

Ovule development at anthesis in Japanese plum (*Prunus salicina* Lindl.) cultivars

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Abstract

The stage of ovule development at anthesis has been studied in two consecutive years in seven major cultivated plum cultivars: 'Red Beaut', 'Fortune', 'Angeleno', 'Santa Rosa', 'Larry Ann', 'Son Gold' and 'Golden Japan'. Ovules examined were in general delayed. In much of them mature embryo sacs were not found at time of anthesis. Differences among cultivars were found and these differences were consistent during both years, indicating a genetic determination. In addition, a high heterogeneity of the developmental stage of the ovules examined in each cultivar was observed. The year influenced the developmental stages of ovules at anthesis, and ovules were more delayed in 2002 than in 2003. Cultivars which showed a more advanced ovule development were those that flowered later, in spite of the fact that chilling requirements for breaking of rest were adequately fulfilled during both years. This work gives interesting information regarding the stage of ovule maturity at anthesis in Japanese plum cultivars, which is closely related to their fruit set viability.

Additional key words: intervarietal variation, megagametophyte, ovule maturity, year-by-year variation

Resumen

Desarrollo del óvulo en antesis en cultivares de ciruelo japonés (*Prunus salicina* Lindl.)

El estado de desarrollo del óvulo en antesis fue estudiado en dos años sucesivos sobre siete de los principales cultivares de ciruelo japonés: 'Red Beaut', 'Fortune', 'Angeleno', 'Santa Rosa', 'Larry Ann', 'Son Gold' y 'Golden Japan'. Los óvulos examinados presentaron en general un estado retrasado y en muchos de ellos no se observaron los sacos embrionarios maduros en antesis. Los resultados obtenidos muestran importantes diferencias entre cultivares, que fueron consistentes durante los dos años de estudio, lo que parece indicar una determinación genética del carácter. Además, los resultados muestran una elevada heterogeneidad respecto al estado de madurez de los óvulos en antesis en cada cultivar. Las condiciones climáticas de cada año influyeron sobre el estado de desarrollo de los óvulos en antesis. Así, en el año 2002 los óvulos mostraron un estado de desarrollo más atrasado que en 2003. Los cultivares que mostraron un estado de los óvulos más avanzado fueron aquellos de floración más tardía, a pesar de que los requerimientos de frío para la salida del letargo fueron satisfechos adecuadamente en ambos años. Este trabajo proporciona una interesante información respecto al estado de madurez de los óvulos en antesis en cultivares de ciruelo japonés, lo cual está íntimamente relacionado con su viabilidad de fructificación.

Palabras clave adicionales: madurez del óvulo, megagametofito, variación anual, variación intervarietal.

Introduction

The knowledge of the stage of ovule maturity at anthesis time, when, generally, the flower stigmata can receive the pollen, is very illustrative of the possibilities of a flower to become a fruit. If the ovule is too advanced

at anthesis, depending on temperatures post-anthesis, a risk, more or less accentuated, exists that the megagametophyte will be degenerated when the pollen tube arrives at the ovary (Eaton, 1962; Marro, 1976; Stösser and Anvari, 1982, 1983). On the other hand, retardation in embryo sac development may also be responsible

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Abbreviations used: CU (chill units), FAA (formaldehído, ácido acético glacial y etanol), TBA (tertiary butyl alcohol).

for reduced fruit set (Dorsey, 1929; Daubeny *et al.*, 1967; Furokawa and Bukovac, 1989; Albuquerque *et al.*, 2002). Recent work has deepened our knowledge of these events. Shimizu and Okada (2000), working with *Arabidopsis* mutants where development of the female gametophyte was delayed, found that pollen tubes guidance was affected, because the tubes lost their way just before entering the micropyle and elongated in random directions. Hülskamp *et al.* (1995) showed that none of the ovules with arrested growth during megasporogenesis or early stages of embryo sac development were associated with a pollen tube. These results show that, at some time during embryo sac development, competence to attract pollen is acquired. Similar results were reported by Ray *et al.* (1997). Control by the embryo sac over pollen tube growth in avocado (*Persea americana* Mill) was suggested by Sedgley (1976). In *Prunus* sp. changes in the female tissues that orient and direct the pollen tube to set the right course had been described (Herrero, 2000, 2001).

Differences in the developmental stage of ovules at anthesis among cultivars of the same species have been reported previously in several species such as apple (*Malus domestica* Borkh) (Sato *et al.*, 1988), cherry (*Prunus avium* L.) (Eaton, 1962; Beppu *et al.*, 1997), almond [*Prunus dulcis* (Miller) D.A. Webb] (Pimienta and Polito, 1983; Egea and Burgos, 2000) or apricot (*Prunus armeniaca* L.) (Ruiz and Egea, 2007). In addition, a high heterogeneity has been found in apricot in relation to the developmental stage of the ovules examined in each variety (Ruiz and Egea, 2007), which could be an adaptation mechanism against possible adverse conditions. Diversity in the stages of ovule development in flowers at the same external stage of development has been reported frequently (Eaton and Jamont, 1965; Pimienta and Polito, 1983; Costa and Mackenzie, 1990; Albuquerque *et al.*, 2002).

Although ovule development at anthesis has a genetic determination, differences related to climate have been found (Egea and Burgos, 1994, 2000). This knowledge could be an index of the possibilities of adaptation of cultivars to different areas, mainly in relation with the effect of post-anthesis temperatures on fruit set, with those varieties with advanced ovules at anthesis showing poor adaptation to cold areas (Thompson and Liu, 1973; Keulemans and Van Laer, 1987).

Little work concerning ovule maturity has been done in *Prunus salicina* Lindl., or closely related species. During the first 10 days from the onset of full bloom, ovules of five plum cultivars showed a high viability

in a cold area (Cerovic *et al.*, 2000). Thompson and Liu (1973) found that 'Italian' prune (*Prunus domestica* L.), a cultivar widely recognised as having erratic bearing habits, had mature ovules at anthesis. 'Brooks', a consistently productive prune cultivar, showed a longer ovule viability compared to 'Italian', under both field and growth chamber conditions.

The present work deals with the establishment of the stage of ovule development at anthesis during two consecutive years of unequally cold winter temperatures for seven major plum cultivars. Variability among plum cultivars and year-by-year variation has been evaluated.

Material and methods

The plum cultivars 'Golden Japan' (USA, Obtentor L. Burbank, 1889), 'Santa Rosa' (USA, Obtentor L. Burbank, 1907), 'Larry Ann' (South Africa, 1995), 'Red Beaut' (USA, Obtentor F. Anderson, 1965), 'Angeleno' (USA, Obtentor J.M. Garabedian, 1967), 'Fortune' (USA, USDA, 1971) and 'Son Gold' (South Africa, ARC, 1970) were studied. All cultivars were grafted on 'Mariana 2624' rootstock, with a planting distance of 5 m (between rows) × 4 m (between trees). The trees were 10-years-old when the experiment began. All trees were cultivated in the same experimental orchard (South East Spain, 38°N latitude, 1°W longitude, and 300 m altitude) according to common plum orchard management.

For two consecutive years (2002 and 2003), flowers at the Baggliolini (1952) E-F stage (just-opening flowers), or flowers in stage 59-60 according BBCH scale (Meier *et al.*, 1994), were picked at random as described by Eaton (1962). Pistils were sampled from five different trees for cultivar and immediately fixed in FAA (90% ethanol at 70%, 5% formaldehyde at 40% and 5% glacial acetic acid). The ovaries were placed in 70% ethanol to remove the fixer. The pistils were dehydrated using a tertiary butyl alcohol series (TBA) and then embedded in Paraplast (Paraplast Plus, Sherwood Medical Co., St Louis, MO, USA). Serial sections of 10 µm were mounted on slides impregnated with an adhesive of gelatine, glycerine and 3% formaldehyde. Samples were stained as described by Gerlach (1969) and observed under an Olympus BH2 microscope. The different stages of development of ovules have been described elsewhere (Egea and Burgos, 2000). In this work, six ovule development stages were considered (no megaspore; megaspore to tetrad; two nuclei; four

nuclei; eight nuclei; eight organized nuclei). Twenty-four ovules per cultivar were examined each year. The largest and most developed ovule at anthesis was considered the primary ovule (Rodrigo and Herrero, 1998). The ovule size was calculated using the following formula: $V = 4/3 \cdot \pi \cdot r_1 \cdot r_2^2$, where r_1 is the largest radius of the ovule and r_2 is the smallest (Rodrigo and Herrero, 1998). When there were no differences in size, the ovule with the embryo sac in an earlier stage of development was considered the secondary ovule. Primary and secondary ovules were studied and the ovule development stage was established in each case. In order to facilitate the evaluation of results, a value was assigned to the six ovule development stages established (1: no megaspore; 2: megaspore to tetrad; 3: embryo sac with two nuclei; 4: embryo sac with four nuclei; 5: embryo sac with eight nuclei; 6: embryo sac with eight organised nuclei), and the ovule development stage of each cultivar was expressed as the average value of the ovules studied in the sample.

In order to characterise the climatic conditions of both years, hourly temperatures were collected with an automatic data-logger (Escort® Datalogging Systems, Buchanan, Virginia, USA, 2002). Chill units (CU) between November 1st and February 28th were calculated with the method described by Richardson *et al.* (1974). Flowering time for each cultivar was established as the date when 50% of flowers (F_{50}) were open.

Differences between type of ovules, cultivars and years were determined by ANOVA analysis using SPSS 17.0 for Windows (Chicago, IL).

Table 1. Flowering time (F_{50}) of seven plum cultivars

Cultivar	Flowering time (F_{50})	
	2002	2003
Red Beaut	18 Feb	3 Mar
Fortune	20 Feb	6 Mar
Angeleno	21 Feb	6 Mar
Santa Rosa	23 Feb	9 Mar
Larry Ann	25 Feb	10 Mar
Son Gold	27 Feb	12 Mar
Golden Japan	27 Feb	13 Mar

Results

The CU accumulated in 2002 and 2003 were 1,605 and 1,353 respectively. In 2002, flowering time for all cultivars occurred during the second fortnight of February, while in 2003 flowering time occurred in the first fortnight of March (Table 1). The delayed flowering in 2003 was mainly the result of a warmer late autumn and early winter in 2002-03 than in 2001-02 (Fig. 1), which retarded the satisfaction of chilling requirements in that vegetative cycle. Flowering time was earlier in 'Red Beaut', 'Fortune' and 'Angeleno' than in 'Larry Ann', 'Son Gold' and 'Golden Japan'. 'Santa Rosa' showed an intermediate position. Differences between early and late flowering cultivars were 9-10 days approximately (Table 1).

Significant differences between primary and secondary ovules concerning ovule development stage were found for the two years studied, in the set of evaluated

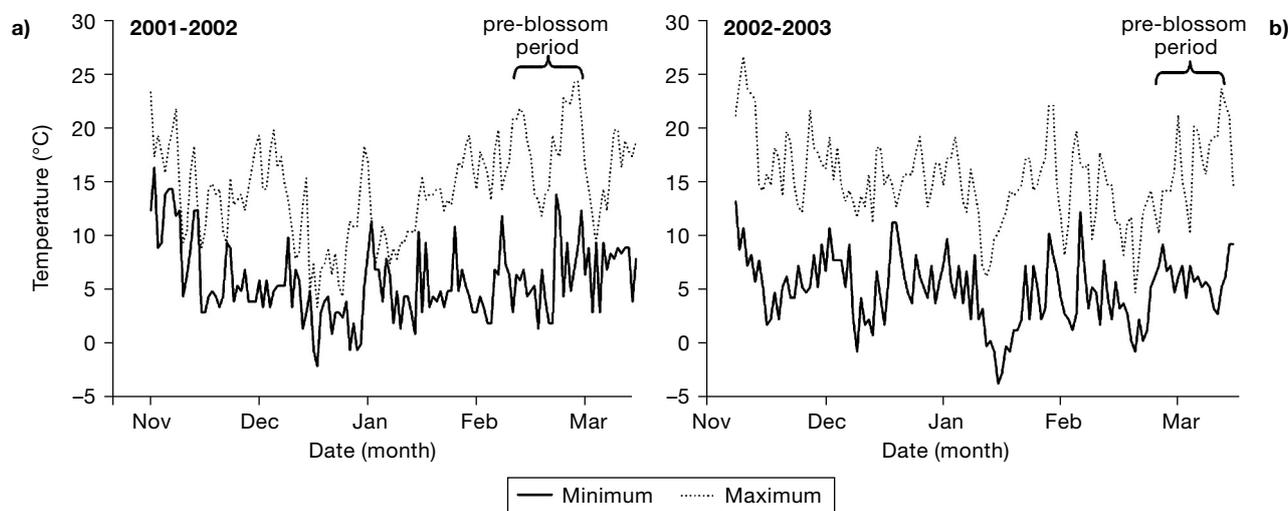


Figure 1. Daily (minimum and maximum) temperatures recorded from November 1st to March 15th during 2001-02 (a) and 2002-03 (b). Minimum and maximum temperatures during pre-blossom period (7 days before anthesis for all cultivars) are reported.

Table 2. *F*-values obtained in the ANOVA for the ovule development stage

Variable	DF ^a	MS ^b	F-value	P
<i>Type of ovule^c</i>				
2002	1	21.44	17.34	0.000
2003	1	7.26	5.66	0.019
<i>Primary ovule</i>				
Cultivar	6	8.90	8.58	0.000
Year	1	17.55	16.93	0.000
Cultivar × Year	6	1.02	0.98	0.440
Error	136	1.04		
<i>Secondary ovule</i>				
Cultivar	6	4.82	8.03	0.000
Year	1	28.38	47.27	0.000
Cultivar × Year	6	2.00	3.33	0.005
Error	120	0.60		

^a DF: degrees of freedom. ^b MS: mean square values. ^c Differences between primary and secondary ovules in two different years.

cultivars (Table 2). Stages of development of secondary ovules were, in all cases, more delayed than for primary ovules. In general, the secondary ovule showed a tendency in its annual development similar to that of the primary ovule.

Results concerning ovule maturity in 2002 and 2003 are shown in Table 3. Although flowers were collected at the same external stage of development in each cultivar in both years, a high heterogeneity of the developmental stage of the ovules was observed in each cultivar (Table 3). This variability was especially accentuated in ‘Son Gold’ in 2003, when ovules in five different stages of development were found. Ovules examined were in general delayed, and in much of them mature embryo sacs were not found at time of anthesis. The general delay of ovule development at anthesis was emphasized by the fact that no cultivar had mature ovules at this time, and only three of them, ‘Fortune’, ‘Son Gold’ and ‘Golden Japan’, exhibited 8-nucleate ovules in 2002, with only ‘Son Gold’ doing so in 2003 (Table 3).

Significant differences among cultivars were found regarding the developmental stage of primary ovules (Table 2). Thus, while in 2002 ‘Larry Ann’ and ‘Son Gold’ had developed sacs in 100% and 83% of primary ovules (at least two-nucleate) respectively, in ‘Angeleno’ and ‘Santa Rosa’ only 33% of primary ovules had developed sacs. The other cultivars showed intermediate stages of development (Table 3). Differences were also observed

in 2003. For example, ‘Son Gold’ had 83% of primary ovules with an embryo sac and ‘Golden Japan’ 75%, whereas ‘Santa Rosa’ had only 8% of ovules with an embryo sac. The percentage of ovules without an embryo sac was a result of both the first and second stages of development. In all cases the embryo sacs were immature.

Significant differences among cultivars also were found regarding the developmental stage of secondary ovules (Table 2). Secondary ovules were more developed in 2002 (Table 3) although 56% of secondary ovules did not show an embryo sac at anthesis. However, in 2003 this value was as high as 80%. As in primary ovules, in both years the secondary ovules exhibited, at anthesis, differences in the developmental stage in just-picked flowers at the same external stage. A low percentage of primary ovules without megaspore were observed in the two studied years, whereas high percentages of secondary ovules at this developmental stage were found, especially in 2003, where secondary ovules without megaspore ranged from 45.4% in ‘Son Gold’ to 91.6% in ‘Santa Rosa’ (Table 3).

Although in both years a delay in ovule development was apparent, there were significant differences between years (Table 2), showing a clear effect of the year over ovule development in plum. Results indicated a higher development stage of primary ovules in 2002 than in 2003 (Table 3). So, in 2002, all cultivars except ‘Santa Rosa’ had primary ovules with a megasporocyte. However, in 2003, all cultivars but ‘Golden Japan’ showed some primary ovules without a megasporocyte. In general, 2002 was a more favourable year concerning ovule development. Regarding the effect of the year on the developmental stage of primary ovules at anthesis, the most remarkable aspect was that in most cases the percentages without a megaspore were higher in year 2003 than in 2002 (Table 3). On the contrary, in all the cultivars, excepting ‘Golden Japan’ in the case of four-nucleate, the presence of four or eight-nucleate ovules was equal or higher in 2002 (Table 3).

On the other hand, no interaction between year and cultivar has been observed in primary ovules (Table 2), for which the effect of year on development of primary ovules is similar for each cultivar.

Discussion

Although differences in size between primary and secondary ovules were not detected at anthesis, differences in the developmental stage of the embryo sac

Table 3. Development stage (%) of primary and secondary ovules at anthesis of seven plum cultivars in two different years

Cultivar		No megaspore	Megaspore to tetrad	Two nuclei	Four nuclei	Eight nuclei	Eight org nuclei	Average ^a
<i>2002^b</i>								
Red Beaut	PO ^c	0	41.7	50	8.3	0	0	2.6 (0.7)
	SO	36.3	45.4	18.2	0	0	0	1.7 (0.6)
Fortune	PO	0	27.3	27.3	18.2	27.3	0	3.3 (1.3)
	SO	20	50	30	0	0	0	2.1 (0.7)
Angelino	PO	0	66.7	16.7	16.7	0	0	2.7 (0.8)
	SO	16.6	66.6	16.6	0	0	0	1.8 (0.4)
Santa Rosa	PO	25	41.7	8.3	25.1	0	0	2.5 (1.1)
	SO	30	60	10	0	0	0	1.6 (0.5)
Larry Ann	PO	0	0	28.6	71.4	0	0	3.7 (0.5)
	SO	0	16.7	66.7	16.6	0	0	2.8 (0.4)
Son Gold	PO	0	16.7	0	33.3	50	0	4.3 (1.4)
	SO	16.6	0	16.6	33.3	33.3	0	3.5 (1.4)
Golden Japan	PO	0	27.3	27.3	18.2	27.3	0	3.5 (1.2)
	SO	0	33.3	16.6	16.6	33.3	0	3.5 (1.4)
Mean	PO	3.6	31.5	22.5	27.3	14.9	0	3.1 (1.1)
	SO	17.1	38.8	24.9	9.5	9.5	0	2.3 (1.1)
<i>2003^b</i>								
Red Beaut	PO ^c	16.6	41.7	33.3	8.3	0	0	2.3 (0.9)
	SO	50	41.6	8.3	0	0	0	1.4 (0.5)
Fortune	PO	16.7	41.7	25	16.7	0	0	2.4 (1.0)
	SO	50	16.7	25	8.3	0	0	1.5 (0.7)
Angelino	PO	25	33.3	25	16.7	0	0	2.3 (1.1)
	SO	66.6	25	8.3	0	0	0	1.4 (0.7)
Santa Rosa	PO	75	16.7	8.3	0	0	0	1.3 (0.7)
	SO	91.6	8.3	0	0	0	0	1.1 (0.3)
Larry Ann	PO	25	16.7	58.3	0	0	0	2.3 (0.9)
	SO	60	30	10	0	0	0	1.4 (0.5)
Son Gold	PO	8.3	8.3	16.6	33.3	33.3	0	3.8 (1.4)
	SO	45.4	9.1	18.2	18.2	9.1	0	2.2 (1.5)
Golden Japan	PO	0	25	41.7	33.3	0	0	3.1 (0.9)
	SO	55.5	22.2	22.2	0	0	0	1.3 (0.5)
Mean	PO	23.8	26.2	29.7	15.4	4.8	0	2.5 (1.2)
	SO	59.9	21.8	13.1	3.8	1.3	0	1.5 (0.8)

^a Average value of ovule development at anthesis: 1: no megaspore; 2: megaspore to tetrad; 3: embryo sac with two nuclei; 4: embryo sac with four nuclei; 5: embryo sac with eight nuclei; 6: embryo sac with eight organised nuclei. Standard deviations in parentheses. ^b Percentage (%) ovule development at anthesis. ^c PO: Primary ovule; SO: Secondary ovule.

were frequently observed. The ovule development was more delayed in secondary ovules. In apricot 'Constant', differences between primary and secondary ovules were only apparent two days after anthesis (Eaton and Jamont, 1965, but in sour cherries (*Prunus cerasus* L.) the secondary ovule showed fluorescence in all cases at anthesis, indicating a senescent stage (Cerovic and

Micic, 1999). Cerovic *et al.* (2000), studying a group of plum cultivars, found that the secondary ovule atrophies at the onset of full bloom. In 'Non Pareil' almond it is difficult to distinguish between the two ovules at anthesis. In general, the secondary ovule aborts, leaving only the viable primary ovule to be fertilised (Pimienta and Polito, 1982).

Although it has been frequently shown that ovule maturity at anthesis is cultivar-dependent, there are some indications that species follow a general tendency in this respect. So, according to Williams (1970) and Costa and Mckenzie (1990), in diploid pear (*Pyrus communis* L.) and apple cultivars maturity of the embryo sac usually coincides with flower opening. On the other hand, apricot shows retarded ovules at anthesis (Ruiz and Egea, 2007) and this tendency was especially accentuated in some varietal groups (Egea and Burgos, 1994; Alburquerque *et al.*, 2002). Stösser and Anvari (1990) concluded that ovule viability in cherries is shorter than in plums. In sweet cherries, Eaton (1962) found that a group of cultivars had ovules in advanced stages of development at anthesis. In almond, contradictory results have been found (Pimienta and Polito, 1982; Egea and Burgos, 2000). According to our results, all examined plum cultivars showed delayed ovules at anthesis, which means that, in Mediterranean climatic conditions, this could be the tendency for plum species.

Significant differences among plum cultivars were found regarding the developmental stage of primary ovules (Table 2). Differences in the developmental stage of ovules at anthesis among cultivars of the same species have been frequently reported. In a previous study with sour cherry cultivars, Eaton (1962) found that all cultivars had advanced ovules, but differences between them were clear. However, Beppu *et al.* (1997) found that the sweet cherry cv. 'Satonhnishiki' showed delayed embryo sacs at anthesis. Sato *et al.* (1988) studied the developmental stage of ovules in two apple cultivars, finding more immature ovules in one of them. Sun *et al.* (1991) found that ovule longevity after anthesis in the 'Brooks' prune cultivar was higher than in the 'Italian' prune. In almond, while a high percentage of ovules at anthesis showed an advanced stage of development in 'Ferragnes', other cultivars showed an intermediate stage (Egea and Burgos, 2000). On the other hand, Pimienta and Polito (1983) reported that most ovules of 'Non Pareil' were in the megaspore mother cell stage at the time of flower opening. A recent work has found significant differences among cultivars in apricot species (Ruiz and Egea, 2007).

With the exception of 'Santa Rosa', the group of later-blooming cultivars studied in the present work showed a more advanced stage of embryo sacs. Eaton (1962) found similar results in sweet cherries. However, in apricot, the ovules of the earlier-flowering cultivars showed a more advanced stage of embryo sacs (Alburquerque *et al.*, 2002).

On the other hand, a high heterogeneity of the developmental stage of the ovules was observed in each cultivar (Table 2). Diversity in the stages of ovule development in flowers at the same external stage of development has been frequently reported in other species (Eaton and Jamont, 1965; Pimienta and Polito, 1983; Costa and Mackenzie, 1990; Alburquerque *et al.*, 2002; Ruiz and Egea, 2007). This characteristic could be an adaptation mechanism against possible adverse conditions which could affect the ovule development and, consequently, the fruiting.

Year-by-year differences in the developmental stages of ovules at anthesis have been reported in several species (Pimienta and Polito, 1983; Egea and Burgos, 1994; Cerovic and Micic, 1999; Ruiz and Egea, 2007). When ovules of apricot were examined at anthesis in different years and locations, differences in the stage of development were found (Egea and Burgos, 1998). This is in agreement with our results in plum, and means that, while there is a genetic determination, environmental conditions also affect ovule development. In our climatic conditions, significant differences between years were found (Table 2). In addition, no interaction between year and cultivar has been found in primary ovules (Table 2) which show a homogeneous effect of the year over the ovule development in different plum cultivars. Plum ovules were more advanced in 2002 (Table 3) when chill accumulation was higher, this factor appeared to have an important effect over the variation. In addition, 2003 was characterized by slightly higher pre-blossom maximum temperatures than 2002 (Fig. 1). The high temperatures before anthesis could produce a lack of synchrony between external phenological stage and ovule development, resulting in more delayed ovules at anthesis, as it was observed in apricot (Rodrigo and Herrero, 2002). However, other authors have found that temperatures in the «after rest to bloom» period have a clear influence over year-by-year differences in the developmental stages of ovules (Tromp, 1986).

Delayed ovules in almond did not affect fruit set (Pimienta *et al.*, 1983). According to these authors, when pollen tubes arrive at the base of the style, their growth slows greatly or stops and then the final stages of embryo sac differentiation are completed. There is, however, evidences that when ovules are retarded excessively at anthesis their fertilisation may be affected (Furokawa and Bukovac, 1989; Alburquerque *et al.*, 2000). Delayed and irregular megagametophyte development may be a characteristic of abortive ovules (Pimienta and Polito, 1982). A new line of evidence

developed in different species shows that, in mutants with a delayed embryo sac, pollen tube guidance is affected, because they lose their way just before entering the micropyle, elongating in random directions (Shimizu and Okada, 2000; Higashiyama, 2002). Depending on the stage of ovule development at anthesis, post-pollination temperatures can be determinants for fruit set (Thompson and Liu, 1973; Keulemans and Van Laer, 1987).

The erratic bearing habit of plum 'Santa Rosa' (Egea and García, 1995), one of the most important cultivars grown in Spain, could be a consequence of its excessively-delayed ovules at anthesis, as it was found in this work.

In conclusion, the ovule development at anthesis in Japanese plum cultivars was in general delayed, and mature embryo sacs were not found in much of them. Differences among cultivars were found, indicating a genetic determination. In addition, a high heterogeneity of the developmental stage of the ovules examined in each cultivar was observed. Cultivars which showed a more advanced ovule development were those that flowered later, in spite of the fact that chilling requirements for breaking of rest were adequately satisfied during both years. A significant effect of the year over ovule development has been found as well as no interaction between year and cultivar. It proves the influence of environmental conditions over ovule development in plum, which is closely related to fruit set viability.

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