

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

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10
11 **Abstract**

12 The stage of ovule development at anthesis has been studied in two consecutive
13 years in seven major cultivated plum cultivars: ‘Red Beaut’, ‘Fortune’, ‘Angeleno’,
14 ‘Santa Rosa’, ‘Larry Ann’, ‘Son Gold’ and ‘Golden Japan’. Ovules were always
15 delayed. In much of them mature embryo sacs were not found. Differences among
16 cultivars were found and these differences were consistent during both years. In
17 addition, a high heterogeneity of the developmental stage of the ovules examined in
18 each variety was observed. The year influenced the developmental stages of ovules at
19 anthesis but differences were not accentuated, indicating a genetic determination.
20 Cultivars which showed a more advanced ovule development were those that flowered
21 later, in spite of the fact that chilling requirements for breaking of rest were adequately
22 satisfied during both years.

23 **Additional key words:** genetic diversity, megagametophyte, ovule maturity, *Prunus*
24 *salicina* Lindl, year-by-year variation

Resumen

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

El estado de desarrollo del óvulo en antesis fue estudiado en dos años sucesivos sobre siete de las principales variedades cultivadas de ciruelo japonés: ‘Red Beaut’, ‘Fortune’, ‘Angeleno’, ‘Santa Rosa’, ‘Larry Ann’, ‘Son Gold’ y ‘Golden Japan’. Los óvulos presentaron en general un estado retrasado y en muchos de ellos no se observaron los sacos embrionarios. Los resultados obtenidos muestran importantes diferencias entre variedades, que fueron consistentes durante los dos años de estudio. Además, los resultados muestran una elevada heterogeneidad respecto al estado de madurez de los óvulos en antesis en cada variedad. Las condiciones climáticas de cada año influyeron sobre el estado de desarrollo de los óvulos en antesis, aunque las diferencias no fueron muy acentuadas, lo que parece indicar una determinación genética del carácter. Las variedades que mostraron un estado de los óvulos más avanzado fueron aquellas de floración más tardía, a pesar de que los requerimientos de frío para la salida del letargo fueron satisfechos adecuadamente en ambos años.

Palabras clave adicionales: diversidad genética, megagametofito, madurez del óvulo, *Prunus salicina* Lindl, variación anual

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 (Stösser and Anvari, 1982, 1983; Eaton, 1962; Marro, 1976). On the other hand, retardation in embryo sac development may also be responsible for reduced fruit set (Daubeney *et al.*, 1967; Dorsey, 1929; 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,

1 found that pollen tubes guidance was affected, because the tubes lost their way just
2 before entering the micropyle and elongated in random directions. Hülkamp *et al.*
3 (1995) showed that none of the ovules arrested during megasporogenesis or early stages
4 of embryo sac development were associated with a pollen tube. These results show that,
5 at some time during embryo sac development, competence to attract pollen is acquired.
6 Similar results were reported by Ray *et al.* (1997). Control by the embryo sac over
7 pollen tube growth in avocado was suggested by Sedgley (1976). In prunus changes in
8 the female tissues that orient and direct the pollen tube to set the right course had been
9 described (Herrero, 2000, 2001).

10 Differences in the developmental stage of ovules at anthesis among cultivars of
11 the same species have been reported previously in several species such as apple (Sato *et al.*
12 *al.*, 1988), cherry (Eaton, 1962; Beppu *et al.*, 1997), almond (Pimienta and Polito, 1983;
13 Egea and Burgos, 2000) or apricot (Ruiz and Egea, 2007). In addition, a high
14 heterogeneity has been found in apricot in relation to the developmental stage of the
15 ovules examined in each variety (Ruiz and Egea, 2007), which could be an adaptation
16 mechanism against possible adverse conditions. Diversity in the stages of ovule
17 development in flowers at the same external stage of development has been reported
18 frequently (Eaton and Jamont, 1965; Costa and Mackenzie, 1990; Albuquerque *et al.*,
19 2002; Pimienta and Polito, 1983).

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

26 Little work concerning ovule maturity has been done in *Prunus Salicina* Lindl.,
27 or closely related species. During the first 10 days from the onset of full bloom, ovules
28 of five plum cultivars showed a high viability in a cold area (Cerovic *et al.*, 2000).
29 Thompson and Liu (1973) found that ‘Italian’ prune (*Prunus domestica*, L.), a cultivar
30 widely recognised as having erratic bearing habits, had mature ovules at anthesis.
31 ‘Brooks’, a consistently productive prune cultivar, showed a longer ovule viability
32 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’, ‘Santa Rosa’, ‘Larry Ann’, ‘Red Beaut’, ‘Angeleno’, ‘Fortune’ and ‘Son Gold’ were studied. All cultivars were grafted on ‘Mariana 2624’ rootstock, with a planting distance of 5m (between rows) x 4m (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 habitual apricot orchard management.

For two consecutive years (2002 and 2003), flowers at the Baggiolini (1952) E-F stage (just-opening flowers) were picked at random as described by Eaton (1962). Pistils were sampled from 5 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). 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). Primary and secondary ovules were studied and the ovule development stage was established in each case.

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 years were determined by simple chi-squared test (differences for each cultivar) and by t-Test for correlated samples (differences between years considering all cultivars) by using SPSS 13.0 for Windows (Chicago, IL).

Results

The CU accumulated in 2002 and 2003 were 1605 and 1353 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 (Figure 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).

Results concerning ovule maturity in 2002 and 2003 are shown in Table 2 and Table 3. Although in both years a delay in ovule development was apparent, there were differences between them. 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 (Table 3), the most remarkable aspect was that in most cases the percentages without a megaspore were higher in year 2003, with significant differences between years. 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 higher in 2002. Although results do not show significant differences between years, presumably due to important variability between cultivars, the differences were almost significant ($p = 0.055$) and results indicated a higher development stage of primary ovules in 2002 .

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 2).

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 variety (Table 2). This variability was especially accentuated in ‘Son Gold’ in 2003, when ovules in five different stages of development were found.

Important differences among cultivars were found regarding the developmental stage of primary ovules. 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. 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.

Concerning the secondary ovule, differences in size with respect to the primary ovule at anthesis were not found, but stages of development were frequently more delayed. The largest and most developed ovule at anthesis was considered the primary ovule (Rodrigo and Herrero, 1998). In general, the secondary ovule showed a tendency in its annual development similar to that of the primary ovule. Secondary ovules were more developed in 2002 (Table 2) 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 was observed in the two studied years, whereas high percentages of secondary ovules at this developmental stage were found, especially in 2002, where more than 33 % of secondary ovules were at this stage.

Discussion

Although differences in size between primary and secondary ovules were not detected at anthesis, differences in the developmental stage of the embryo sac were frequently observed. The ovule development was more delayed in secondary ovules. In apricot cv. ‘Constant’, differences between primary and secondary ovules were only apparent two days after anthesis (Eaton and Jamont, 1964), but in sour cherries 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

1 cultivars, found that the secondary ovule atrophies at the onset of full bloom. In ‘Non
2 Pareil’ almond it is difficult to distinguish between the two ovules at anthesis. In
3 general, the secondary ovule aborts, leaving only the viable primary ovule to be
4 fertilised (Pimienta and Polito, 1982).

5 Although it has been frequently shown that ovule maturity at anthesis is cultivar-
6 dependent, there are some indications that species follow a general tendency in this
7 respect. So, according to Costa and Mckenzie (1990) and Williams (1970), in diploid
8 pear and apple cultivars maturity of the embryo sac usually coincides with flower
9 opening. On the other hand, apricot shows retarded ovules at anthesis (Ruiz and Egea,
10 2007) and this tendency was especially accentuated in some varietal groups
11 (Albuquerque *et al.*, 2002; Egea and Burgos, 1994). Stösser and Anvari (1990)
12 concluded that ovule viability in cherries is shorter than in plums. In sweet cherries,
13 Eaton (1962) found that a group of cultivars had ovules in advanced stages of
14 development at anthesis. In almond, contradictory results have been found (Pimienta
15 and Polito, 1982; Egea and Burgos, 2000). According our results, all examined plum
16 cultivars showed delayed ovules at anthesis, which means that, in our climatic
17 conditions, this could be the tendency for plum species.

18 Important differences among plum cultivars were found regarding the
19 developmental stage of primary ovules (Table 2). Differences in the developmental
20 stage of ovules at anthesis among cultivars of the same species have been frequently
21 reported. In a previous study with sour cherry cultivars, Eaton (1962) found that all
22 cultivars had advanced ovules, but differences between them were clear. However,
23 Beppu *et al.* (1997) found that the sweet cherry cv. ‘Satohnishiki’ showed delayed
24 embryo sacs at anthesis. Sato *et al.* (1988) studied the developmental stage of ovules in
25 two apple cultivars, finding more immature ovules in one of them. Sun *et al.* (1991)
26 found that ovule longevity after anthesis in the ‘Brooks’ prune cultivar was higher than
27 in the ‘Italian’ prune. In almond, while a high percentage of ovules at anthesis showed
28 an advanced stage of development in ‘Ferragnes’, other cultivars showed an
29 intermediate stage (Egea and Burgos, 2000). On the other hand, Pimienta and Polito
30 (1983) reported that most ovules of ‘Non Pareil’ were in the megaspore mother cell
31 stage at the time of flower opening. A recent work has found significant differences
32 among cultivars in apricot species (Ruiz and Egea, 2007).

1 With the exception of ‘Santa Rosa’, the group of later-blooming cultivars
2 studied in the present work showed a more advanced stage of embryo sacs. Eaton
3 (1962) found similar results in sweet cherries. However, in apricot, the ovules of the
4 earlier-flowering cultivars showed a more advanced stage of embryo sacs
5 (Alburquerque *et al.*, 2002).

6 On the other hand, a high heterogeneity of the developmental stage of the ovules
7 was observed in each variety (Table 2). Diversity in the stages of ovule development in
8 flowers at the same external stage of development has been frequently reported in other
9 species (Eaton and Jamont, 1965; Pimienta and Polito, 1983; Costa and Mackenzie,
10 1990; Alburquerque *et al.*, 2002; Ruiz and Egea, 2007). This characteristic could be an
11 adaptation mechanism against possible adverse conditions which could affect the ovule
12 development and, consequently, the fruiting.

13 Year-by-year differences in the developmental stages of ovules at anthesis have
14 been reported in several species (Pimienta and Polito, 1983; Egea and Burgos, 1994;
15 Cerovic and Micic, 1999; Ruiz and Egea, 2007). When ovules of apricot were examined
16 at anthesis in different years and locations, differences in the stage of development were
17 found (Egea and Burgos, 1998). This is in agreement with our results in plum, and
18 means that, while there is a genetic determination, environmental conditions also affect
19 ovule development. In our climatic conditions, plum ovules were more advanced in
20 2002 (Table 3) when chill accumulation was higher, this factor appeared to have an
21 important effect over the variation. In addition, 2003 was characterized by slightly
22 higher pre-blossom maximum temperatures than 2002 (Figure 1). The high
23 temperatures before anthesis could produce a lack of synchrony between external
24 phenological stage and ovule development, resulting in more delayed ovules at anthesis,
25 as it was observed in apricot (Rodrigo and Herrero, 2002). However, other authors have
26 found that temperatures in the “after rest to bloom” period have a clear influence over
27 year-by-year differences in the developmental stages of ovules (Tromp, 1986).

28 Delayed ovules in almond did not affect fruit set (Pimienta *et al.*, 1983).
29 According to these authors, when pollen tubes arrive at the base of the style, their
30 growth slows greatly or stops and then the final stages of embryo sac differentiation are
31 completed. There is, however, evidences that when ovules are retarded excessively at
32 anthesis their fertilisation may be affected (Furokawa and Bukovac, 1989;
33 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 cultivar grown in Spain, could be a consequence of its excessively-delayed ovules at anthesis, as it was found in this work.

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2 **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

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1 **Table 2.** Development stage of primary and secondary ovules at anthesis of seven plum
2 cultivars

Cultivar		No megaspore	Megaspore to tetrad	Two nuclei	Four nuclei	Eight nuclei	Eight org nuclei
% Development Stage 2002							
Red Beaut	P.O. ^a	0	41.7	50	8.3	0	0
	S.O.	36.3	45.4	18.2	0	0	0
Fortune	P.O.	0	27.3	27.3	18.2	27.3	0
	S.O.	20	50	30	0	0	0
Angeleno	P.O.	0	66.7	16.7	16.7	0	0
	S.O.	16.6	66.6	16.6	0	0	0
Santa Rosa	P.O.	25	41.7	8.3	25.1	0	0
	S.O.	30	60	10	0	0	0
Larry Ann	P.O.	0	0	28.6	71.4	0	0
	S.O.	0	16.7	66.7	16.6	0	0
Son Gold	P.O.	0	16.7	0	33.3	50	0
	S.O.	16.6	0	16.6	33.3	33.3	0
Golden Japan	P.O.	0	27.3	27.3	18.2	27.3	0
	S.O.	0	33.3	16.6	16.6	33.3	0
Mean	P.O.	3.6	31.5	22.5	27.3	14.9	0
	S.O.	17.1	38.8	24.9	9.5	9.5	0
% Development Stage 2003							
Red Beaut	P.O. ^a	16.6	41.7	33.3	8.3	0	0
	S.O.	50	41.6	8.3	0	0	0
Fortune	P.O.	16.7	41.7	25	16.7	0	0
	S.O.	50	16.7	25	8.3	0	0
Angeleno	P.O.	25	33.3	25	16.7	0	0
	S.O.	66.6	25	8.3	0	0	0
Santa Rosa	P.O.	75	16.7	8.3	0	0	0
	S.O.	91.6	8.3	0	0	0	0
Larry Ann	P.O.	25	16.7	58.3	0	0	0
	S.O.	60	30	10	0	0	0
Son Gold	P.O.	8.3	8.3	16.6	33.3	33.3	0
	S.O.	45.4	9.1	18.2	18.2	9.1	0
Golden Japan	P.O.	0	25	41.7	33.3	0	0
	S.O.	55.5	22.2	22.2	0	0	0
Mean	P.O.	23.8	26.2	29.7	15.4	4.8	0
	S.O.	59.9	21.8	13.1	3.8	1.3	0

3 ^aP.O. Primary ovule; S.O. Secondary ovule

Table 3. Effects of year on the developmental stage of primary ovules at anthesis of seven plum cultivars

Cultivar	Year	No megaspore	Megaspore to tetrad	Two nuclei	Four nuclei	Eight nuclei	Eight org nuclei
% Development stage							
Red Beaut	2002	0	41.7	50	8.3	0	0
	2003	16.6	41.7	33.3	8.3	0	0
	Significance ¹	*	NS	NS	NS	NS	NS
Fortune	2002	0	27.3	27.3	18.2	27.3	0
	2003	16.7	41.7	25	16.7	0	0
	Significance ¹	*	NS	NS	NS	*	NS
Angeleno	2002	0	66.7	16.7	16.7	0	0
	2003	25	33.3	25	16.7	0	0
	Significance ¹	*	*	NS	NS	NS	NS
Santa Rosa	2002	25	41.7	8.3	25.1	0	0
	2003	75	16.7	8.3	0	0	0
	Significance ¹	*	*	NS	*	NS	NS
Larry Ann	2002	0	0	28.6	71.4	0	0
	2003	25	16.7	58.3	0	0	0
	Significance ¹	*	*	*	*	NS	NS
Son Gold	2002	0	16.7	0	33.3	50	0
	2003	8.3	8.3	16.6	33.3	33.3	0
	Significance ¹	*	NS	*	NS	NS	NS
Golden Japan	2002	0	27.3	27.3	18.2	27.3	0
	2003	0	25	41.7	33.3	0	0
	Significance ¹	NS	NS	NS	NS	*	NS
Mean	2002	3.6	31.5	22.5	27.3	14.9	0
	2003	23.8	26.2	29.7	15.4	4.8	0
	Significance ²	*	NS	NS	NS	NS	NS

¹ NS, *: Nonsignificant or significant at P < 0.05 by the simple chi-squared test.

² NS, *: Nonsignificant or significant at P < 0.05 by the t-Test for correlated samples.

Figure 1. Daily (minimum and maximum) temperatures recorded from November 1st to March 15th during 2002-03 (a) and 2003-04 (b). Minimum and maximum temperatures during pre-blossom period (7 days before anthesis for all cultivars) are reported.

