

Habitat-related germination behaviour and emergence phenology in the woodland geophyte *Anemone ranunculoides* L. (*Ranunculaceae*) from northern Italy

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Abstract

This study examined whether the restricted habitat preference of the spring-flowering woodland geophyte *Anemone ranunculoides* L., compared with that of *A. nemorosa* growing in the same woodlands in northern Italy, could be explained by subtle differences in germination preference and emergence phenology. Immediately after harvest, seeds of *A. ranunculoides* were either sown on agar in the laboratory under simulated seasonal temperatures or placed in nylon mesh sachets and buried in the wild. Embryos, undifferentiated at the time of seed dispersal, grew during summer in the laboratory and in the wild, culminating in radicle emergence in the autumn, when temperatures fell to *c.* 15°C. Shoot emergence was delayed under natural conditions until soil temperature had dropped further to *c.* 10°C. Compared with populations of the closely related *Anemone nemorosa* L. occupying the same woodland habitat, which have been reported to have non-dormant radicles, *A. ranunculoides* displayed a narrower temperature tolerance for radicle emergence and high levels of germination were possible only after prolonged exposure to summer conditions, indicating physiological dormancy. However, unlike *A. nemorosa*, shoot emergence in *A. ranunculoides* was not dependent on winter temperatures, suggesting weaker epicotyl morphophysiological dormancy. Under a regime of diurnal temperature alternation, simulating the microclimate where there is little plant cover, germination failed almost completely; this could explain the absence of *A. ranunculoides* in open habitats.

Keywords: *Anemone ranunculoides*, ecological adaptation, embryo growth, epicotyl morphophysiological dormancy, radicle and shoot emergence, seed germination

Introduction

A number of studies have shown how variation in the germination requirements between species may represent adaptation to the local habitat conditions. Baskin *et al.* (1999) suggested that the wide range of habitat preferences amongst *Leptochloa* species might be explained by species differences in dormancy and germination behaviour. Schutz (1997) also reported marked differences in germination requirements between forest and open-habitat sedges. In a subsequent study of 32 temperate *Carex* species, Schutz and Rave (1999) found that although broadly similar germination patterns were evident, species differences in germination requirements were related to habitat preference. Evidence for a similar pattern of behaviour between related species of *Papaver* but with differences in germination preference and depth of dormancy was reported by Karlsson and Milberg (2007). The same authors have also recently reported a similar pattern of behaviour in four annual *Lamium* species and suggested that local adaptations arise through differences in dormancy strength (Karlsson and Milberg, 2008). Habitat-related differences in germination behaviour have also been reported amongst *Rumex* species (Van Assche *et al.*, 2002) and Vandeloos *et al.* (2008) have shown that subtle differences in germination requirements amongst four species of *Caryophyllaceae* correlate with habitat preferences.

Anemone ranunculoides L. ssp. *ranunculoides* is a native European woodland geophyte (Tutin *et al.*, 1964; Hulthén and Fries, 1986). While often present in the same woodland habitats as the closely related *A. nemorosa* L., *A. ranunculoides* has a more restricted geographical distribution and habitat preference. In northern Italy these two species often grow together in shady and damp deciduous woodlands in the lowland (Po plain) and in the mountains (northern Apennines and Alps), from 0 to 1500 m above sea level (asl) (Abrami, 1971; Pignatti, 1982). Although quite common in the Alps and Apennines (Pignatti, 1982; Aeschmann *et al.*, 2004), *A. ranunculoides* is rarer in the

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Po plain (Macchi, 2005; Bonali *et al.*, 2006) compared with *A. nemorosa*. On a European scale *A. ranunculoides* is again less common than *A. nemorosa*, and it is rarely found in the Mediterranean and Atlantic regions (Tutin *et al.*, 1964; Jalas and Suominen, 1989). Both species grow in deciduous woodlands (*Quercus-Fagetea*) (Oberdorfer, 1994); however, *A. nemorosa* also occurs in a range of other communities such as hedgerows, heathlands, grasslands and meadows (Bothmer *et al.*, 1971; Pigott, 1982; Shirreffs, 1985; Grime *et al.*, 1988). *A. ranunculoides* is a shade-demanding species (Ellenberg, 1974, 1988) whereas *A. nemorosa* is shade tolerant but also grows successfully in open areas (350–50,000 lux) (Canullo, 1985).

A recent comparative study of the germination and emergence phenology of lowland and mountain populations of *A. nemorosa* from northern Italy revealed that radicles emerge during the autumn following a period of embryo growth in the summer (Mondoni *et al.*, 2008). Under natural conditions, shoot emergence was delayed until temperatures had decreased further; laboratory tests confirmed the presence of epicotyl dormancy and that a period of cold stratification was required to trigger shoot growth.

As far as we are aware, there have been no previous studies of seed germination behaviour in *A. ranunculoides*. Because this species can often be found growing alongside *A. nemorosa*, we examined the hypothesis that *A. ranunculoides* would display a similar pattern of dormancy loss and germination preference to *A. nemorosa* but that subtle differences in behaviour might explain its narrower habitat tolerance.

Materials and methods

Seed collection

Collections of achenes (hereafter referred to as seeds) were made at the time of natural dispersal (Baskin and Baskin, 1998; Hay and Smith, 2003), 5 May 2006, from a lowland population of *A. ranunculoides* growing in the Ticino Natural Park (Po plain; *c.* 79 m asl), northern Italy.

Phenology of embryo growth and of radicle and shoot emergence in the wild

At the time of collection, 25 fine-mesh polyester bags with 50 seeds each were buried approximately 5 cm under leaf litter at the collection site. Sachets were retrieved at intervals of 30 d from May to September and then at weekly intervals until mid-January. Embryo growth, radicle emergence and shoot emergence were monitored throughout. Soil temperature at

the level of the sachets was recorded at hourly intervals using Tiny Tag data loggers (Gemini Data Logger Ltd, Chichester, Sussex, UK).

Phenology of embryo growth and of radicle and shoot emergence in the laboratory

All laboratory experiments involved sowing three replicates of 50 seeds each on 1% distilled water agar held in 9-cm diameter Petri dishes. Treatments were incubated in temperature- and light-controlled incubators using a 12-h daily photoperiod (photosynthetically active radiation 40–50 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

On the day of harvest, seeds were placed into a temperature regime simulating seasonal changes occurring in the west Po plain (Mariani *et al.*, 2001). Summer conditions (May to September) were simulated by 150 d at 20°C; early autumn (October) by 30 d at 15°C; late autumn (November) by 30 d at 10°C; winter (December to February) by 90 d at 4°C; early spring (March) by 30 d at 10°C; and late spring (April) by 30 d at 15°C.

On the same day, 20 seeds were dissected (Kondo *et al.*, 2004; Toshikazu *et al.*, 2004) and the embryo length measured under a binocular microscope equipped with a micrometer. Observations on embryo growth and on root and shoot emergence were made every 30 d during summer and then at 5-d intervals until the end of the simulated cycle.

Constant temperatures were used because the forest cover and surface leaf litter are effective insulators of daily temperature variations (Ellenberg, 1988; Daws *et al.*, 2002; Mondoni *et al.*, 2008) and because it was assumed that seeds would be more responsive to seasonal rather than diurnal temperature variations. However, additional germination tests were set up to investigate the effect of diurnal alternating temperatures. The following regimes were used: 150 d at 25/15°C (summer) followed by 30 d at 20/10°C (early autumn), 30 d at 15/5°C (late autumn), 90 d at 4°C (winter), 30 d at 15/5°C (early spring) and 30 d at 20/10°C (late spring). In each case a 12/12 h thermoperiod was used and illumination was provided during the warm phase.

Controls were also set up to investigate the effects of prolonged incubation at each of the mean seasonal constant temperatures (4, 10, 15 and 20°C). Seeds were checked for root and shoot emergence at regular intervals over 360 d.

Effects of summer and autumn conditions on root emergence

To examine the importance of summer conditions, samples of seeds were held at 20°C for 0, 30, 60, 90, 120 and 150 d. After each period, seeds were moved to

autumn and then winter conditions. The importance of autumn conditions was examined by placing seeds into simulated summer conditions (150 d at 20°C) and then moving them to the other seasonal conditions, skipping the early autumn (15°C) treatment, the late autumn (10°C) treatment, or both. For each test the number of seeds and replications, method of sowing, light conditions and observations were as described above.

The importance of winter for shoot emergence

The aim of this experiment was to investigate whether cold stratification may affect shoot emergence. After the summer and autumn phases of the seasonal sequence, seeds with emerged radicles were given 0, 30, 60 or 90 d of cold stratification (4°C) before transfer to early spring (30 d at 10°C) followed by late spring conditions (30 d at 15°C). The number of seeds and replications for each test and other details were the same as before.

Data analysis

Embryo growth data were analysed using linear regression analysis in Minitab 14 (Minitab Inc., State College, Pennsylvania, USA) on subsets of the data. To describe the progress of germination in the laboratory, the Gompertz function (Brown and Mayer, 1988) was fitted to cumulative germination data using Sigma Plot 7, according to the following formula:

$$y = a * \exp(-\exp(-x - x_0)/b)$$

where y = germination percentage at time x (d), a = final germination, x_0 = germination delay and b = germination rate. In addition, χ^2 tests (maximum likelihood) were carried out in Genstat 11 (VSN International Ltd., Hemel Hempstead, Herts, UK) to compare the final proportions of germinated seeds under different conditions.

Results

Phenology of root and shoot emergence under natural conditions

Embryos of seeds buried in leaf litter at the collection site grew continuously during the summer (May–September; data not shown) but radicle emergence did not occur until mid-October when soil surface temperature had dropped to *c.* 15°C (Fig. 1). The radicle emerged from almost 60% of the seeds within 2 weeks at this temperature, while it emerged from the remaining 40% of the seeds during the next 3 weeks when the temperature had dropped to *c.* 10°C.

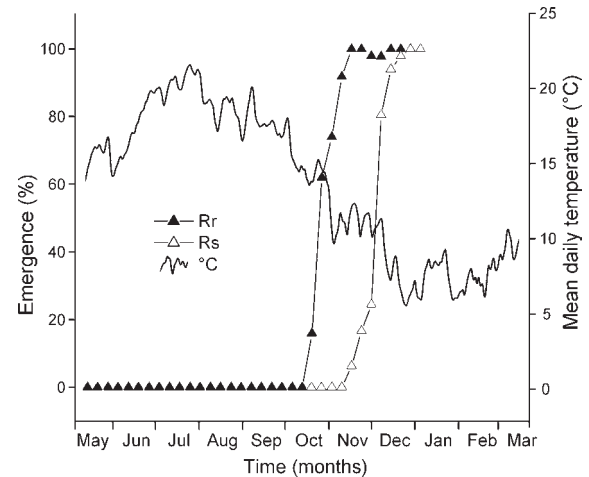


Figure 1. Radicle (Rr) and shoot (Rs) emergence from seeds of *A. ranunculoides* in the wild. Also shown is the mean daily soil temperature (°C) calculated from measurements made at hourly intervals at the study site.

Shoot emergence did not commence until mid-November but then proceeded at a similar rate, with *c.* 95% shoot emergence in 4 weeks, despite the temperature having dropped further to *c.* 5°C (Fig. 1).

Phenology of embryo growth and of root and shoot emergence in the laboratory

Embryos of *A. ranunculoides*, undifferentiated at the time of dispersal, grew at a constant rate under continuous, simulated summer conditions in the laboratory (20°C) from about 0.2 to 0.67 mm after 90 d on agar, when they occupied approximately 32% of the whole endosperm length (2.1 mm; Fig. 2). There was no further significant embryo growth after this point prior to radicle emergence.

Under simulated seasonal temperatures in the laboratory about *c.* 94% of radicles of *A. ranunculoides* had emerged after seeds were transferred from summer conditions (150 d at 20°C) to the early autumn condition (15°C). However, shoot emergence was delayed until seeds were moved to winter conditions (4°C; Fig. 3).

Effects of summer condition on root emergence

In the absence of summer conditions, there was no germination of *A. ranunculoides* seeds. For seeds given 30 d of summer conditions, only *c.* 57% germinated after they had been moved through autumn conditions (30 d at 15°C followed by 30 d at 10°C) and under winter conditions (4°C) for 90 d (Fig. 4). Although the difference in the final germination after 30 or 60 d of summer conditions was not significant ($\chi^2_{(1df)} = 0.01$, $P = 0.907$), germination was faster after 60 d of

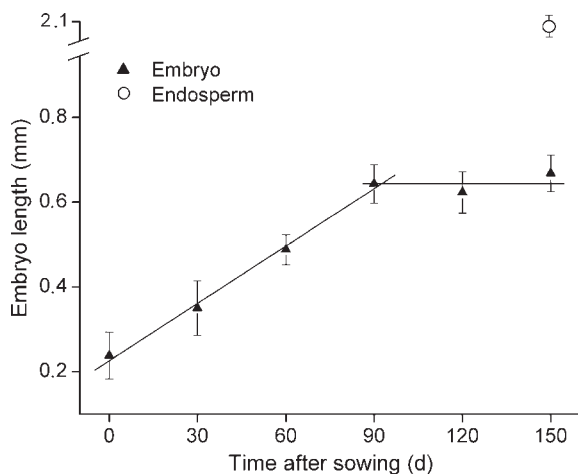


Figure 2. Linear regressions of embryo growth of *A. ranunculoides* in the laboratory at simulated summer conditions (20°C). Bars are \pm SE. Also shown is the length of the mucilaginous endosperm (open circle), within which the embryo develops.

summer conditions. There was 53% radicle emergence after 45 d of autumn conditions for seeds given 60 d of summer conditions, compared with 37% for seeds given 30 d of summer. Increasing the length of simulated summer conditions to 90, 120 or 150 d resulted in further increases in both the total percentage of seeds germinating and the rate of germination, while the time before germination was first observed decreased (Fig. 4).

Effects of autumn condition on root emergence

After 150 d of summer conditions, 63% of the seeds had an emerged radicle after 30 d at 15°C and 92% by the time seeds were transferred to winter conditions

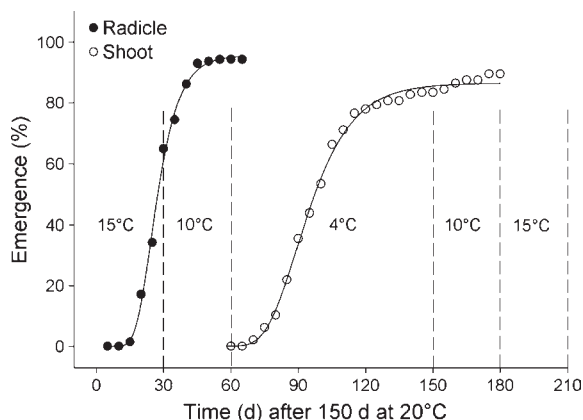


Figure 3. Germination progress curves for seeds of *A. ranunculoides* at simulated seasonal temperatures. Radicle (closed symbols) and shoot (open symbols) emergence after summer conditions (150 d at 20°C). Curves were fitted using the Gompertz function.

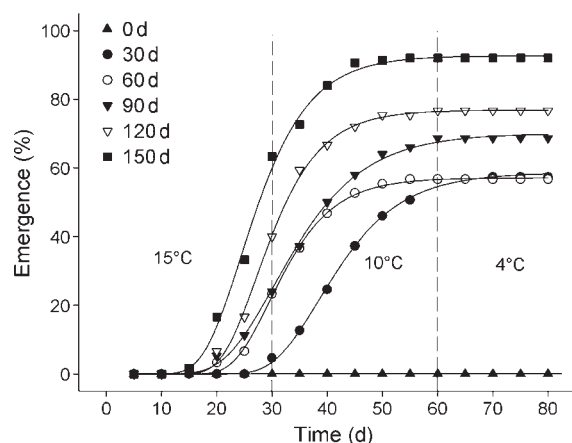


Figure 4. Germination progress curves for seeds of *A. ranunculoides* at simulated seasonal autumn (15 and 10°C) and winter (4°C) temperatures, after increasing durations of summer conditions (0, 30, 60, 90, 120 and 150 d at 20°C, as indicated). Curves were fitted using the Gompertz function.

(4°C), with no more radicle emergence after this time. In contrast, when summer-treated seeds were placed under late autumn conditions (10°C), only low levels of radicle emergence occurred (*c.* 30%; Fig. 5). When seeds were placed directly into winter (4°C), there was no radicle emergence.

The importance of cold stratification for shoot emergence

Differences in final shoot emergence of seeds given 0, 30, 60 or 90 d of winter conditions were not significant ($\chi^2_{(2df)} = 1.93$, $P = 0.380$). Almost complete shoot emergence was observed in *A. ranunculoides* even in the absence of winter conditions (Fig. 6), with 95% of shoots emerging by the time seeds were transferred to the simulated late spring condition (15°C). However, shoot emergence did not occur in the absence of cooler autumn conditions (10°C).

The effect of diurnal alternating temperatures

In the simulated regime of diurnal alternating temperatures, no seeds had emerged radicles at 25/15 or 20/10°C and only *c.* 10% had emerged radicles when seeds were transferred to late autumn conditions (15/5°C) (Table 1).

Continuous constant temperature controls

No seeds germinated at continuous 5, 10 and 20°C. After 80 d at 15°C, however, seeds began to germinate slowly, with 97% radicle emergence recorded after a further 80 d (data not shown). Shoot emergence did not occur at continuous 15°C.

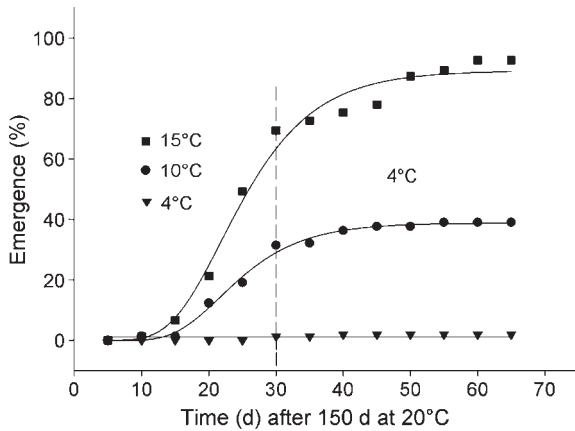


Figure 5. Germination progress curves for seeds of *A. ranunculoides* at simulated seasonal temperatures given 150 d of summer pre-treatment at 20°C and then incubated at temperatures of early autumn (15°C) and winter (4°C) (squares), late autumn (10°C) and winter (4°C) (circles) and winter (4°C) (triangles). Curves were fitted using the Gompertz function.

Discussion

Under natural conditions, the pattern of radicle and shoot emergence phenology in seeds of *A. ranunculoides* was remarkably similar to that recently reported for seeds from the closely related species *A. nemorosa* from identical low-altitude woodland habitats (Mondoni *et al.*, 2008). Like *A. nemorosa*, differentiation and growth of *A. ranunculoides* embryos began immediately after seed dispersal and continued during the summer, culminating in radicle emergence and then shoot emergence when temperatures fell in the autumn.

Although there is clear evidence that shoot emergence under natural conditions is delayed until temperatures fall to late autumn values, c. 80% of

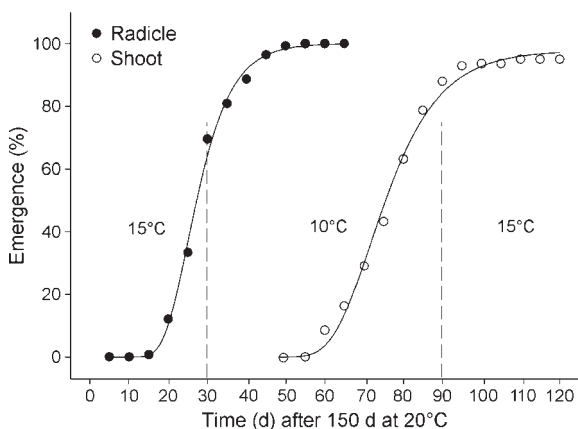


Figure 6. Radicle and shoot emergence progress curves for seeds of *A. ranunculoides* at simulated seasonal temperatures in the absence of winter conditions. Curves were fitted using the Gompertz function.

Table 1. Radicle emergence (%) of seeds of *A. ranunculoides* under simulated seasons using diurnal alternating temperature cycles (except for winter). Seeds were moved progressively through the seasons with germination scored at each step

Temperature (°C)	Time (d)	Radicle emergence (%)
25/15	150	0
20/10	30	0
15/5	30	10.3
4	90	0
15/5	30	0
20/10	30	0

shoots had emerged before temperatures had dropped below 10°C (Fig. 1). By contrast, in all three populations of *A. nemorosa* studied by Mondoni *et al.* (2008), the same level of shoot emergence was not observed until temperatures had dropped well below 10°C. This subtle, but significant difference in temperature response between these two related species was further revealed in laboratory experiments.

Ali *et al.* (2007) and Mondoni *et al.* (2008) concluded that radicles of *A. nemorosa* should be regarded as non-dormant because they grew continuously with no evidence of developmental arrest prior to emergence as temperatures fell in the autumn. Here we have clear evidence of arrest in embryo growth in *A. ranunculoides* after about 90 d under simulated summer conditions (Fig. 2). Compared with *A. nemorosa*, seeds of *A. ranunculoides* also appear to require more prolonged exposure to summer conditions prior to germination. Under simulated seasonal temperatures, radicle emergence occurred in the autumn, but high levels of germination were only recorded after 150 d of summer conditions (Fig. 4). In seeds of *A. nemorosa*, just 30 d of summer conditions were enough to elicit high levels of radicle emergence when they were transferred to simulated autumn temperatures (Mondoni *et al.*, 2008). Moreover, unlike seeds from three populations of *A. nemorosa* from identical woodland habitats that were capable of radicle emergence at simulated early autumn, late autumn and winter temperatures, seeds of *A. ranunculoides* appeared to have a much narrower temperature range for germination, giving high levels of radicle emergence only when seeds were incubated at early autumn temperatures (Fig. 5).

Ellenberg (1974) ascribed so-called indicator values for six ecological factors, including, for example, light and temperature, to the native flora of central Europe. A species given a low value occurs mainly where the factor is less pronounced; species could also be defined as indifferent. In our study the narrower temperature

tolerance of *A. ranunculoides* seeds compared with that of *A. nemorosa* was reflected in the lower Ellenberg index for temperature, based on the mean annual temperatures occurring in the growing areas of the two species in central Europe.

Kos and Poschlod (2007) have recently shown that the germination of canopy-associated species was inhibited by high-amplitude, diurnal-alternating temperatures typical of matrix (open) sites (Ellenberg, 1988). Although this study was based on species from xeric Kalahari savannah, Kos and Poschlod (2007) argue that mechanisms that serve to detect canopy shade, based on sensitivity to the amplitude of diurnal temperatures, might be widespread, especially in bird-dispersed, fleshy-fruited species.

Although seeds of *A. ranunculoides* are neither bird-dispersed nor fleshy-fruited, very few seeds were able to germinate under a regime of diurnal temperature alternation (Table 1). By contrast, unpublished data from our laboratory showed that seeds of *A. nemorosa* were capable of *c.* 90% germination under the same conditions. Although further work is needed, this might help to explain why *A. ranunculoides* is restricted to closed-canopy sites and why *A. nemorosa* can be found in both open- and closed-canopy habitats.

Our laboratory experiments reveal that radicles of *A. ranunculoides* clearly have more stringent germination requirements than those of *A. nemorosa*. Despite the fact that germination of *A. ranunculoides* is possible at a constant temperature of 15°C, the arrest of embryo growth after 90 days at summer temperatures (Fig. 1), combined with the requirement for additional time at these temperatures in order to elicit high levels of germination (Fig. 4), leads us to conclude that the radicle should be regarded as dormant.

Mondoni *et al.* (2008) reported clear evidence that shoots of *A. nemorosa* possess non-deep epicotyl morphophysiological dormancy (*sensu* Baskin and Baskin, 1998). In that study, laboratory experiments confirmed that shoots required a period of cold stratification at winter temperatures or application of GA₃ (to substitute for cold stratification). Here we have shown that shoot emergence in seeds of *A. ranunculoides* is not dependent on exposure to winter temperatures. Supporting the field data, laboratory tests showed that although shoot emergence is possible at winter temperatures (Fig. 3), it can also occur in the absence of winter (Fig. 6), but not in the absence of the cooler autumn temperature (10°C) and thus the level of epicotyl dormancy in seeds *A. ranunculoides* is clearly much weaker than that expressed in seeds of *A. nemorosa* from the same habitat. The general pattern of germination and emergence phenology in *A. ranunculoides* under natural conditions is more or less identical to that of populations of *A. nemorosa* occupying the same woodland habitats in northern Italy. However, seeds

of *A. ranunculoides* complete germination and shoot emergence in the autumn, whereas those of *A. nemorosa* do so during the winter.

Other papers have described similar patterns of behaviour between species in a number of genera across a range of plant families and attributed subtle differences in depth of dormancy or response to specific factors to habitat preference (Schutz, 1997; Baskin *et al.*, 1999; Schutz and Rave, 1999; Daws *et al.*, 2002; Van Assche *et al.*, 2002; Karlsson and Milberg, 2007, 2008; Vandeloos *et al.*, 2008). Moreover, in a comparative study of germination and emergence phenology in three lowland and one mountain population of *A. nemorosa*, Mondoni *et al.* (2008) reported significant differences in germination behaviour and clear evidence that the timing of germination and emergence was well adapted to the climatic differences between the two sites. It is therefore tempting to speculate that the subtle differences in dormancy and germination behaviour between *A. ranunculoides* and *A. nemorosa* growing in the same woodlands might explain the more restricted ecological distribution of *A. ranunculoides*.

High levels of germination and seedling recruitment in the wild have been reported for *A. nemorosa* (Eriksson, 1995; Holderegger, 1996) and it is reasonable to assume that this would be true for *A. ranunculoides*. Therefore, it is likely that the differences in behaviour we have highlighted are ecologically meaningful and may help to predict future changes in the relative abundance of these two species in the woodlands of northern Italy. Seed germination in both species is clearly highly sensitive to seasonal temperatures and the subtle differences between them in the optimum temperatures for radicle and shoot emergence suggest that climate change might affect their relative reproductive success. For example, the absence of a cold stratification requirement for shoot emergence could favour *A. ranunculoides* over *A. nemorosa* in a trend of warmer winters. However, the absence of *A. ranunculoides* in Mediterranean regions suggests that it has a low tolerance to warm, dry summers, which could become a more important limiting factor. Thus, since the climate is expected to become warmer and drier in several mid-latitude European areas, including northern Italy (IPCC, 2007), *A. ranunculoides* may become more scarce.

Laboratory and field studies of seed germination and emergence phenology are clearly an important tool for detecting ecologically meaningful differences between closely related plant species, and thus they may help to predict future trends in plant distribution. However, such studies will need to be backed up by long-term monitoring of plant populations (Pauli *et al.*, 2007) if we are to properly understand the relationship between seed germination events and plant population dynamics in a changing climate.

Acknowledgements

Financial support was provided by the University of Pavia, the *Centro Flora Autoctona* of the Lombardy Region and the MIUR (Italian Ministry for Education, University and Research) through the project no. 2007JNJ7MX.

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Received 3 February 2009
accepted after revision 28 March 2009
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