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Seed dormancy in relation to seed storage behaviour in *Acer*

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Dormancy in seeds of *Acer opalus* is shown to be mainly caused by the seed coats, although a slight embryo dormancy exists in fresh seeds. The ability to germinate after drying indicates that seed storage behaviour is orthodox. Recalcitrant seeds were heavier than orthodox seeds not only within section *Acer* but also within the whole genus after statistical control of phylogeny, through a phylogenetic ANOVA with data from two different *Acer* phylogenies. An evolutionary change from orthodox to recalcitrant behaviour is postulated for genus *Acer*, but this change appears not to have been accompanied by a change in seed dormancy, at least in the taxonomic section in which *Acer opalus* belongs.

ADDITIONAL KEYWORDS: imposed dormancy – orthodox behaviour – phylogenetic ANOVA – seed coat.

INTRODUCTION

The nature and expression of seed dormancy in the genus *Acer* varies, ranging from the non-dormant seeds of *Acer saccharinum* (Suszka & Tomasewska, 1971) to the deeply dormant propagules of species such as *A. ginnala* (Dumbroff & Webb, 1970). Pinfield & Dungey (1985) divided *Acer* species that produce dormant seeds into those whose propagules that have an embryo dormancy (embryos do not germinate even when isolated from seed coats) and those in which dormancy can best be described as testa-imposed (embryos germinate as soon as the seed coats are removed). However, the distinction between these two categories is often not very clear, and seeds of many species may show features associated with both kinds of dormancy (see for example Webb & Dumbroff (1969) for *Acer saccharum,* Dumbroff & Webb (1970) for *Acer ginnala*, Wilson, Hibbs & Fischer (1979) for *Acer pensylvanicum*, Pinfield, Stutchbury & Bazaid (1987) for *Acer pseudoplatanus*). Furthermore, Thomas, Webb & Wareing (1973) found that embryos are dormant during seed maturation in *Acer pseudoplatanus*, a species traditionally classified as having seeds with testa-imposed dormancy. Because seeds of different *Acer* species are morphologically very similar to one another, Thomas *et al.* (1973) proposed that it is probable that they all pass through a similar maturation process. This consists of a sequence of phases that are characterized by modifications of the dormancy present, starting from deep embryo dormancy in the developing seed and reaching a stage of coat-imposed dormancy at the end. The variety of different types of dormancy found in the genus may reflect differences in the duration of particular stages during the maturation process, as well as differences in the timing of fruit dispersal.

Seed storage behaviour has been associated with characters such as the seed shape, weight and moisture content at shedding (Hong & Ellis, 1996). In a study that was particularly focused in *Acer* species, Hong & Ellis (1996) introduced a scheme for estimating seed storage behaviour that is based on two criteria, seed moisture content at maturity (or shedding)

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and the thousand-seed weight. According to this scheme, heavier seeds with a high moisture content are more likely to be recalcitrant (seeds that do not survive if desiccated to levels below 90% relative humidity, RH), whereas the opposite characters are more frequent in orthodox seeds (seeds that tolerate the immediate effects of severe water loss) (Dickie & Pritchard, 2002). The authors pointed out that an association between taxonomy and seed storage behaviour could exist (i.e. recalcitrant species only occur in some taxonomic series), and thus the relationship between seed weight and seed storage behaviour may be the result of common evolutionary ancestry (i.e. all the recalcitrant species within a series had a recalcitrant, heavy-seeded ancestor). Therefore, it is necessary to take into account the lack of statistical independence derived from the phylogenetic relatedness of the species to test the relationship between seed weight and seed storage behaviour (Harvey & Pagel, 1991).

The inter-relationship between the physiological traits of seed dormancy and desiccation (in)tolerance is complex. Some orthodox (desiccation-tolerant) seeds are dormant at shedding/dispersal, but there is no evidence to suggest a causal relationship. In addition, whereas most recalcitrant seeds are non-dormant, some, such as *Acer pseudoplatanus* (sycamore), are dormant (Pinfield *et al*., 1987). In this study we consider whether these traits may have evolved in parallel in the genus *Acer*. We first determine which type of seed dormancy predominates in *Acer opalus* (Aceraceae), then we classify *Acer opalus* seeds in terms of their storage behaviour, and using these data we test the hypothesis that recalcitrant seeds are heavier than orthodox seeds, taking the genus *Acer* phylogeny into account. Finally, we determine whether evolutionary changes in seed storage behaviour are correlated with changes in seed dormancy mechanisms within the section *Acer*, in which *Acer opalus* is located.

MATERIAL AND METHODS

STUDY SPECIES

Acer opalus Mill. (Aceraceae) is a deciduous small tree with usually five-lobed leaves that are very variable in size and shape. Populations are sexually dimorphic, composed of bisexual (monoecious) and unisexual (male) trees. Anthesis takes place before the leaves emerge. The flowers are functionally unisexual because of abortion of the pistil or incapacity of the anthers to open, and are arranged in subsessile corymbs. Both types of flowers are yellowish, actinomorphic and they contain a nectary. Ovaries have two locules with two ovules, but only one develops after fertilization. The indehiscent fruits are samaras, composed of two one-seeded mericarps. Seeds are non-endospermous, and embryos have green cotyledons. Parthenocarpic development of the fruit is very frequent (de Jong, 1976).

The infrageneric classification of the genus *Acer* proposed by van Gelderen, de Jong & Oterdoom (1994) is used in this work. According to this classification, *Acer opalus* belongs to section *Acer*.

SEED DORMANCY

Fresh fruits were collected from ten trees in 'El Boixar', eastern Spain (40°41′N, 0°07′E) in October 2001. The fruits were dried for 11 days at room temperature in a chamber with 6–10% air humidity. The fruits were left in the chamber until they reached a seed water content of *c*. 5% [according to ISTA (1991), a water content between 5 and 10% is suitable for conservation of the fruits]. The water content of the fruits was determined from three samples of 30 fruits each, with a Mettler Toledo HB43 moisture analyser. The temperature selected for drying in the analyser was 105°C, and the disconnection criteria, 1 mg 50 s^{-1}. The dried fruits were then stored at 4°C until the beginning of the experiment 25 days after the fruit collection. Before starting the germination experiment, fruits were disinfected in 1% sodium hypochlorite solution for 15 min. As the frequency of parthenocarpic fruits is high in the species, a flotation test was performed to separate viable seeds from non-viable seeds. Wings of the samaras were cut and the wingless fruits (seeds hereafter) were soaked in distilled water for 24 h, after which seeds that did not sink were discarded (empty seeds).

A two factorial design involving two factors (stratification and excision of the embryo) with two levels each (presence or absence of stratification or excision treatments) was performed in the following way:

1. no stratification + no excision;
2. no stratification + excised embryos;
3. cold stratification + no excision;
4. cold stratification + excised embryos.

The excised embryos treatment involved the removal of the pericarp and testa. In the stratification treatment, the seeds were layered in sand and vermiculite and were stored at 4°C for 3 months. The fruits that received the two treatments were stratified before pericarp and testa were removed.

After these treatments, seeds/embryos were placed in Petri dishes, which were watered with 0.072% Himexazol solution to prevent fungi infection. The incubation was performed in a Sanyo MLR-35O germination chamber (fluorescent lamp of 40 W × 15 ps) operating at 20°C with a 12 : 12-h light/dark photoperiod at a light intensity of 15 µmol m^{-2} s^{-1}. Water was supplied once before the beginning of the germination period. Eight Petri dishes were used per treatment, each one containing 25 seeds. The dishes were checked every 3–4 days for 1 month and the number of seeds/embryos germinated was scored. A seed was considered to have germinated when the radicle emerged and, in the case of the excised embryos, when the radicle had elongated by at least 1 cm. At the end of the assay, seeds that had not germinated were opened to check if the embryo was aborted or if the seed was empty. The time elapsed until reaching 50% germination of the final germination level (T_{50}) was the measure selected to study the germination velocity.

The percentage of germination of seeds in each treatment was analysed in a two-way robust analysis of variance in which seed germination was considered the dependent variable and stratification and excision as independent variables. Variance was zero in the control group because all the seeds failed to germinate. As some variance is necessary to run statistical comparisons, we slightly modified one value from 0 to 0.1%. Similarly, one of the values of the chilling plus excision of embryos treatment, in which all the seeds germinated, was changed from 100 to 99.9%. Robust ANOVA was used to account for the departure of normality and homogeneity of variances of the data (Wilcox, 1997) and was run in the R statistical software (Ihaka & Gentleman, 1996). Means ± 1 standard deviation are shown throughout the text.

SEED STORAGE BEHAVIOUR IN *ACER OPALUS*

The traits proposed by Hong & Ellis (1996) to be associated with storage behaviour were measured. The weight of 1000 fruits was obtained, and the water content at maturity was determined as the difference between the weight of fresh fruits and the weight of dried fruits (fruits were dried at 105°C for 24 h). In addition to this, the germination response after drying of the fruits until 5% moisture content (see germination experiments) was used to evaluate the seed storage behaviour.

PHYLOGENETIC ANALYSIS OF SEED WEIGHT AND STORAGE BEHAVIOUR

Data for seed weight and storage behaviour were obtained from Hong, Linington & Ellis (1996), from Tweddle, Turner & Dickie (2003) and from the present work.

To test the differences in seed weight between recalcitrant and orthodox *Acer* species, we ran two phylogenetic ANOVAs after incorporating the relationships from the following two different *Acer* phylogenies: Hasebe, Ando & Iwatsuki (1998) and Ackerly & Donoghue (1998) (see Fig. 2). The phylogeny proposed by Suh, Heo & Park (2000) for genus *Acer* was not used because it does not include enough recalcitrant species to run the analysis. In the Ackerly & Donoghue phylogeny, it was necessary to consider recalcitrant and intermediate species [species with seeds that tolerate considerable (at least to 30% RH) but not complete drying (Dickie & Pritchard, 2002)] together in the same group to run the statistical test.

The phylogenetic ANOVAs were run in the PDAP software (Garland *et al*., 1993), which creates, by computer simulation, a null distribution incorporating the phylogenetic relationships among species. Equal branch lengths were assumed because *Acer opalus* was grafted into the trees and therefore the original branch lengths could not be used (see Verdú, 2002 for a similar procedure). Seed weight was log-transformed to achieve normality.

RESULTS

SEED DORMANCY

Figure 1 shows the germination behaviour from the four different treatments. Control seeds failed to germinate whereas the removal of the seed coats resulted in a high germination percentage (84.6 ± 6.1%) but at a low speed ($T_{50} = 18 \pm 1$ days). Chilling of the intact fruits accelerated the speed of germination ($T_{50} = 5 \pm 0$ days), although to level of 50.9 ± 8.6% germination at the end of the experiment. Finally, chilling plus embryo excision increased both the speed and the final percentage of germination, yielding 100% of germination and a T_{50} of 5 ± 1 days.

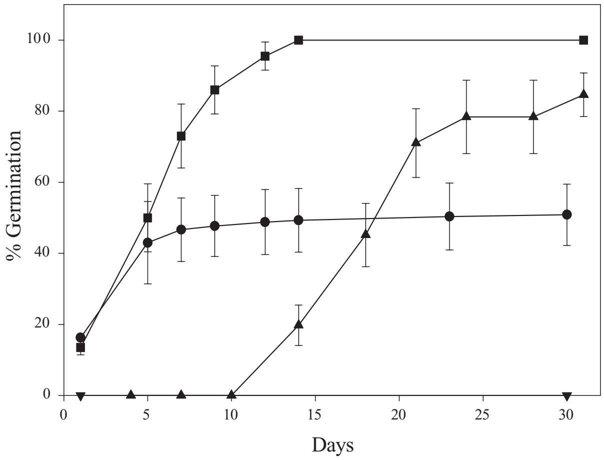

Figure 1. Percentage germination of seeds and isolated embryos treated as described in the Material and Methods section. (●) Chilling, (▲) excised embryos, (■) chilling + excised embryos, (▼) control. The vertical lines show the means ± one standard deviation.

The robust two-way ANOVA on the germination percentage showed a significant effect of the stratification ($t = 313.8$, $P \ll 0.05$), excision ($t = 1279.7$, $P \ll 0.05$) and the interaction between both treatments ($t = 31\,981\,045$, $P \ll 0.05$). This interaction indicates that the level of success of the chilling treatment partly depends on the excision treatment, as the combination of embryo isolation and chilling is clearly the most successful dormancy-breaking treatment.

SEED STORAGE BEHAVIOUR IN *ACER OPALUS*

The moisture content of the seeds at maturity was 23%, and the weight of 1000 seeds was 37.5 g. The low water content as well as the quite low weight of the seeds indicate that *Acer opalus* seeds are very close to the area of orthodox seeds in the scheme proposed by Hong & Ellis (1996). Additional evidence of the orthodox behaviour of *Acer opalus* seeds comes from the fact that although the seeds were dried to about 5% moisture content, 100% germination was obtained in one of the treatments performed, a result that would have been impossible in recalcitrant seeds, which do not survive drying to any large degree (Dickie & Pritchard, 2002).

PHYLOGENETIC ANALYSIS OF SEED WEIGHT AND STORAGE BEHAVIOUR

Non-orthodox seeds in the genus *Acer* were significantly heavier than orthodox seeds after controlling for phylogeny. This result was consistent across the two published phylogenies of the genus *Acer* ($F_{1,24} = 11.7$, $P = 0.0019$ for Hasebe *et al.*'s phylogeny and $F_{1,26} = 11.8$, $P = 0.0001$ for Ackerly & Donoghue's phylogeny) (Fig. 2).

DISCUSSION

Seeds of *Acer opalus* were dormant at the time of dispersal, as all the untreated seeds failed to germinate after 1 month incubation at 20°C. Dormancy imposed

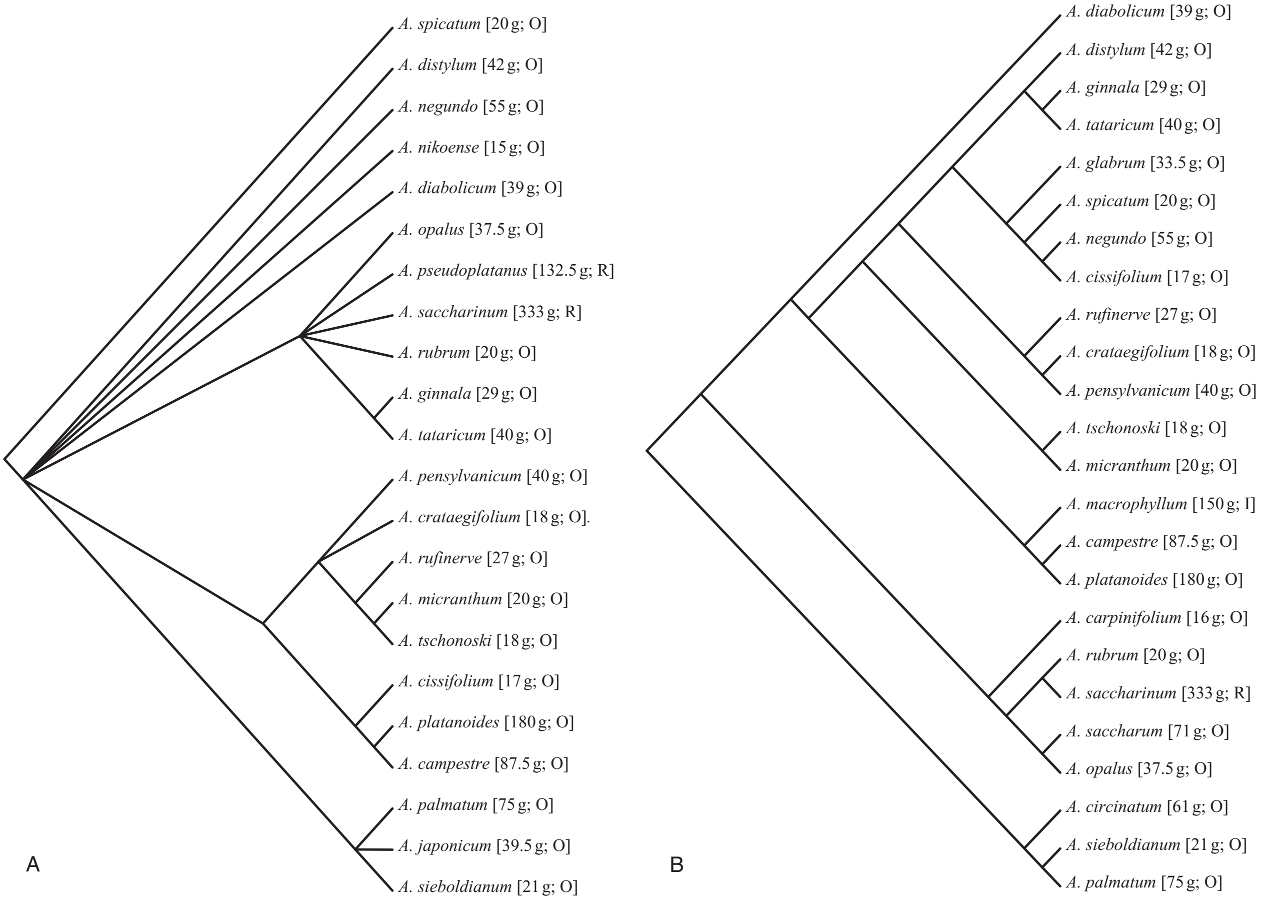

Figure 2. Phylogenetic trees of *Acer* species proposed by (A) Hasebe *et al*. (1998) and (B) Ackerly & Donoghue (1998) in which the evolutionary relationship between seed weight and seed storage behaviour has been analysed. The thousand seed weights and the storage behaviour (O: orthodox, I: intermediate, R: recalcitrant) are shown in parentheses.

by the structures covering the embryos appears to be the main factor preventing the germination of fresh fruits. Removal of the seed coats considerably increased the percentage of germination up to 80% in 1 month, suggesting that the integuments that enclose the embryos play a principal role in the prevention of germination. Seed-coat-imposed dormancy has also been reported within section *Acer* for *Acer pseudoplatanus* (Pinfield & Stobart, 1972; Webb & Wareing, 1972).

Cold stratification accelerated the speed of germination, but was not sufficient completely to break dormancy in 3 months. Three months of low temperatures may not be enough to break dormancy in all *Acer opalus* seeds, as chilling treatment resulted in only 50% germination. This would explain the germination behaviour observed in the wild, where some of the seeds produced one year may require two periods of low temperatures to germinate (L. Gómez, pers. comm.). The fact that the germination of a single crop can be dispersed over at least two seasons was also observed for *Acer rubrum* (Farmer & Cunningham, 1981).

Cold stratification plus embryo excision increased the percentage of germination to 100% and the speed to a T_{50} value of 5 days. This fact confirms that a double dormancy mechanism is acting on *Acer opalus* seeds: a slight physiological dormancy in the embryo and a seed-coat-imposed dormancy. Thomas *et al.* (1973) also reported the presence of a transient phase of embryo dormancy existing only for a short period in *Acer* species in which dormancy is imposed by the seed coat. A similar incomplete embryo dormancy was found in *Acer pseudoplatanus* (Pinfield *et al.*, 1987).

Regarding the storage behaviour, the low moisture content and weight of the *Acer opalus* seeds situate this species in the intermediate area, but very close to the orthodox behaviour in the scheme proposed by Hong & Ellis (1996). Results of the present study support the orthodox behaviour of *Acer opalus*, as long as seeds are able to germinate up to 100% after seed water content was reduced to 5%. This fact fits well, at least within the section *Acer*, with the hypothesized relationship between seed weight and storage behaviour, because the weight of the recalcitrant seeds of *A. pseudoplatanus* is 3.5-fold that of the orthodox *A. opalus* seeds (Hong & Ellis, 1996; Kermode & Finch-Savage, 2002). Furthermore, this relationship is not only valid within one single section but also within the whole genus *Acer*, as the phylogenetic ANOVAs performed in two different phylogenies revealed. Very few species within the genus *Acer* are recalcitrant, suggesting the most parsimonious evolutionary hypothesis that recalcitrant behaviour is a derived trait in the genus. The same hypothesis has been proposed for all angiosperms, where the recalcitrant behaviour is considered an apomorphic state (Dickie & Pritchard, 2002).

Nevertheless, the evolutionary changes that occurred in seed storage behaviour within the section *Acer* from orthodox to recalcitrant seeds may not be apparently correlated with changes in seed dormancy mechanisms, at least within the section *Acer*. In this section, the dormancy mechanisms found in the orthodox *A. opalus* are extremely similar to those described in the recalcitrant *A. pseudoplatanus*. Thus, surprisingly seed storage behaviour seems to have evolved independently from seed dormancy mechanisms. We suggest further work is focused on elaborating this hypothesis.

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