

Laboratory germination and seed bank storage of *Ranunculus peltatus* subsp. *baudotii* seeds from the Tuscan Archipelago

A. CARTA¹, G. BEDINI¹, B. FOGGI² AND R.J. PROBERT³

¹ Department of Biology, Botanic Garden, University of Pisa, via Luca Ghini 5, I-56125, Pisa, Italy
(E-mail: acarta@biologia.unipi.it)

² Department of Evolutionary Biology, Laboratories of Botany, University of Florence, via La Pira, 4, I-50121, Florence, Italy

³ Seed Conservation Department, Royal Botanic Gardens, Kew, Wakehurst Place, Ardingly, West Sussex, RH17 6TN, UK

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Summary

Germination requirements and suitability for *ex situ* conservation were investigated for seeds from the only population of *Ranunculus peltatus* Schrank subsp. *baudotii* (Godron) Meikle ex C.D.K. Cook (Ranunculaceae) from the Tuscan Archipelago (Italy). A $2 \times 2 \times 3$ factorial experiment investigated the interactive effects of light, alternating temperatures and mean temperature on the germination of seeds sown on agar. Logistic regression analysis showed significant effects of both temperature ($P < 0.0001$) and light ($P < 0.05$). Alternating temperatures improved germination but only at mean temperatures of 20 and 15°C. The results indicate that seeds of *R. baudotii* exhibit morphological dormancy and a phenological adaptation to seasonal ponds. Drying led to a significant reduction in germination in seeds collected in both 2009 and 2010 ($P < 0.05$); the reduction was larger for 2009. The better quality of the 2010 seed was probably due to a post-harvest treatment that enabled continued maturation of the seeds. Storage of seeds for one year under seed bank conditions (-20°C) did not reduce viability. Seeds of *R. baudotii* possess orthodox storage behaviour and are therefore suited to *ex situ* conservation in seed banks; however, further research is needed to confirm their long term storage prospects.

Introduction

Biodiversity declines are far greater in fresh waters than in terrestrial ecosystems (Sala *et al.*, 2000). As a consequence, in many countries, aquatic plants are among the most threatened groups of organisms (Preston and Croft, 2001). *Ex situ* conservation through seed banks will play an important role in safeguarding aquatic species (UNEP, 1992), thus an understanding of seed germination requirements is essential for designing propagation protocols and appropriate restoration techniques (Bowes, 1999).

Ranunculus peltatus Schrank subsp. *baudotii* (Godron) Meikle ex C.D.K. Cook (Ranunculaceae; hereafter *R. baudotii*) is a hydrophyte widely distributed throughout West and South Europe (Tutin *et al.*, 1993), where it grows in shallow water bodies (Passarge, 1992) characterised by alternate flooding and drying (Hartog and Segal, 1964). The habitat where it grows is of conservation interest in the European Community (Habitats Directive 92/43/CEE).

The only population in the Tuscan Archipelago (Italy) became extinct in the wild in 2010 due to habitat loss after the invasion of the pond by tall helophytes. An integrated *in situ / ex situ* conservation project, supported by the Tuscan Archipelago National Park and launched in 2001 (Foggi *et al.*, 2001), allowed for restoration of the pond and the *ex situ* conservation of the plant (Carta *et al.*, 2009).

Seeds of numerous Ranunculaceae have underdeveloped embryos (Martin, 1946; Engell, 1995). In seeds with an underdeveloped embryo, the embryo has to grow to a critical length before visible germination can occur; this lag period is often referred to as “morphological dormancy” (Baskin and Baskin, 1998). In some Ranunculaceae species there is no additional mechanism preventing embryo growth and seed germination (Baskin and Baskin, 1998). However, in seeds of other Ranunculaceae, an additional physiological mechanism delays embryo growth (Mondoni *et al.*, 2008).

Detailed studies related to the seed germination and *ex situ* conservation of this taxon are still lacking, although low temperature requirements for seedling establishment have been reported (Bonis *et al.*, 1996) as has seed desiccation tolerance (Hay *et al.*, 2000).

Mediterranean ponds are characterised by periods of inundation during winter and spring and exposure to dry conditions in summer (Keeley and Zelder, 1998). Moreover, seeds of aquatic plant species tend to be sensitive to light and require specific temperatures for germination (Baskin and Baskin, 1998). Consequently, we hypothesise a positive germination response of *R. baudotii* to decreasing temperatures and to light conditions.

In this study, embryo elongation and the effects of light, temperature and temperature regime on the germination of *R. baudotii* seeds were investigated using laboratory experiments. Also, seed germination following one year of cold, dry storage under seed bank conditions was tested. The results are discussed from an ecological and conservation perspective.

Materials and methods

Field site and population characteristics

The “Stagnone”, located on Capraia Island at 321 m a.s.l., is the only natural pond in the Tuscan Archipelago. It is fed by freshwater and is approximately 100 m in length and 50 m in width. Before the invasion of tall helophytes such as *Typha angustifolia* L., *T. latifolia* L. and *Phragmites australis* (Cav.) Trin. ex Steud. it was covered by rhizophytic floating vegetation, dominated by *R. baudotii* and *Myriophyllum alterniflorum* DC. (Lastrucci *et al.*, 2009).

Recordings of water temperatures are not available for the Stagnone, however water depth rarely exceeds 1 m and therefore water temperature probably fluctuates through the year following the trend of monthly average air temperatures. The temperature regime on the Island is typically Mediterranean, characterized by mild winters and hot summers (average temperature of 10 and 26°C respectively). Pinna (1991) observed that autumn and winter rainfall predominate (62% of annual rainfall) and during the summer, marked aridity coupled with high summer temperatures may lead to water scarcity in the pond. Thus, the pond shows a semi-permanent hydrology (Keeley and Zelder, 1998).

Seed collection and processing

The *ex situ* conservation phase of the project was planned and implemented before the start of the restoration activities. During May 2009, 10 plants of *R. baudotii*, at least 5 m apart from one another, were sampled from the *in situ* population at the late flowering stage, and taken to the Botanical Garden of Florence, where they were grown in a pool. Collections of achenes (hereafter referred to as seeds) were made from these plants in June 2009 just before the time of natural dispersal, when the achenes were about to be shed from the parental plants, by gently shaking the fruit heads (Baskin and Baskin, 1998). Seeds were then taken to the Pisa Botanical Garden Seed Bank (table 1), where they were rapidly dried to a low moisture content in a controlled environment room (15°C, 15% RH) or tested for viability immediately by means of a germination test. A water activity station (Rotronic) was used to check whether seeds had equilibrated to the room conditions. Dried seeds were then hermetically sealed in a single laminated aluminium foil bag and maintained at -20°C. Seedlings from the initial germination test were grown to establish a second *ex situ* living collection at the Botanic Garden of Pisa.

Table 1. Details of *R. baudotii* material used in the investigations: seed collections, year of collection, post-harvest treatment, weight of seeds and number of seeds used in each experiment. A = embryo growth; B = effect of temperature and light on germination; C = effect of stratification on germination; D = effect of drying and storage on germination.

Collection	Year of collection	Post-harvest treatment ¹	100 seed dry weight (g)	Experiment ² (number of seeds)			
				A	B	C	D
Pisa BG	2010	Yes	0.071	200	600	100	150
Florence BG	2009	No	0.060	100	0	0	150

¹Seeds placed in water held in polythene boxes after collection; ²experiments B and C conducted using fresh seeds.

In May 2010, seeds were collected from these plants (table 1). The seed lot was subjected to the following treatment, mimicking natural post-dispersal conditions: (1) seeds were kept for 2 weeks in water held in polythene boxes (c. 25/15°C), where the seeds initially floated then sank; (2) these seeds were then either directly placed in a dry room (15°C, 15% RH) or subjected to drying conditions chosen so that the rate of water loss matched the rate that would occur during maturation drying *in situ* (seeds maintained in the laboratory at average conditions of approximately 20°C and 50% RH). Experiments with fresh seeds collected in 2010 were started within 1 month of harvesting.

In this study a standard sample size of 50 seeds for germination experiments was allowed due to the small number of seeds available from the small population studied.

Temperature requirements for embryo growth

An experiment in controlled conditions was performed to study the effect of low and high temperatures on embryo elongation. Dried seeds collected in 2010 (Pisa BG) were germinated in Petri dishes containing 1% distilled water-agar, incubated at a constant temperature of either 10 or 20°C with 12 h photoperiod.

The average embryo length to seed length ratio (E:S ratio) was determined following the method of Forbis *et al.* (2002). Observations on embryo growth were made at 5 day intervals on samples of 10 seeds with intact seed coat. Using a binocular microscope to aid dissection, embryo and seed length was determined using an ocular micrometer. The critical E:S ratio was computed as the average E:S ratio of 10 seeds with split seed coats and no radicle protrusion.

An additional test to measure E:S ratio was performed using dried seeds collected in 2009 (Florence BG) placed at constant temperature of 10°C and exposed to a 12 h daily photoperiod.

Effect of temperature and light on germination

A 2 × 2 × 3 factorial design was used to test the interactive effects of light, alternating temperatures and mean temperature on germination. Each treatment consisted of two samples of 25 fresh seeds collected in 2010. All experiments were carried out in 90 mm diameter Petri dishes containing 1% distilled water agar. For dark treatments, Petri dishes were wrapped in aluminium foil. Seeds to be exposed to light were left unwrapped. Samples were maintained in temperature ($\pm 1^\circ\text{C}$) and light controlled incubators using 12 h daily photoperiod and thermoperiod (for alternating temperatures). Seeds were exposed to temperature conditions simulating seasonal temperatures in Capraia Island: constant temperatures (10, 15 and 20°C) simulating germination under flooded conditions, and diurnal alternating temperatures (5/15, 10/20 and 15/25°C) simulating wet conditions of the pond margins. The experiment lasted for 50 days, during which germinated seeds were counted and removed every two days. Germination was recorded when the radicle had emerged from the pericarp by at least 1 mm.

Effect of stratification on germination

R. baudotii seeds are dispersed during spring, suggesting that a period at high temperatures might be required to break dormancy. Therefore, we examined the effect of moist storage at high temperature on subsequent germination at lower temperatures. Seeds were sown in Petri dishes containing agar and placed at 20°C in the dark for 1 month, before being transferred to 10 and 15°C in the light. After transferring to lower temperatures, germinated seeds were counted and discarded every two days.

Effect of drying and storage on germination

To test seed germination following exposure to seed banking conditions, seeds from both collections (Florence BG and Pisa BG) were tested for germination after drying (15°C, 15% RH) and storage (-20°C) for 1 year. Germination tests were carried out with two replicates of 25 seeds at 10°C in the light; germinated seeds were counted and removed every two days.

Data analysis

Seed germination data were analysed using a generalized linear model in R (R Development Core Team, 2011), with a logit link function and a binomial error structure, followed by a likelihood test. For each experiment, a full model including all main factors

and interactions was fitted. In addition, χ^2 test were carried out to compare the final proportions of fresh germinated seeds from both collection. At the end of the experiment a cut test was performed to determine the number of ungerminated viable seeds; defective seeds (i.e. empty or damaged) were excluded from the experiments.

Results

Temperature requirements for embryo growth

Ripe seeds of *R. baudotii* collected in 2010 (Pisa BG) had an initial E:S ratio of 0.4 ± 0.02 (mean \pm SE). Growth of the embryo started immediately in seeds incubated at 10°C in the light. After 10 days, 80% of the seeds had reached the critical E:S ratio and had germinated (figure 1). The small standard error during the course of the experiment indicated that the embryo elongated at a similar rate in all seeds. None of the seeds that were incubated at 20°C in the light had reached the critical E:S ratio (0.8 ± 0.01) after 45 days of incubation. The average E:S ratio at the end of the incubation period was 0.4 ± 0.02 . Seeds of *R. baudotii* collected in 2009 (Florence BG) had an initial E:S ratio of 0.3 ± 0.02 (mean \pm SE). Growth of the embryos started immediately; however, due to their shorter length, they required one more week to reach the critical E:S ratio compared with seeds collected in 2010 (Pisa BG). The smaller embryo size suggested they may have been less mature. The difference in time taken for embryos to reach critical length is matched by greater variation in seed size in the 2009 seed lot (higher SE).

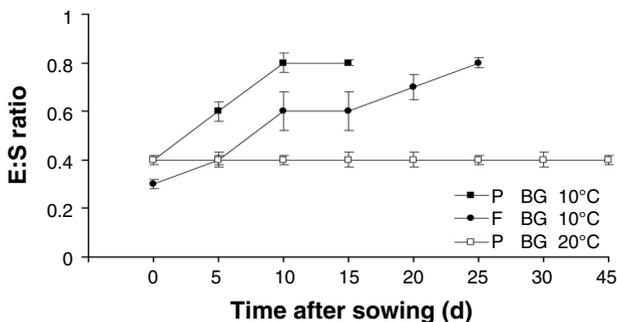


Figure 1. Embryo growth of *R. baudotii* seeds collected at Pisa BG (closed squares) and at Florence BG (closed circles) incubated at 10°C in the light. Embryo growth for seeds collected at Pisa BG incubated at 20°C in the light (open squares). Vertical bars represent SE; $n = 10$.

Interactive effects of light, alternating temperature and temperature on germination

There was high germination ($> 75\%$) in most germination treatments (figure 2). Logistic regression analysis showed that the main effects of temperature range and light were significant ($P < 0.0001$ and $P < 0.05$ respectively) and there was a highly significant first order interaction effect of temperature regime and mean temperature ($P < 0.001$). That is to say the effect of alternating temperatures depended on the mean temperature, with a marked effect at warm temperatures (mean 20°C) but no effect at cool temperatures (mean 10°C).

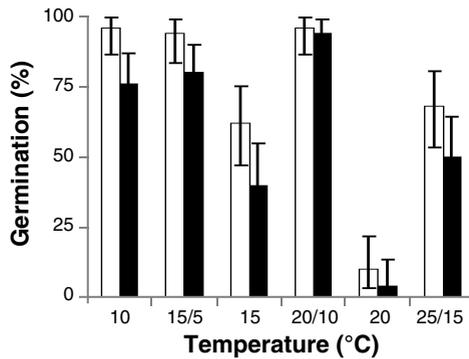


Figure 2. Germination percentage ($\pm 95\%$ binomial confidence intervals) of fresh seeds incubated in the light (open columns) or in the dark (closed columns).

Highest germination occurred at 10°C and 15°C in the light, and at $20/10^{\circ}\text{C}$, in the light and dark with no significant differences between these conditions ($P > 0.05$). However, seeds germinated fastest at 10°C , where germination started after 8 days and up to 80% of the seeds had germinated by 10 days (figure 3).

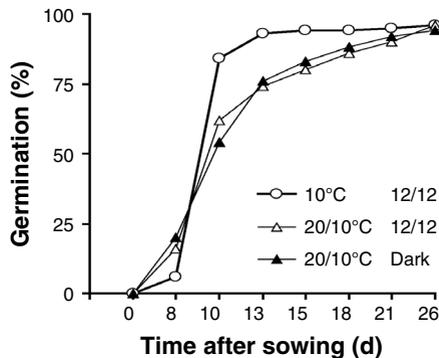


Figure 3. Germination time course for fresh seeds of *R. baudotii* at 10°C in the light (open circles), at $20/10^{\circ}\text{C}$ in the light (open triangles) and at $20/10^{\circ}\text{C}$ dark (closed triangles).

Effect of stratification on germination

Pre-treating the seeds at 20°C (dark) for 1 month did not enhance germination rather the final germination percentage in the light was reduced from 96% to 86%.

Effect of drying and storage on germination

Drying and storage led to a significant reduction in germination in seeds collected in 2010 at Pisa Botanic Garden ($P < 0.05$) and to a highly significant reduction ($P < 0.001$) in seeds collected in 2009 at Florence BG (figure 4).

Germination levels for each corresponding treatment were lower in 2009 compared with 2010. For example, the germination of fresh seeds in 2009 (82%) was significantly lower than the germination of fresh seeds in 2010 (96%) ($P < 0.05$). For both collections, storage of dried seed for one year had no effect on germination ($P > 0.05$).

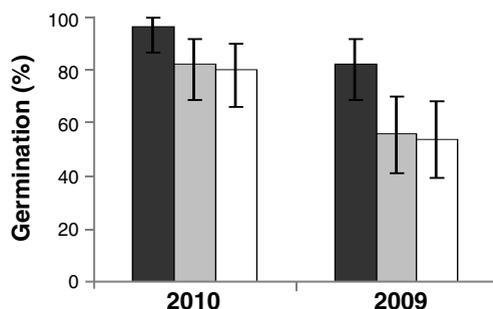


Figure 4. Germination percentage ($\pm 95\%$ binomial confidence intervals) at 10°C in the light of fresh (dark), dried (gray) and stored (white) seeds of *R. baudotii* collected in 2010 (Pisa BG) and 2009 (Florence, BG).

Discussion

As is the case for other Ranunculaceae species (Engell, 1995), this study has shown that embryos of *R. baudotii* are underdeveloped at the time of natural dispersal. After completion of embryo elongation, seeds germinated quickly, within 30 days without any pre-treatment. Warm stratification actually reduced final germination percentage, as in other Ranunculaceae (Young *et al.*, 1992; Vandeloos *et al.*, 2009). Therefore according to the classification of seed dormancy (Baskin and Baskin, 1998; 2004) seeds of *R. baudotii* can be described as having morphological dormancy (MD). However, recent reports on the germination phenology of other species where embryo development continues seamlessly under natural condition until radicle emergence have argued that such seeds should be regarded as non-dormant (Mondoni *et al.*, 2008; Carasso *et al.*, 2011). *Ranunculus* L. species adapted to the aquatic environment have been recognised as a taxonomic entity ascribed to Subgen. I. *Batrachium* (DC) A. Gray. (Johansson, 1998) and are considered as the most advanced group within the genus (Diosdado *et al.*, 1993). However, the underdeveloped embryos of seeds in *R. baudotii* suggest that the species has maintained at least one ancestral character (Forbis *et al.*, 2002; Baskin and Baskin, 2004).

For aquatic plants worldwide, temperature preferences for germination below 15°C are uncommon (Baskin and Baskin, 1998). The present investigation found that a temperature around 10°C appears to be optimal for germination and this is in agreement with the results of Bonis *et al.* (1996) obtained from soil sample experiments. At this temperature, seeds are insensitive to diurnal variation in temperature (figure 1) indicating that in the winter seeds could germinate equally well when fully submersed or on the pond margins where diurnal variation in temperature would be experienced. At higher temperatures and particularly when the mean temperature was 20°C germination was strongly influenced by alternating temperatures suggesting that germination would only occur in late summer or autumn if seeds were not fully immersed. It is speculated that this could be an adaptation to prevent germination during periods when oxygen levels in the water are low which would result in seedling death before establishment could take place. Overall, as with other species occurring in habitats characterised by alternate inundation and drying under

a mediterranean climate (Tuckett *et al.*, 2010), a requirement of cool temperatures for germination are preferred but not essential, suggesting that germination may occur in any of the months from late autumn to early spring.

The seeds of *R. baudotii* studied here were comparatively insensitive to light; although seeds consistently germinated better in the light (figure 1), relatively high germination levels were possible in the dark. Although other populations may respond differently to light, the ability to germinate under low light levels suggests that seeds of *R. baudotii* of Capraia are unlikely to form a persistent soil seed bank (Fenner and Thompson, 2005). Considering the frequency of *R. baudotii* in temporary pools in the Mediterranean and the very large fluctuations in environmental conditions between years in these habitats (Grillas *et al.*, 1993), it is surprising that the only protection detected in this study preventing germination of fresh seeds at the wrong time is low germination at high constant temperatures. Summer drought and high temperature may induce a change in the physiological behavior of seeds (Probert, 2000); for example, a reduction in responsiveness to light (Pons, 1991) could prevent germination and cause the accumulation of the seeds in the soil.

Although seeds of *R. baudotii* are suited to *ex situ* conservation in seed banks, it is possible that *R. baudotii* seeds are relatively short-lived. The closest related wetland species for which viability constants are available is *Ranunculus sceleratus* L. Using the Ellis-Roberts viability equations (Ellis and Roberts, 1980), seeds of this species would be predicted to survive under seed bank conditions operating to international standards (drying to 15% RH at 15°C and storage at -20°C) for several decades. Evidence from unpublished viability monitoring data at Kew's Millennium Seed Bank revealed that a collection of seeds of *R. baudotii* from England showed no significant decline in viability ($P > 0.05$) after 10 years of storage under seed bank conditions.

Whilst this is promising and suggests that seeds of *R. baudotii* may survive at least as well as *R. sceleratus*, detailed controlled ageing experiments are needed to confirm this.

Embryo length and growth suggest that the 2009 collection was less mature and of lower quality compared with the 2010 collection. Placing seeds in boxes filled with water will allow seed collectors to harvest seeds prior to natural dispersal with no loss of quality. Supporting a general view that seeds of wild plants often benefit from slow or delayed drying treatments that encourage seed ripening after harvest (Probert *et al.*, 2007), this treatment will improve the general quality of *R. baudotii* conservation collections and probably those of many other aquatic plants.

The results of this study are encouraging for both the *ex situ* and *in situ* conservation of *R. baudotii* from the Tuscan Archipelago. The species does not exhibit physiological dormancy and the seeds germinate readily over a broad range of conditions. It will therefore be relatively easy to establish plants from stored seeds for re-introduction to the wild. Subject to confirmation by further research, it is likely that loss of the natural population can be insured against by *ex situ* conservation of seed collections in seed banks.

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References

- Baskin, C.C. and Baskin, J.M. (1998). *Seeds. Ecology, Biogeography, and Evolution of Dormancy and Germination*. Academic Press, San Diego, USA.
- Baskin, J.M. and Baskin, C.C. (2004). A classification system for seed dormancy. *Seed Science Research*, **14**, 1-16.
- Bonis, A., Lepart, J. and Laloe, F. (1996). Effet de la température sur l'installation et la croissance des plantes annuelles de marais temporaires méditerranéens. *Canadian Journal of Botany*, **74**, 1086-1094.
- Bowes, B.G. (1999). *A Colour Atlas of Plant Propagation and Conservation*. Manson Publishing Ltd, London.
- Carasso, V., Hay, F.R., Probert, R.J. and Mucciarelli, M. (2011). Temperature control of seed germination in *Fritillaria tubiformis* subsp. *moggridgei* (Liliaceae) a rare endemic of the South-west Alps. *Seed Science Research*, **21**, 33-38.
- Carta, A., Puggioni, G. and Bedini, G. (2009). *Ranunculus peltatus* subsp. *baudotii* C.D.K. Cook (Ranunculaceae) dell'isola di Capraia (Arcipelago toscano): coltivazione e conservazione ex situ all'Orto Botanico di Pisa. [*Ranunculus peltatus* subsp. *baudotii* C.D.K. Cook (Ranunculaceae) from Capraia Island (Tuscan Archipelago): ex situ cultivation and conservation at the Botanic Garden of Pisa.]. *Atti Società Toscana Scienze Naturali, Memorie, Serie B*, **116**, 27-32.
- Diosdado, J.C., Fernandez, J., Pastor, E. and Valdes, B. (1993). Contributions to the karyological study of the genus *Ranunculus* L. subgenus *Batrachium* (DC) A. Gray, from the Iberian Peninsula. *Biological Journal of the Linnean Society*, **112**, 75-87.
- Ellis, R.H. and Roberts, E.H. (1980). Improved equations for the prediction of seed longevity. *Annals of Botany*, **45**, 13-30.
- Engell, K. (1995). Embryo morphology of the Ranunculaceae. *Plant Systematics and Evolution*, Supplement **9**, 207-216.
- Fenner, M. and Thompson, K. (2005). *The Ecology of Seeds*. Cambridge University Press, London.
- Foggi, B., Sposimo, P., Grigioni, A. and Sanesi, G. (2001). Interventi per la conservazione della biodiversità: Capraia e piccole isole dell'Arcipelago toscano. [Actions for conservation of biodiversity: Capraia and small islands in the Tuscan Archipelago]. *Informatore Botanico Italiano*, **33**, 152-155.
- Forbis, T.A., Floyd, S.K. and De Queiroz, A. (2002). The evolution of embryo size in angiosperms and other seed plants: implications for the evolution of seed dormancy. *Evolution*, **56**, 2112-2125.
- Grillas, P., García-Murillo, P., Geertz-Hansen, O., Marbá, N., Montes, C., Tan-Ham, L. and Grossman, A. (1993). Submerged macrophyte seed bank in a Mediterranean temporary marsh: abundance and relationships with established vegetation. *Oecologia*, **94**, 1-6.
- Hartog, C. and Segal, S. (1964). A new classification of the water-plant communities. *Acta Botanica Neerlandica*, **13**, 367-393.
- Hay, F., Probert, R., Marro, J. and Dawson, M. (2000). Towards the ex situ conservation of aquatic Angiosperms: a review of seed storage behaviour. In *Seed Biology: Advances and Applications*, (eds. M. Black, K.J. Bradford, J. Vázquez-Ramos), pp. 161–177, CAB International, Wallingford, UK.
- Johansson, J.T. (1998). Chloroplast DNA restriction site mapping and the phylogeny of *Ranunculus* (Ranunculaceae). *Plant Systematics and Evolution*, **213**, 1-19.

- Keeley, J.E. and Zelder, P. (1998). Characterisation and Global Distribution of Vernal Pools. In *Ecology, Conservation, and Management of Vernal Pool Ecosystems*, (eds. C.W. Witham, E.T. Bauder, D. Belk, Jr W.R. Ferren and R. Ornduff), Proceedings from a 1996 Conference, pp. 1-14, California Native Plant Society, Sacramento, CA.,
- Lastrucci, L., Foggi, B., Mantarano, N., Ferretti, G., Calamassi, R. and Grigioni, A. (2009). La vegetazione del laghetto «Lo Stagnone» (Isola di Capraia, Toscana). [The vegetation of the lake «Lo Stagnone» (Capraia Island, Tuscany)]. *Atti Società Toscana Scienze Naturali, Memorie, Serie B*, **116**, 17-25.
- Martin, A.C. (1946). The comparative internal morphology of seeds. *American Midland Naturalist*, **36**, 513-660.
- Mondoni, A., Probert, R.J., Rossi, G., Hay, F.R. and Bonomi, C. (2008). Habitat-correlated seed germination behaviour in populations of wood anemone (*Anemone nemorosa* L.) from Northern Italy. *Seed Science Research*, **18**, 213-222.
- Passarge, H. (1992). Mitteleuropäische Potamogetonetea I. *Phytocoenologia*, **20**, 489-527.
- Pinna, M. (1991). Sui caratteri climatici dell'Arcipelago Toscano. [Climatic conditions of the Tuscan Islands]. *Rivista Geografica Italiana*, **98**, 303-344.
- Pons, T.L. (1991). Induction of dark dormancy in seeds: its importance for the seed bank in the soil. *Functional Ecology*, **5**, 669-675.
- Preston, C.D. and Croft, J.M. (2001). *Aquatic Plants in Britain and Ireland*. Harley Books, Colchester, Essex.
- Probert, R.J. (2000). The role of temperature in the regulation of seed dormancy and germination. In *Seeds: The Ecology of Regeneration in Plant Communities, 2nd edition* (eds. Fenner, M.), pp. 261-292, CAB International, Wallingford, UK.
- Probert, R.J., Adams, J., Coneybeer, J., Crawford, A.D. and Hay, F.R. (2007). Seed quality for conservation is critically affected by pre-storage factors. *Australian Journal of Botany*, **55**, 326-335.
- R Development Core Team (2011). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, ISBN 3-900051-07-0, Vienna, Austria. URL <http://www.R-project.org/>.
- Sala, O.E., Chapin III, F.S., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo R., Huber Sanwald, E., Huenneke, L.F., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D.M., Mooney, H.A., Oesterheld, M., Leroy Poff, N., Sykes, M.T., Walker, B.H., Walker, M. and Wall, D.H. (2000). Global biodiversity scenarios for the year 2100. *Science*, **287**, 1770-1774.
- Tuckett, R.E., Merritt, D.J., Hay, F.R., Hopper, S.D. and Dixon, K.W. (2010). Dormancy, germination and seed bank storage: a study in support of ex situ conservation of macrophytes of southwest Australian temporary pools. *Freshwater Biology*, **55**, 1118-1129.
- Tutin, T.G., Burges, A.O., Chater, J.R., Edmonson, J.R., Heywood, V.H., Moore, D.M., Valentine, D.H., Walters, S.M. and Webb, D.A. (1993). *Flora Europea*, 2nd edn., Vol. 1, Cambridge University Press.
- Vandelook, F., Lenaerts, J. and Van Assche, J. (2009). The role of temperature in post-dispersal embryo growth and dormancy break in seeds of *Aconitum lycoctonum* L. *Flora*, **204**, 536-542.
- UNEP. (1992). Convention on biological diversity. United Nations Environmental Programme.
- Young, J. A., Martens, E. and West, N.E. (1992). Germination of bur buttercup seeds. *Journal of Range Manage*, **45**, 358-362.