A) Esporogenous tissue. (B) Pollen mother cells surrounded by callose at prophase I. (C) Pollen Mother Cells surrounded by callose at Telophase II. (D) Tetrad. (E) Pollen grains. Squashed anthers double stained with Aniline Blue and DAPI.

In both cultivars and in the five years, the anthers presented the sporogenous tissue with pollen mother cells during endodormancy, as it has been reported in sour cherry (Felker and Robitaille, 1985) and apricot (Julian et al., 2011). While in apricot (Julian et al., 2011) and peach (Citadin et al., 2002), meiosis timing was reported to occur just after the chilling fulfilment, first changes in sweet cherry occurred three weeks after chilling fulfillment as prelude of anther meiosis, showing a longer period of ecodormancy than other *Prunus* sp.

The length of ecodormancy was highly variable depending on cultivars and years (Table 1). The timing of meiosis showed few variations between years, occurring in a threshold of ten days at early March for cultivar 'Bing' (Figure 3 A), and about a week before for cultivar 'Burlat' (Figure 3 B). The period from chilling fulfilment to pollen meiosis lasted about six weeks, which is considerably longer than that reported for apricot (Julian et al., 2014). Once meiosis occurred a conserved period of 25-26 days elapsed until flowering. Blooming time showed a variation of two weeks between years, occurring at the end of March and early April for both cultivars (Figure 3).

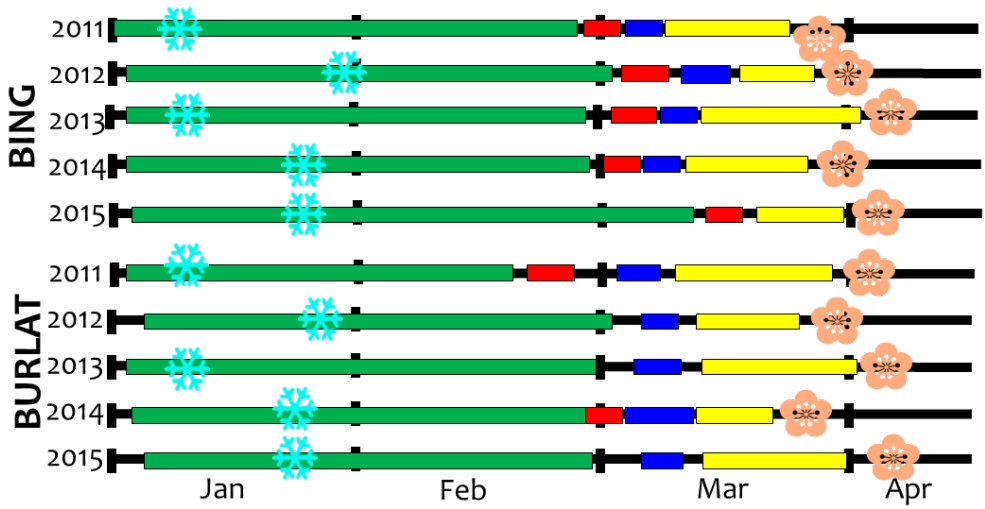

Figure 3: Phases of pollen development in two sweet cherry cultivars over five years. Esporogenous tissue (green); meiosis (red), tetrad (blue) and pollen grains (yellow); chilling fulfilment (snowflakes) and full bloom (pink flower).

Despite of the high variations detected in the number of days from dormancy breaking to blooming (coefficients of variation (CV) between years for each cultivar ranging 30 - 40%), the heat requirements were significantly consistent (CV = 12%) in both cultivars: 6339 ± 535 for 'Bing', and 6411 ± 377 for 'Burlat' (Table 1). These heat requirements were similar to those reported for different sweet cherry cultivars grown under low winter chilling conditions (Alburquerque et al., 2008; Gannouni et al., 2017), suggesting a consistent heat requirements for sweet cherry cultivars. These values are remarkably higher than heat requirements reported for peach (Richardson et al., 1974) and apricot (Julian et al., 2014). Positive correlations were found between the GDH accumulated from chilling fulfilment to pollen meiosis (R_{Bing} = 0.577; R_{Burlat} = 0.624) and full bloom (R_{Bing} = 0.678; R_{Burlat} = 0.645) for both cultivars.

Table 1: Number of days and GDH counted from chilling fulfilment to bloom (heat requirements), from chilling fulfilment to meiosis and from meiosis to bloom for cultivars 'Bing' and 'Burlat'.

	Bing				Burlat			
	Days	CV (%)	GDH	CV (%)	Days	CV (%)	GDH	CV (%)
From chilling fulfilment to bloom	68 ± 19	28	6339 ± 535	8	68 ± 20	29	6411 ± 377	6
From chilling fulfilment to meiosis	42 ± 18	48	3269 ± 429	13	41 ± 18	44	3208 ± 399	12
From meiosis to bloom	25 ± 3	12	3070 ± 381	12	26 ± 3	11	3202 ± 298	9

Values shown are the means ± standard deviation

CONCLUSIONS

During endodormancy, in both cultivars and all years the anthers showed the sporogenous tissue formed by the pollen mother cells. After chilling fulfilment, flower buds were able to growth under suitable conditions during ecodormancy, but no significant changes were observed in the anthers during the next weeks. First changes detected were

associated with meiosis and occurred after a highly variable period of time between years. Thus, in the mild-winter years, meiosis occurred later than in the cold-winter years, showing the influence of chilling winter temperatures on pollen meiosis time in sweet cherry. However, both meiosis and blooming occurred after a constant accumulation of heat from chilling fulfilment.

ACKNOWLEDGEMENTS

This research was supported by Ministerio de Economía, Industria y Competitividad (MEIC) - European Regional Development Fund, European Union (AGL2012-40239); Instituto Nacional de Investigación Agraria (INIA) (RTA2014-00085-00, RFP2015-00015-00); Gobierno de Aragón - European Social Fund, European Union (Grupo Consolidado A-43). E. F. was supported by a grand BES-2010-037992.

Literature cited

Alburquerque, N., García-Montiel, F., Carrillo, A., and Burgos, L. (2008). Chilling and heat requirements of sweet cherry cultivars and the relationship between altitude and the probability of satisfying the chill requirements. Environ. Exp. Bot. *64*, 162–170.

Bargioni, G. (1996). Sweet cherry scions: characteristics of the principal commercial cultivars, breeding objectives and methods. In Cherries: Crop Physiology, Production and Usesp Physiology, A.D. Webster, and N.E. Looney, eds. (Wallingford), pp. 73–112.

Brown, D.S., and Kotob, F.A. (1957). Growth of flower buds of apricot, peach, and pear during the rest period. Proc. Am. Soc. Hortic. Sci. *69*, 158–164.

Brukhin, V., Hernould, M., Gonzalez, N., and Chevalier, C. (2003). Flower development schedule in tomato *Lycopersicon esculentum* cv . sweet cherry. Sex Plant Reprod. *15*, 311–320.

Citadin, I., Bassols, M.C., Gilberto, F., Baptista, J., Centellas, A., and Posser, C.A. (2002). Meiosis stage as an indicator for peach endodormancy. Rev. Bras. Frutic. *24*, 23–28.

Currier, H.B. (1957). Callose substance in plant cells. Am. J. Bot. *44*, 478–488.

Dennis, F. (2003). Problems in standardizing methods for evaluating the chilling requirements for the breaking of dormancy in buds of woody plants. HortScience *38*, 347–350.

Fadón, E., Herrero, M., and Rodrigo, J. (2017). Flower bud development and chilling requirements in cherry cv. "Bing." Acta Hortic. *1161*, 361–366.

Felker, F.C., and Robitaille, H.A. (1985). Chilling accumulation and rest of sour cherry flower buds. Jounal Am. Soc. Hortic. Sci. *110*, 227–232.

Fishman, S., Erez, A., and Couvillon, G.A. (1987). The temperature dependence of dormancy breaking in plants : mathematical analysis of at two-step model involving a cooperative transition. J. Theor. Biol. *124*, 473–483.

Gannouni, T.A., Campoy, J.A., Quero-García, J., Barreneche, T., Arif, A., Albouchi, A., and Ammari, Y. (2017). Dormancy related traits and adaptation of sweet cherry in Northern Africa: A case of study in two Tunisian areas. Sci. Hortic. (Amsterdam). *219*, 272–279.

Julian, C., Rodrigo, J., and Herrero, M. (2011). Stamen development and winter dormancy in apricot (*Prunus armeniaca*). Ann. Bot. *108*, 617–625.

Julian, C., Herrero, M., and Rodrigo, J. (2014). Anther meiosis time is related to winter cold temperatures in apricot (*Prunus armeniaca* L.). Environ. Exp. Bot. *100*, 20–25.

Koltunow, a. M., Truettner, J., Cox, K.H., Wallroth, M., and Goldberg, R.B. (1990). Different temporal and spatial

gene expression patterns occur during anther development. Plant Cell *2*, 1201–1224.

Kurokura, T., Mimida, N., Battey, N.H., and Hytönen, T. (2013). The regulation of seasonal flowering in the Rosaceae. J. Exp. Bot. *64*, 4131–4141.

Lang, G.A., Early, J.D., Martin, G.C., and Darnell, R.L. (1987). Endodormancy, paradormancy, and ecodormancy - Physiological terminology and classification for dormancy research. HortScience *22*, 371–377.

Owen, H.A., and Makaroff, C.A. (1995). Ultrastructure of microsporogenesis and microgametogenesis in *Arabidopsis thaliana* (L .) Heynh . ecotype Wassilewskija (Brassicaceae). Protoplasma *185*, 7–21.

Perry, T.O. (1971). Dormancy of trees in winter. Science *171*, 29–36.

Richardson, E.A., Seeley, S.D., and Walker, D.R. (1974). A model for estimating the completion of rest for "Redhaven" and "Elberta" peach trees. HortScience *9*, 331–332.

Sedgley, M., and Griffin, A.R. (1989). Sexual Reproduction of Tree Crops (London: Academic Press).

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. Plant Cell *2*, 755–767.

Weinberger, J.H. (1950). Chilling requirements of peach varieties. Proc. Am. Soc. Hortic. Sci. *56*, 122–128.

Williams, J.H.W., Friedman, W.E.F., and Arnold, M.L.A. (1999). Developmental selection within the angiosperm style: Using gamete DNA to visualize interspecific pollen competition. Proc. Natl. Acad. Sci. *96*, 9201–9206.