The relationship between the mitotic activity and moisture content of recalcitrant seeds of *Acer saccharinum* (L.) during maturation, post-maturation drying and germination

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Abstract

The decline of embryo moisture content from approx. 82 to 53% in 1997 and 56% in 1998 in recalcitrant seeds of *Acer saccharinum* during maturation was accompanied by decreased mitotic activity in the meristems and an increase in the percentage of cells in the G_1 phase of the cell cycle. DNA synthesis and mitosis in the root apex ceased at approx. 53% embryo moisture content, and 67% of the cells were arrested in the G_1 phase. During post-maturation drying, cell division in the shoot apex and embryonic leaves continued as long as the embryo moisture content was higher than 50 and 45%, respectively. Mitotic activity in the drying embryo may be controlled by its moisture level. Increased proliferation of the root, shoot and leaf meristems of the mature seeds was already recorded at 24 h of germination, before the root protruded through the seed coat. However, the increase in the embryo moisture content was small – from 56 to 59%. In the 3 d seedlings (10–15 mm long) the mitotic index reached 8% in the root apex and 12% in the shoot apex with leaves. Placing mature seeds in moist conditions might be necessary for the switch from proliferation decline towards its activation. Thus, in contrast with orthodox seeds, the preservation of cell division capacity and increased mitotic activity may be essential for rapid germination immediately after shedding in mature *Acer* seeds.

Keywords: *Acer saccharinum*, germination, maturation, mitotic activity, moisture content, recalcitrant seed

Introduction

Seeds have been classified as orthodox or recalcitrant depending on their storage behaviour (Roberts, 1973). Orthodox seeds can be dried to a low moisture content without damage. They naturally undergo desiccation at maturity. Seeds with recalcitrant behaviour are shed from the mother plant at high moisture contents and cannot survive desiccation below a relatively high moisture content.

In orthodox seeds mitotic activity in the meristems progressively decreases during maturation drying. For *Triticum durum* Avanzi *et al*. (1969) have shown that cell cycle arrest occurs asynchronously in different meristem tissues during desiccation: first in shoot apex at approx. 63% seed moisture content, then in primordial leaves at 50% and in the root apex during further drying. DNA synthesis declines earlier than mitotic activity. Thus, in the radicle meristems of ripening *Vicia faba* seeds, DNA synthesis ceased at a moisture content below 75% in the cotyledons and mitotic activity below 65% moisture content (Brunori, 1967). The water content drop in seeds during maturation might cause inhibition of DNA synthesis (Deltour, 1985) and result in the accumulation of cells in the G_1 phase of the cell cycle. In many species, meristem cells of quiescent embryos are completely arrested in the pre-synthesis G_1 phase (Deltour and Jacqmard, 1974; Bino *et al*., 1993; for review, Bewley and Black, 1994). However, in mature embryos of other species, G_2 cells have also been detected (Bino *et al*., 1993; Finch-Savage *et al*., 1998).

A mitotic cycle reactivation during orthodox seed germination occurs after a period of imbibition. DNA synthesis precedes mitosis, because most meristem cells of mature seeds have 2C DNA content. The lag period prior to DNA replication is species-specific and ranges from 6 h after imbibition in *Hordeum vulgare* (Arnason *et al*., 1966) to 45 h in *Zea mays* (Deltour and Jacqmard, 1974). This pre-replicative period has a complex nature and concludes the transition from the quiescence state (G_0) to the G_1 phase of the first cell cycle (Troyan *et al*., 1984).

In the highly recalcitrant seeds of *Avicennia marina* there is also an arrest of DNA replication, but it lasts no more than 24 h around shedding (Berjak, Dini and

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Osborne, unpublished results, cited in Pammenter and Berjak, 1999). Mature *Azadirachta indica* seeds exhibit a 2C DNA level almost to the exclusion of 4C, corresponding to a cell cycle arrest in the G_1 phase (Sacandé *et al*., 1997). A predominance of G_1 cells is also observed in the mature recalcitrant seeds of *Castanea sativa* (Bino *et al*., 1993). Since a characteristic feature of this seed type is an absence of maturation drying, the authors suggested that cell cycle arrest in the G_1 phase is not directly linked to seed water status. However, most recalcitrant seeds undergo a partial decrease of moisture content during maturation (Chin *et al*., 1984; Hong and Ellis, 1996; Finch-Savage *et al*., 1998). The mature dormant recalcitrant *Acer pseudoplatanus* seeds contain nuclei arrested both at the 2C and 4C levels (Finch-Savage *et al*., 1998). However, a relationship between embryo moisture level and proliferative activity of meristematic cells in such seeds has not been reported. The aim of the work presented here was to investigate the effect of embryo moisture content on the mitotic activity and relative DNA contents in cells of maturing and germinating *Acer saccharinum* seeds having recalcitrant storage behaviour (Hong and Ellis, 1996).

Materials and methods

Acer saccharinum (L.) samaras were gathered from one group of trees in a city park in Kiev at the final stages of seed development in 1997 and 1998. In the first year's experiment, samaras were collected on 14–26 May 1997 at 28, 35 and 41 d (maturity) after anthesis. Mature samaras were picked from the trees when natural shedding had begun. Embryo axes were excised within an hour after samara harvest and fixed immediately. For mitotic activity studies, the 28, 35 and 41 d axes were fixed in absolute ethanol/glacial acetic acid (3:1, v/v). For cytological determination of meristem areas, a portion of the 28 and 41 d embryo collection was fixed in FAA [4% formalin/ethanol/glacial acetic acid, 3:1:0.3, v/v] and stored in 70% ethanol at 4°C until required. To investigate the influence of post-maturation drying on mitotic activity and germinability of seeds, mature samaras were naturally dried at room temperature (19 ± 1°C, without air mixing) in thin layers on plastic trays. After 4 and 17 d drying, embryo axes of the seeds were excised and fixed for mitotic activity studies as above. Seeds at the maturation stages and mature seeds dried for 2, 4, 7, 10, 14 and 17 d were tested for germination (radicle >2 mm long). The isolated seeds were germinated on moist paper in Petri dishes in the dark at 26 ± 1°C for 3 d. Chloramphenicol (10 mg ml^{-1}) was added to the water in order to prevent microbial growth. Each germination test was comprised of three replicates of 30 seeds each. Axes of the 3 d seedlings were fixed for mitotic activity studies as above.

In the second year's experiment, a more detailed determination of cell division arrest points, as well as cell cycle reactivation in root, shoot apices and embryo leaves was carried out. A flow cytometric evaluation of nuclear DNA levels in embryo axes was conducted during seed drying. Samaras were collected on 12–18 May 1998 at 42, 45 and 48 d (maturity) after anthesis. For the drying treatment, a portion of the mature samaras was naturally dried at room temperature as above (19 ± 1°C) for 1, 2 and 3 d. Another portion of the mature seeds was germinated for 3, 6, 9, 15 and 24 h. Axes were excised from the maturing seeds and mature seeds, dried or germinated, and fixed for mitotic activity studies as above. Embryo axes of seeds collected at 42 and 48 d after anthesis and mature seeds dried for 24 h were used to study relative DNA contents by flow cytometry.

In both years, embryo moisture content was determined gravimetrically on three replicates of 10 embryos by oven drying for 17 h at 103 ± 2°C (ISTA, 1993). Results were calculated on the fresh weight basis (w.b.).

For cytological analysis, the axes fixed in FAA were embedded in paraffin. Longitudinal sections were cut to thicknesses of 10 μm and stained with hematoxylin. Sections were observed by bright field microscopy using an NU-2 microscope (Zeiss, Germany). Cell length in the radicle was measured in the second and third subepidermal cell rows of the cortex on seven axes.

For study of mitotic index changes, the axes fixed in ethanol/acetic acid were hydrolysed in 1 N HCl at 60°C for 5 min before being stained in acetoorcein. Squash preparations were made of 0.5 mm long root apices and 0.5 mm long shoot apices either together with embryo leaves (in 1997) or separately (in 1998). Each mitotic index was based on the mean of 6–10 samples; 1500–3000 cells were analysed in each sample.

Results were analysed using one-way analysis of variance (ANOVA, Statistica for Windows, 5.0 A, StatSoft, Inc.) at $P < 0.05$.

For flow cytometric analysis of the nuclear DNA levels, the embryo axes were fixed in a 4% (w/v) solution of formaldehyde in Tris buffer [10 mM Tris (hydroxymethyl)aminomethane, 10 mM Na_2-EDTA, 100 mM NaCl, pH 7.4 (Hamada and Fujita, 1983)] containing 0.1% (v/v) Triton X100 at 4°C for 20 min and then washed twice in the buffer (Sgorbati *et al*., 1986). Using a razor blade and a dissecting needle, the fixed axes were dissected into 1 mm long root tip and the axis without a root tip. A sample was prepared by squashing of 8–10 root tips or 8–10 axes without a

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root in 1 ml of cooled Tris buffer and 0.1% Triton X100 for 1–2 min in a chilled mortar. The suspensions of nuclei were passed through two nylon meshes of 60 and 20 µm pore size. Nuclei were stained with propidium iodide (Sigma; 0.05 mg ml^{-1}) for 30 min and subsequently analysed with a Becton Dickinson FACStar Plus fluorescence-activated cell sorter using a 488 nm line from an argon-ion laser. Ten thousand cells were analysed from each sample. Two independent samples were used for each variant. Results were displayed as a histogram of the number of nuclei versus the relative DNA content generated by a Hewlett-Packard computer attached to the flow cytometer and an appropriate program (X Consort 30, HP). Young leaves of *A. saccharinum* trees were used to isolate nuclei for a control of the 2C-peak (Fig. 1). The percentage of cells in G_1, S, and G_2 plus mitosis of the cell cycle was determined using a computer program (CellFIT Cell Cycle Analysis Software, HP).

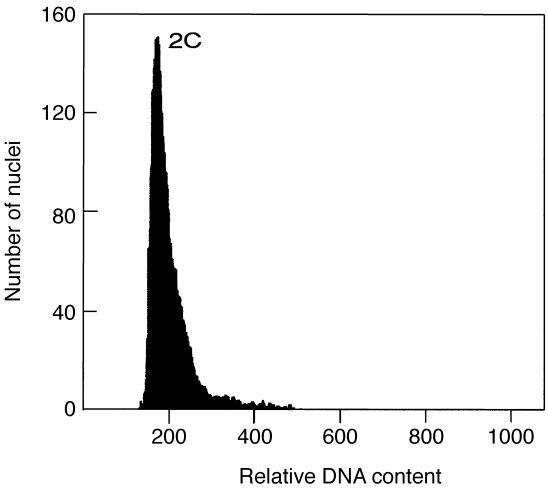

Figure 1. Histogram of flow cytometric analysis of nuclei released from young leaves of *Acer saccharinum*. Nuclei from the leaves show a peak at the 2C DNA level (channel 170).

Results

Studies of *A. saccharinum* seeds were carried out at the final stages of development when their moisture declined. Embryo moisture content of the seeds at shedding differed slightly in each harvest year: 53% in 1997 and 56% in 1998.

During the period of intensive growth (20–26 d after anthesis), the embryo axes of *A. saccharinum* seeds have the cell structure of meristematic tissue: a thin cell wall, central position of the nucleus and the presence of juvenile organelles (Musatenko *et al*., 1995). Similarly, we observed mitoses throughout the axes at 28 d after anthesis. To determine meristem areas of mature embryos, central longitudinal sections of axes were studied. Lengthening of the radicle cortex cells from 10.24 ± 0.22 µm to 15.56 ± 0.41 µm was observed at a distance 429 ± 20 µm from the radicle tip. The area before this point might be considered as a root meristem. Clear changes of cell sizes in the shoot meristem were not observed. For the study of mitotic activity, the first 0.5 mm of radicle and the first 0.5 mm of shoot with embryonic leaves were used.

The percentage of cells in mitosis declined during maturation of the *A. saccharinum* seeds. In 1997 (Table 1), the mitotic index in root apices dropped from 3.1% in the immature seeds (at 82% embryo moisture content) to 0% in the mature seeds (at 53% embryo moisture content) and in shoot apices with embryonic leaves from 5.4 to 3.3%. Cell division in the latter was arrested during dehydration of mature embryos to 45% moisture content. In 1998 (Table 2), the arrest of cell division in the root was also observed at 53% embryo moisture content, similar to that in 1997,

Table 1. Moisture contents and mitotic activities in meristems of *A. saccharinum* embryos at successive maturation stages and in dried or germinated mature seeds (1997 harvest). Values are means ± $SD_{0.05}$

Sampling time	Embryo moisture content, % (w.b.)	Mitotic index, %	
		Root apex	Shoot apex with embryo leaves
28 d after anthesis	82 ± 1	3.1 ± 0.5	5.4 ± 0.8
35 d after anthesis	60 ± 2	1.8 ± 0.3	4.5 ± 1.0
41 d after anthesis (mature seeds)	53 ± 1	0.0	3.3 ± 0.6
4 d drying of mature seeds	45 ± 2	0.0	0.0
3 d germination of mature seeds[a]	62 ± 1	8.0 ± 0.7	12.3 ± 1.5

[a] Mature seeds were tested for germination immediately after harvest. Only germinated seeds were used for the embryo moisture content and mitotic index determinations; germination percentage was 97.

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Table 2. Moisture contents and mitotic activities in meristems of *A. saccharinum* embryos at successive maturation stages and in dried or germinated mature seeds (1998 harvest). Values are means ± $SD_{0.05}$

Sampling time, days after anthesis	Embryo moisture content, % (w.b.)	Mitotic index, %		
		Root apex	Shoot apex	Embryo leaf
42	66 ± 3	0.7 ± 0.1	1.3 ± 0.3	2.9 ± 0.4
45	59 ± 3	0.2 ± 0.1	1.8 ± 0.4	2.8 ± 0.7
48 (mature seeds)	56 ± 2	0.1 ± 0.1	0.4 ± 0.1	1.9 ± 0.5
Duration of drying of mature seeds, d				
1	53 ± 1	0.0	0.1 ± 0.1	0.7 ± 0.2
2	51 ± 1	0.0	0.1 ± 0.0	0.5 ± 0.1
3	50 ± 1	0.0	0.0	0.3 ± 0.2
Germination time, h[a]				
3	56 ± 1	0.1 ± 0.0	0.4 ± 0.1	2.4 ± 1.0
6	56 ± 1	0.3 ± 0.1	0.1 ± 0.0	3.6 ± 0.8
9	58 ± 1	0.2 ± 0.1	0.2 ± 0.1	3.4 ± 0.7
15	58 ± 1	0.2 ± 0.1	0.3 ± 0.0	2.7 ± 0.4
24	59 ± 1	2.2 ± 0.9	2.3 ± 0.8	5.5 ± 1.0

[a] Mature seeds were tested for germination immediately after harvest.

although seeds had matured and been shed earlier (at 56% embryo moisture content). Mitotic activity in shoot apices and embryonic leaves was analysed separately this time. The mitotic index in the embryo leaves of mature seeds was higher (1.9%) than in the shoot apices (0.4%). During further seed dehydration, mitosis was arrested in the shoot apices at about 50% embryo moisture content, whereas some mitotic activity was observed in embryonic leaves.

Flow cytometry was used to study nuclear DNA levels in the axes during late seed development and post-maturation drying only in 1998. Three cell classes characteristic of proliferating meristems were observed: 2C-peak (corresponding to the pre-synthesis G_1 phase), 4C-peak (post-synthesis G_2 phase) and 2C–4C intermediate values (S phase) (Fig. 2). The G_1 cell population rose in the axes simultaneously with the decrease of mitotic activity during seed drying. In the root meristems the relative frequency of cells in the S phase decreased. When the embryo moisture content dropped to 53%, S cells in the root meristems were not detected, and 33% of the cells were arrested in the G_2 phase (Fig. 2E). In the embryo axes without root tips, the relative frequency of cells in S phase remained at approx. 16%, and the percentage of G_2 cells decreased during this period.

Almost one-fifth of the immature seeds harvested at 28 d after anthesis (1997) at about 82% embryo moisture content was able to germinate for 3 d, but germination was delayed (Figs 3A, B). Germinability increased considerably during the late development. The mature seeds germinated quickly and produced the longest seedlings. Drying of mature samaras to 48% embryo moisture content resulted in even more intensive seed germination, but subsequent drying led to the progressive reduction of germinability. At 34% moisture content the seeds did not germinate and were covered with moist necrotic stains.

A rapid (re)activation of meristematic cell cycling was observed during mature seed germination assayed immediately after harvest. An increased mitotic percentage was recorded at 24 h after imbibition: in the root apex to about 2.2%, in the shoot apex to 2.3% and in the embryonic leaves to 5.5% (Table 2). This increase was detected both in germinated (Table 2) and ungerminated seeds after 24 h (data not shown), but the rise of moisture content was small – from 56 to 59%. In 3 d seedlings (10–15 mm long) the mitotic index reached 8.0% in the root apices and 12.3% in the shoot apices with embryonic leaves (Table 1).

Discussion

During *A. saccharinum* seed development, when embryo moisture content was approx. 82% and mitoses were observed all over the axis, the embryo was capable of germination, but the capacity for high germinability was attained during maturation. Embryo moisture contents in this period decreased to 53–56%. Mature seeds germinated quickly in moist conditions. Slight dehydration of mature seeds intensified their subsequent germination. Such a response of recalcitrant seeds to slight drying may be ascribed to a continuation of maturation processes

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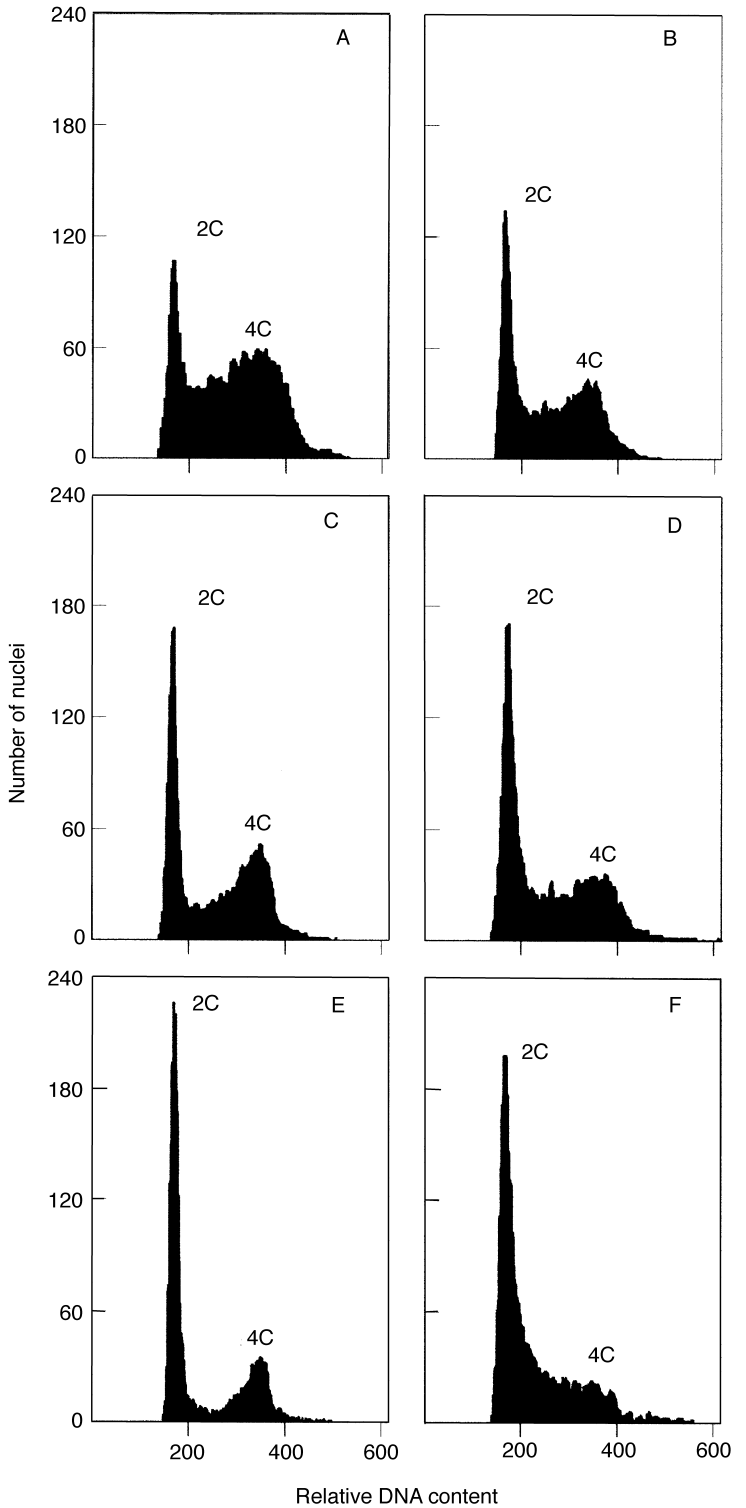

Figure 2. Histograms of flow cytometric analysis of nuclei released from 1 mm long root tips (A, C, E) and axes without root tips (B, D, F) of *Acer saccharinum* seeds (1998 harvest): immature (A, B), mature (C, D) and mature seeds naturally dried in a thin layer of samaras at room temperature (19 ± 1°C, without air mixing) for 24 h (E, F). Nuclei show peaks at the 2C and 4C DNA level (channels 170 and 340, respectively). The number of nuclei used for each determination was about 10,000.

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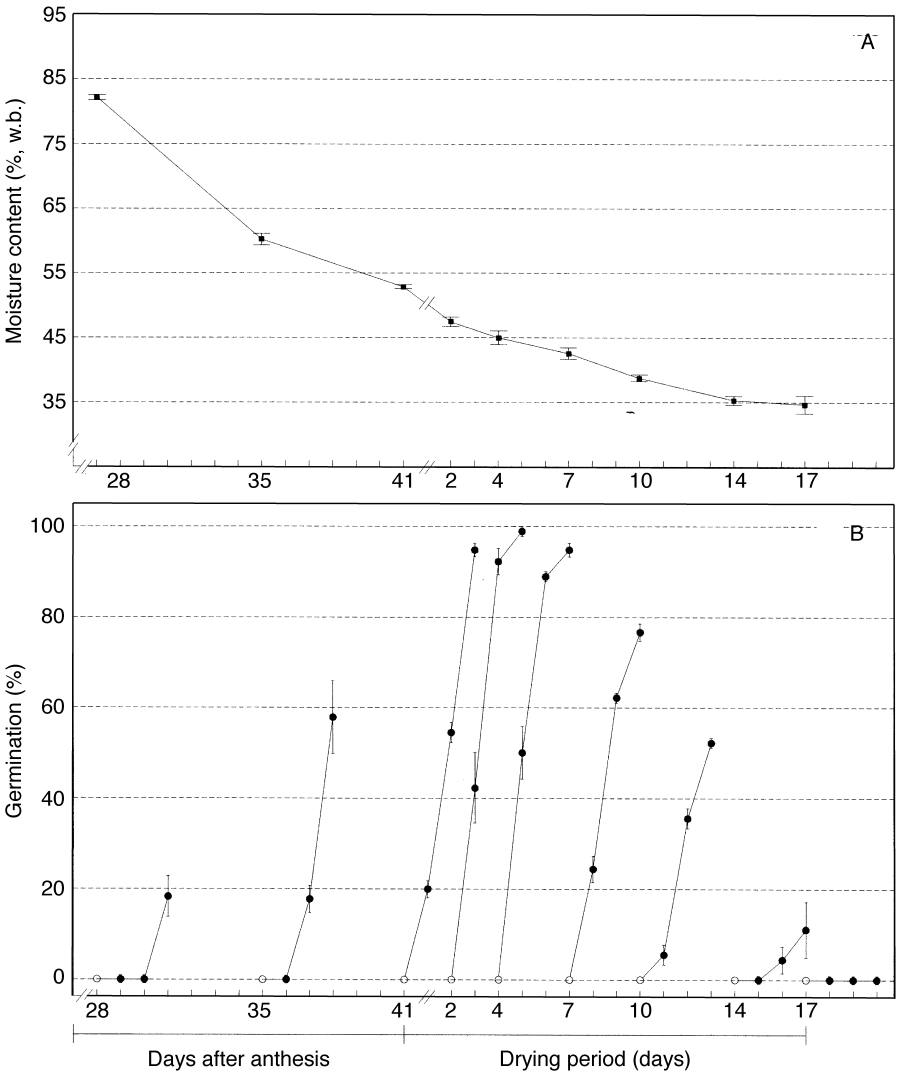

Figure 3. Decline in the embryo moisture content (%, w.b.) (A) and germination (B) of *Acer saccharinum* seeds during maturation and post-maturation drying (1997 harvest). For the post-maturation drying treatment, the mature samaras were naturally dried in a thin layer at room temperature (19 ± 1°C, without air mixing) for 2, 4, 7, 10, 14 and 17 d. For the germination test, seeds harvested at 28, 35 and 41 d after anthesis and the mature seeds (41 d after anthesis) following the drying periods were used. The seeds were assayed immediately after harvest or drying period (○) and tested for germination (radicle >2 mm long) at 26 ± 1°C for 3 d (●). The bars represent the $SD_{0.05}$.

(Pammenter and Berjak, 1999). Further drying of the *A. saccharinum* seeds in samaras resulted in the progressive decline of germinability. The absence of seed germination at 34% moisture content, as well as the appearance of surface necrotic stains under moist conditions, may indicate a non-viable state. Such dependence of viability on moisture content, characteristic of desiccation-sensitive seeds, is in contrast to orthodox seeds that naturally undergo desiccation to low moisture contents at maturity and remain viable for extended periods.

In maturing orthodox seeds, delayed mitotic activity was observed at seed moisture contents from 65 to 48% (Brunori, 1967; Avanzi *et al*., 1969), but in the highly recalcitrant seeds of *Avicennia marina*, there was only 24 h arrest of DNA replication around shedding (Berjak, Dini and Osborne, unpublished results, cited in Pammenter and Berjak, 1999). In *A. saccharinum* seeds mitotic activity in the embryo meristems also declined in the last period of the development when their moisture content decreased. However, when samaras were shed, the embryos contained dividing cells, i.e. continued growing. In the ecology of the species, it could be important for seeds to germinate immediately after shedding and form new plants during the same vegetative season.

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In 1997, when the mass samara abscission took place at 53% embryo moisture content, cell division was observed in shoot apices with embryonic leaves, whereas proliferation in root meristems had ceased. But in 1998, mitotic activity in the mature seeds (56% embryo moisture content) occurred at a low level in the root as well as shoot and leaf meristems. Thus, these results support the view (Berjak *et al*., 1989) that recalcitrant seeds can continue development after shedding.

The arrest of cell division in the drying *A. saccharinum* seeds first occurred in the root apices at about 53% embryo moisture content in both experiments. Water status of the seed may play, therefore, a leading role in the cell cycle arrest. Mitosis ceased in shoot apices at about 50% and in embryonic leaves at 45% embryo moisture content. Mitotic activity in orthodox seeds of *Triticum durum* was arrested at a similar range of moisture contents (Avanzi *et al*., 1969). However, the order of proliferation arrest in meristems was different: first in shoot meristem and primordia and then in the root meristem.

Mitotic activity delay in post-maturation dried seeds of *A. saccharinum* might be regarded as a part of a general response to water stress. Thus, a 30–35% decrease of moisture content in the water-stressed wheat shoot meristems halted mitosis (Shmatko and Kabluchko, 1980). Apical meristem cells of the dried maize roots were viable until they lost 65% of the water (Nir *et al*., 1969).

The decline in mitotic activity in the *A. saccharinum* embryo axes was accompanied by the rise of cells in the G_1 phase to over 60%. Possibly, the physiological significance of G_1 cell accumulation might be a necessity to preserve as many meristem cells as possible during partial drying when the seed has to survive after abscission, before appearance of favourable conditions for its germination. Cells in the G_1 phase exhibit high viability for extended periods of time (Yanishevsky and Stein, 1981) and are more resistant to stress conditions (Deltour, 1985). However, in the mature *A. saccharinum* seeds a significant percentage of G_2 cells was also detected. Similarly, in recalcitrant *Acer pseudoplatanus* seeds during late development, the percentage of nuclei with 2C DNA level increased to over 60% (Finch-Savage *et al*., 1998), but most of the cells were arrested in the G_1 phase in mature recalcitrant seeds of *Castanea sativa* (Bino *et al*., 1993) and *Azadirachta indica* (Sacandé *et al*., 1997). Apparently, the variation in C levels in recalcitrant seeds might be tissue- and species-specific, similar to that for orthodox seeds (Bino *et al*., 1993), or dependent on environmental conditions during maturation. Pammenter and Berjak (1999) have taken into consideration a species provenance. In addition, a high relative frequency of G_2 cells in non-proliferating meristems has been suggested to be connected with rapid drying of seeds: a slow drying treatment of carrot embryoids was necessary for decreased numbers of cells in the G_2 phase (Tetteroo *et al*., 1995). Similarly, a rapid drying of the wheat seedlings during drought resulted in the arrest of cycling in most of the shoot meristem cells in the G_2 phase (Shmatko and Kabluchko, 1980).

A resumption of DNA replication during orthodox seed germination has to follow the pre-replicative period of the cell cycle necessary for cell transition from quiescence state (G_0), DNA repair (Elder and Osborne, 1993), chromatin decondensation (Crève-coeur *et al*., 1976), and preparation for DNA synthesis (Troyan *et al*., 1984). In contrast, the presence of dividing cells in the embryo of mature *A. saccharinum* seeds may indicate that immediate germination does not require the pre-replicative period.

A decrease of moisture level in maturing recalcitrant *A. saccharinum* seeds was accompanied by the progressive diminution of mitotic activity of the meristem cells and accumulation of G_1 cells. The post-maturation drying of the seeds resulted in the arrest of cell division. The data suggest that the embryo moisture content may control the extent of mitotic activity in the meristems of maturing seed. The strategy of immediate germination of recalcitrant *A. saccharinum* seed includes preservation of cell division at low levels in the embryo of mature seed and high mitotic activity in the meristems of germinating seed.

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