Seed Science and Biotechnology ©2007 Global Science Books

Ripe Fruits of *Sisymbrium officinale* L. Contain Heterogeneous Endospermic Seeds with Different Germination Rates

Raquel Iglesias-Fernández[1] • Angel J. Matilla[1*] • Iñigo Pulgar[2] • Francisco de la Torre[1]

[1] Departments of Plant Physiology and Botany, [2] Faculty of Pharmacy, University of Santiago de Compostela, 15782-Santiago de Compostela, Spain

Corresponding author: * bvmatilla@usc.es

ABSTRACT

The seeds of *Sisymbrium officinale* are largely heterogeneous in mass and colour within the ripe fruit. In order to initiate the characterization of the germinative process from these heterogeneous seeds, we have used two different seed lots (i.e. brown and light-brown), and studied several physical and physiological properties and their possible influence on the germination rate. The most notable features from this work were: (i) the mass of the brown seeds was higher than the light-brown ones, which imbibed more quickly; (ii) light seeds contain much less mucilage (myxospermy) than do the brown; (iii) under optimal germination conditions for the brown seeds, the light-brown ones germinated 6-fold less; and (iv) very similar profiles of germination stimulation was found in the presence of gibberellins (i.e. GA_{4+7}) and ET in brown seeds, but both hormones were not capable of stimulating the germination in light-brown ones. As a general conclusion, we have demonstrated the coexistence in ripe fruits of *S. officinale* of two seed lots with remarkable physiological and physical heterogeneity that could be used as a tool to increase our knowledge about the germination process.

Keywords: ethylene, gibberellins, heterogeneous seeds, imbibition rate, myxospermy, seed density

INTRODUCTION

The appearance of the seed, the propagule chosen by spermatophytic plants for their dispersion and propagation, has great evolutionary implications, since this organ represents the survival and perpetuation of the mother plant (Boesewinkel and Bouman 1995). A typical seed of an angiosperm contains an embryo (diploid) surrounded by an endosperm (triploid; with two-thirds of its genome of maternal origin) and a seed coat (diploid) (Raghavan 1986; Torres-Ruiz 1998). The endosperm is formed by live cells with high metabolic activity, while the seed coat is comprised of a tissue of maternal origin (i.e. ovular tissue), the cells of which die during late seed maturation. Depending on the species, the endosperm is visible in dry seed (i.e. endosperm seeds) or else completely or partially disappears during zygotic embryogenesis (i.e. non-endospermic seeds), the cotyledons exercising the nutritional role of the embryo (Friedman 1998; Forbis *et al*. 2002).

The seeds of most angiosperms are dormant at maturity, and dormancy must be lost before germination can occur (Bewley 1997a, 1997b; Finch-Savage and Leubner-Metzger 2006). The seed transformation from dormant (i.e. failure of an intact, viable seed to complete germination under favourable conditions) to non-dormant stage is associated with changes in gene expression and protein patterns (Cadman *et al*. 2006; Chibani *et al*. 2006; Lee *et al*. 2006). The germination of a mature, viable, and non-dormant seed begins with the gradual uptake of water, which has three phases: rapid initial uptake (phase I, i.e. imbibition) followed by a plateau in water uptake (phase II) and a further increase after germination is completed, as the embryo axes elongate (phase III) (Bewley 1997a; Manz *et al*. 2005). Dormant seeds do not complete germination and therefore lack phase III. The germination process ends when the radicle tip has protruded through its covering structures (i.e. endosperm and seed coat in endospermic seeds) (Bewley 1997b; Kucera *et al*. 2005). Seed coat and endosperm rupture occur at the micropylar level. Cell elongation of the radicle is necessary and possibly sufficient for the completion of protrusion. However, cell division is not essential (Barroco *et al*. 2005). That is, the radicle protrusion depends on embryo expansion, which is a process driven by water uptake and cell-wall loosening (Koornneef *et al*. 2002). In most endospermic seeds (e.g. coffee, lettuce, tobacco, or tomato) the tissue that constitutes the endosperm is a germination-limiting barrier, and so that germination is completed the growth potential of the radicle must be high enough to overcome the resistance of the endospermic tissue (Bewley 1997a; Koornneef *et al*. 2002; Leubner-Metzger *et al*. 2006). The role of the seed coat as a germination constraint has been studied by using testa mutants of Arabidopsis (Debeaujon and Koornneef 2000; Koornneef *et al*. 2002). The seed coat and endosperm rupture are temporally separated in model plants as tobacco and Arabidopsis (Liu *et al*. 2005; Leubner-Metzger *et al*. 2006; Müller *et al*. 2006).

Environmental factors (i.e. light and temperature) and phytohormones (i.e. gibberellins, GAs; abscisic acid, ABA; and ethylene, ET) have been associated with the promotion of germination of endospermic and non-endospermic seeds (Hilhorst and Toorop 1997; Matilla 2000; Toorop *et al*. 2000; Koornneef *et al*. 2002; Kucera *et al*. 2005; Müller *et al*. 2006). Thus, two functions for embryonic GAs have been proposed to be needed during endospermic-seed germination: (i) to increase the embryo growth potential; and (ii) to overcome the mechanical restraint conferred by the seed-covering layers by weakening of the tissues surrounding the radicle (Hilhorst and Downie 1995; Koornneef *et al*. 2002; Leubner-Metzger 2003; Bassel *et al*. 2004; Yamauchi *et al*. 2004; da Silva *et al*. 2005). Nevertheless, GAs can conduct the germination positively by means a yet well known cross-talk with ABA and environmental factors (i.e. light or temperature). Thus, light (i.e. red light) and low temperature (i.e. 4ºC) can modulate the spatial expression pattern of GA biosynthetic genes. Recently, Yamauchi *et al*. (2004) demonstrated that a gene involved in GA biosynthesis in seeds of *A. thaliana* was activated by cold stratification at 4ºC. The increase in tissue sensitivity to GAs during

Received: 11 July, 2007. Accepted: 6 September, 2007.

Original Research Paper

cold stratification is another factor that may be involved in controlling seed germination (Derkx and Karssen 1993; Koornneef *et al*. 2002). In some seeds (e.g. *Nicotiana attenuata*), GAs can replace red-light to trigger dark-germination (Schwachtje and Baldwin 2004). From recent evidences in Arabidopsis it can be concluded that the GA requirement for seed germination is determined by seed-coat characteristics, embryonic growth potential and by embryonic ABA (Debeaujon and Koornneef 2000; Debeaujon *et al*. 2000). The above and other recent data indicate that GAs release coat and embryo dormancy, promote germination and counteract inhibitory ABA effects, directly or indirectly.

Also, it has been demonstrated that ABA inhibits: (i) phase-III water uptake but does not inhibit phase I or initial embryo-extension growth (Homrichhausen *et al*. 2003; Frey *et al*. 2004; Manz *et al*. 2005; Müller *et al*. 2006); (ii) the embryo growth potential and endosperm cap weakening during coffee-seed germination (da Silva *et al*. 2004); (iii) the endosperm rupture but not seed coat rupture in tobacco and petunia seeds (Toorop *et al*. 2000; Petruzzeli *et al*. 2003; Kucera *et al*. 2005). In seeds in which the seed-coat and endosperm rupture is not easy to detect visually (e.g. tomato), germination is also inhibited by ABA (Wu *et al*. 2000; Leubner-Metzger 2003); but deficiency in ABA in *sit*^{w} mutant is associated with a thinner seed coat (Hilhorst and Downie 1995; Toorop *et al*. 2000). On the other hand, during development and germination of some seeds, ABA and ET action appear to be antagonistic. Thus, it was proposed that ABA increases tissue sensitivity to ET, affecting seed germination, whereas ET may suppress seed dormancy by inhibiting ABA action (Beaudoin *et al*. 2000; Grossmann and Hansen 2001; Koornneef *et al*. 2002).

ET promotes germination and counteracts ABA effects in many seeds (Kepczynski and Kepczynska 1997; Beaudoin *et al*. 2000; Ghassemian *et al*. 2000; Matilla 2000; Koornneef *et al*. 2002) and its production is higher in non-dormant compared to dormant seeds (Matilla 2000). However, even though great progress has been made in elucidating ET signalling, the mechanism(s) of ET action remains to be explained in dormant and germinating seeds (Kucera *et al*. 2005; de la Torre *et al*. 2006). ET biosynthesis and sensitivity are both important for the germination (Beaudoin *et al*. 2000; Siriwitayawan *et al*. 2003; Matilla *et al*. 2005) and transcripts as β-1,3-glucanase or ACC-oxidase, into others, are ET-altered during germination of some seeds (Leubner-Metzger *et al*. 1998; Pretruzzeli *et al*. 2003a, 2003b; Puga-Hermida *et al*. 2003; Rodríguez-Gacio *et al*. 2004). Summarily, although all findings until now published support the view that ET can promote seed germination and counteract ABA inhibitory effects by interfering with ABA signalling, cross-talk between ET and GAs cannot be discarded (Kucera *et al*. 2005).

Fig. 1 Adult plant of *S. officinale*.

In the present study, wild mature dry seeds of *Sisymbrium officinale*, collected in north-western Spain, were used. In this species, seed-coat and endosperm rupture are temporally separated at the beginning of germination and the hormonal control of both process can be studied. This plant is common in fields and path borders and can be identified by its yellow flowers, basal leaves divided with a large and round terminal lobe, and erect fruits held close to the stem. *S. officinale* produces endospermic seeds housed into a fruit which contains numerous heterogeneous in mass and colour seeds (**Fig. 1**). Although the heterogeneity appears to be related in some cases to survival, little is known about how the seeds are affected physiologically (Puga-Hermida *et al*. 2003; Matilla *et al*. 2005). To evaluate the impact of heterogeneity on the germination process of *S. officinale* seeds, in this work we used two lots (brown and light-brown) of the above seeds to investigate some of theirs morphologic characteristics and response to ET and GAs.

MATERIALS AND METHODS

Chemicals

Aminoethoxyvinylglycine (AVG), 1-aminocyclopropane-1-carboxylic acid (ACC), paclobutrazol, silver nitrate, ruthenium red, GA_{4+7}, and Co_2Cl were purchased from Sigma-Aldrich (Spain), whereas potassium nitrate was from Merck (Germany).

Plant material

Ripe wild seeds of *Sisymbrium officinale* were collected in Galicia

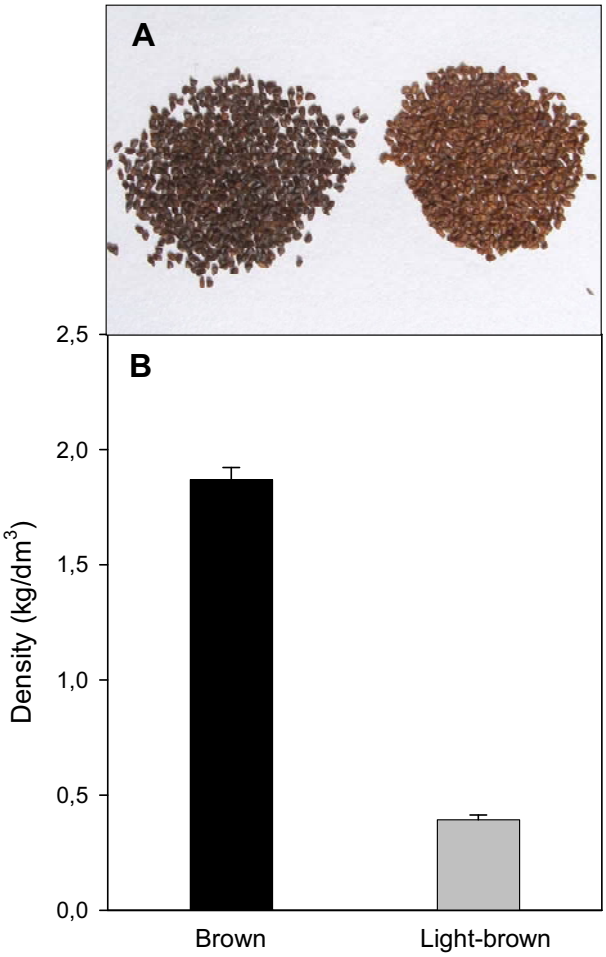

Fig. 2 External aspect (A) and density (B) of brown and light-brown lots of *S. officinale* dry seeds. Data are the means ± standard error (S.E.) of 3-4 independent experiments. Differences in density between lots were highly significant ($P<0.001$) as determined by the LSD test.

(north-western Spain) during July-August, 2006. Seed samples were separated from ripe fruit residues (i.e. pedicel, replum and valve) by means of a suitable sieve, divided into two populations (i.e. brown and light-brown; see **Fig. 2A**) according to the colour of seed coat, weighed in lots of 100 seeds (21.8 ± 0.3 and 13.3 ± 0.6 mg for dark and light, respectively) and stored at 21 ± 0.2ºC before use in the germination tests. The brown:light-brown seed ratio inside mature fruits was around 1.8 ± 0.1.

Germination experiments

Fifty seeds were put into plastic Petri dishes on two Whatman No. 1 filter paper discs moistened with 3 ml of 20 mM KNO_3 (control) supplemented with solutions of gibberellin (GA_{4+7}) and etephon (ET), inhibitors of GAs (PC, paclobutrazol) and ET (AVG, Co_2Cl) synthesis and the inhibitor of ET signalling (NO_3Ag). The germination percentage was determined in a climate room (24ºC, 16 h-light/8 h-dark photoperiod). Seeds were not sterilized in order to avoid influencing their dormancy status; however, by using light microscope fungal infection was not detected. Radicle emergence was taken as evidence of germination. For each experimental condition, three replicates at least were used.

Water uptake

To study the water uptake rate, we took known weights of dark as well as light-brown dry seeds and surface-washed them for 15 s in distilled water. The seeds were then immediately placed onto filter paper dampened with distilled water and kept under conditions identical to those for seed germination. The seeds were rapidly surface dried and weighed hourly, and then immediately replaced to continue the imbibition phase. This process was repeated for 5 h. The water uptake for each lot was considered as fresh weight (FW) minus dry weight (DW) per mg of seeds.

Tetrazolium test

Intact seeds were incubated in a 1% (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride (Merck, Germany) at 30ºC in darkness for 2 d. Tetrazolium salts are metabolically reduced to highly coloured end products called formazans by NADH-dependent reductases of the endoplasmic reticulum (Berridge *et al*. 1996).

Ruthenium red staining to seed mucilage

Mucilage was detected in the seed basically as described by Western *et al*. (2000). Whole seeds were allowed to imbibe on moist filter paper for between 5 min and 1 h, before the application of 0.2% (w/v) aqueous ruthenium red solution. Seeds were photographed with an Olympus B061 stereomicroscope.

Statistical treatment

The data obtained here are the mean of at least three replicates. The statistical treatment was based on a variance analysis and the averages/means were compared using the least-significant- difference (LSD) test at $P<0.05$ (Steel and Torrie 1982).

RESULTS AND DISCUSSION

Seed development is not temporally uniform in any given population, even when mother plants are grown in identical environments. In short, a type of seed heterogeneity develops (Matilla *et al*. 2005). As a result of such heterogeneity, populations of seeds with morphological differences (e.g. colour, size, weight, or shape) appear, and some of these differences can affect physiological properties (e.g. storage capacity or level of dormancy) (Debeaujon *et al*. 2000). Thus, some crucifer seeds do not mature synchronously inside the silique, but sequentially, and some pods shatter before harvesting, resulting in a substantial loss of seeds (Matilla 2007). Given that the heterogeneous character of some seeds can affect germination (Khan *et al*. 1996; Kantar *et al*. 1996; Puga-Hermida *et al*. 2003; Matilla *et al*. 2005; Luzuriaga *et al*. 2006), these heterogeneous lots of seeds constitute a valuable tool in seed physiology to study the endogenous factors that regulate germination. However, the physiological and molecular basis of seed heterogeneity is at present unknown.

Morphological and structural heterogeneity in *S. officinale* seeds

The fruits of *S. officinale*, as with other crucifers described previously (Imbert 2002), contain heterogeneous seeds that we have divided into two lots to study (brown and light-brown) attending to the colour of the outermost part of the seed coat. The colouration of the seed coat in other crucifer seeds studied is due preferentially to the accumulation of flavonoid-type seed compounds (e.g. condensed tannins as proanthocyanidins), which notably influence the seed-coat-imposed dormancy in some species (Debeaujon and Koornneef 2000; Debeaujon *et al*. 2000). The brown seeds of *S. officinale* have a density of approximately 4.7-fold that of the light-brown ones (**Fig. 2B**). Although the volume of the light-coloured seeds is higher than that of the dark ones, the great difference observed in the density is due primarily to different weights between the two lots (i.e. 21.8 ± 0.3 and 13.3 ± 0.6 mg/100 seeds for brown and light-brown, respectively). Once embryogenesis ends, the seed is a completely autonomous organism. Nevertheless, in plant species that produce a large quantity of seeds, this autonomy is not equal in each seed. That is, as seeds are heterogeneous, certain differences affect physiological properties (e.g. storage capacity and dormancy level) that can alter the speed and timing of dispersion (Kigel 1995). The tetrazolium test made in the brown lot of *S. officinale* showed that 100% of the seeds were alive, while in the light-brown lot, only 40 ± 7% were alive. This fact appears to indicate that the light seeds had difficulties in their embryogenic process. Several studies have demonstrated that such difficulty may be determined by, among other factors, a different intensity of nutrient unloading from the mother plant to the embryo (i.e. seed filling) due to a positional effect of the seed in the fruit, thereby affecting seed-size and viability (Fenner 1991; Gutterman 1992; Fenner 1993; Luzuriaga *et al*. 2006). That is, seed-size variability is considered a phenotypical maternal effect (Moles and Westoby 2006).

The brown and light-brown seeds of *S. officinale* are also heterogeneous with respect to each other, given that the former contain much more mucilage in the seed coat than do the light ones. This characteristic (i.e. myxospermy) is manifested in seed imbibition of both lots for a short amount of time in the presence of ruthenium red, a colorant which specifically dyes the mucilage (**Fig. 3**). The mucilage-producing cells are located in epidermal tissue of the seed coat of some species, including those of the family

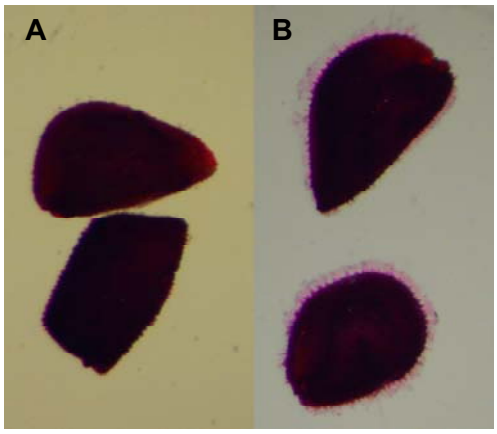

Fig. 3 Structure of light-brown and brown seeds of *S. officinale*. Whole seeds were stained with ruthenium red as described in the Material and Methods. Note red staining capsule of mucilage surrounding dark brown seeds (**B**). No mucilage is visible around light-brown seeds (**A**).

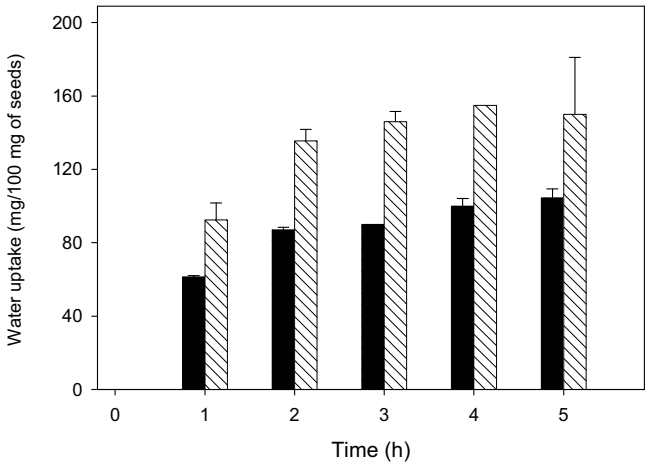

Fig. 4 Hourly water uptake during the first 5 h of imbibition of brown (■) and light-brown (□) lots of *S. officinale* seeds. Data are means ± standard error (S.E.) of 3 independent experiments. Differences between lots were significant (P<0.05) as determined by the LSD test.

Brassicaceae (Boesewinkel and Bouman 1995; Western *et al*. 2000, 2004). When a dry myxospermous seed is placed in an aqueous environment, the mucilage, extremely hydrophilic and pectin-rich (Western *et al*. 2000), is released and completely envelops the seed coat (**Fig. 3**). It was suggested that mucilage extrusion results from the rapid expansion of dried mucilage upon hydration, leading to a local breakage of the cell wall. The nature of mucilage prior to release upon imbibition and its functions when extruded around seed are, at present, unknown aspects. However, mucilage may be related to imbibition, seed dispersal, germination or repair embryo DNA, among other ways (Huang and Gutterman 1998; Huang *et al*. 2000; Western *et al*. 2000, 2004; Huang *et al*. 2007).

The germination capacity of some seeds is altered by seed-coat characteristics (Debeaujon and Koornneef 2000; Debeaujon *et al*. 2000). A controlled imbibitional water-uptake allowed by seed coat components is necessary to initiate the normal germination process. The study of the water-uptake rate in brown and light-brown seeds indicates that the light lot imbibed water more quickly than did the dark lot (**Fig. 4**). The imbibition profile in the brown seeds was linear and increased gradually over the study period (i.e. 5 h); however, the profile of the light seeds was sigmoidal, reaching, at 60 and 180 min, values 1.3- and 1.6-fold greater, respectively, than those obtained for brown seeds (**Fig. 4**). Water uptake profiles from both lot seeds were asintotic after five hours. One of the causes that could favour such a sudden uptake of water in the light seeds could be related to the absence of mucilage. Seed mucilage is a natural example of a hydrogel and is an efficient absorber of water. It was hypothesized that mucilage takes up water to increase and stabilize water potential surrounding the seed, thereby promoting efficient germination and seedling establishment (Penfield *et al*. 2001). If the imbibition behaviour of the brown seeds is as in *A. thaliana* (Penfield *et al*. 2001; Western *et al*. 2000, 2004), the initial hydration of *S. officinale* seeds leads to the immediate release of mucilage, an event correlated with breakage of the outer tangential cell wall of the epidermal cell due to the rapid expansion of dried mucilage upon hydration. This very located cell-wall burst, together with the mucilaginous external envelope, could facilitate a slow and controlled water entry. For demonstrating this, a detailed microscopy study in the seed-coat would be necessary. Besides the presence of mucilage, it is worth mentioning that, although the volume of the light-brown and brown seeds was very similar, the density of the two lots was very different due to the notable mass of the brown seeds (**Fig. 2B**). This flaccidity in light seeds could provoke a quick imbibition in comparison to *S. officinale* brown seeds, and thus the sigmoidal profile of water uptake (**Fig. 4**) should be justified. Very rapid imbibition alters cell permeability and prompts losses of solutes to the external medium, triggering damage that can diminish seed vigour and viability (Legesse and Powell 1992; Puga-Hermida *et al*. 2003).

The heterogeneity in *S. officinale* seeds affects germination and hormonal sensitivity

The experiments to optimise the germination test are shown in **Table 1**. This table reflects the strong dependence on light and the presence of nitrate for rapid radicle emergence. Light cannot be replaced by a stratification period (4ºC), even if nitrate (20 mM) is present (**Table 1**). Nitrate (0.8 mM), in association with light, promotes germination in many species as *Lolium rigidum* Gaud. (Ellery *et al*. 2003). Radicle emergence in *S. officinale* can occur in the absence of nitrate if light and ET are present; however, less time is needed for emergence (**Table 1**). In this work, it has been demonstrated that the ability to germinate under optimal conditions (20 mM KNO_3, 24ºC and 16h light/8h dark photoperiod) differs within two lots of *S. officinale* seeds stu-

Table 1 Effect of several treatments on germination percentage of *S. officinale* seeds. Data are mean values of three replicates ± S.E. Significant differences between values as assessed by LSD test (P<0.05) are shown as different letters.

Treatment	Germination (%)		
	24 h	48 h	72 h
- light			
- nitrate (20 mM)			15 ± 3 a
- nitrate (20 mM) + ET (10 µM)			17 ± 2 a
+ nitrate (20 mM)		50 ± 4 b	100 c
+ nitrate (20 mM) + ET (10 µM)			100 c
+ nitrate (20 mM) + ET (50 µM)			100 c
+ nitrate (20 mM) + ST	n.f.		
+ light			
- nitrate (20 mM)		100 c	
- nitrate (20 mM) + ET (10 µM)		100 c	
+ nitrate (20 mM)	70 ± 8 d		
	13 ± 6 a (LB)		
+ nitrate (20 mM) + ET (10 µM)	95 ± 4 c		
	8 ± 3 a (LB)		
+ nitrate (20 mM) + ACC (10 µM)	98 ± 2 c		
+ nitrate (20 mM) + GA_{4+7} (100 µM)	100 c		15 ± 3 a (LB)
+ nitrate (20 mM) + GA_{4+7} (100 µM) + ET (10 µM)			15 ± 3 a (LB)

LB: light-brown lot; ST: stratification (ed, 4ºC); n.f.: not found.

died. Thus, at 24 h 6-fold more of the brown seeds germinated than the light-brown ones, and the ET and/or the ACC had a positive effect on the brown lot and negative over the light. However, GA_{4+7} strongly stimulated germination in the brown seeds while hardly altering that of the light ones despite that ET was present (**Table 1**). The low percentage of germination in the light lot did not increase by stratification, both in the absence as well as in the presence of GA_{4+7}, nor by a short scarification. Stratification in some seeds affects the expression of genes involved in the synthesis of physiologically active GAs (Yamauchi *et al*. 2004), and also intensifies sensitivity to GAs (Derkx and Karssen 1993; Koornneef *et al*. 2002). The absence of stimulation of the germination of stratified light-brown seeds could imply that GAs did not play a major part in the process or else that these seeds have difficulty synthesising active GAs. By molecular approaches currently under way in our laboratory, we will soon be able to confirm or exclude this possibility.

With the aim of making a thorough study of the effect of GA and ET on the germination rate of brown *S. officinale* seeds, we quantified the radicle emergence in short time periods. The protrusion began to be detectable between 17 and 18 h, reaching 100% at 25-26 h. This demonstrates the speed of the emergence process in this lot. The presence of GA_{4+7} strongly stimulated the germination between 21 and 23 h; in fact, at 23 h the germination was 3.5-fold greater than in control. However, at 26 h, germination reached 100% both in control as well as in the presence of GA_{4+7} (**Fig. 5A**). Conversely brown seeds, light ones have a great positive response to GA_{4+7} reaching in its presence germination percentages of 2.5-8.5–fold higher than the control (**Fig. 5B**). The results of **Figs. 5** and **6** indicate that both lots of seeds present different sensitivity to GA and probably

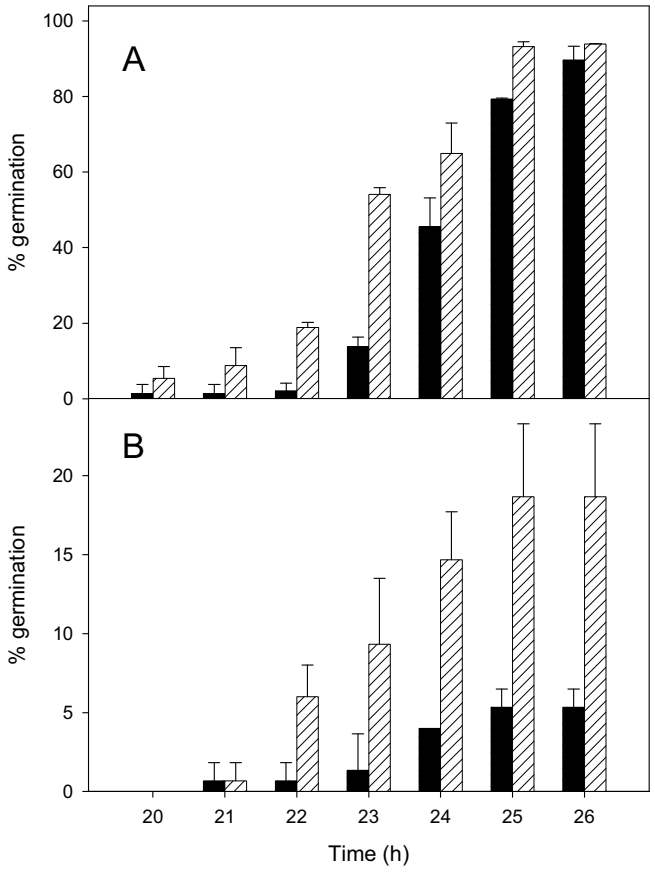

Fig. 5 Germination percentage of brown (A) and light-brown seed lot (B) of *S. officinale* seeds in presence (striped bars) or absence (black bars) of GA_{4+7}. Data are means ± standard error (S.E.) of 3 independent experiments. Differences between GA treated and non-treated seeds in the brown lot were significant (P<0.05) until 25 h; for the light-brown lot, differences were significant except at 21 h (P<0.05) as determined by LSD test.

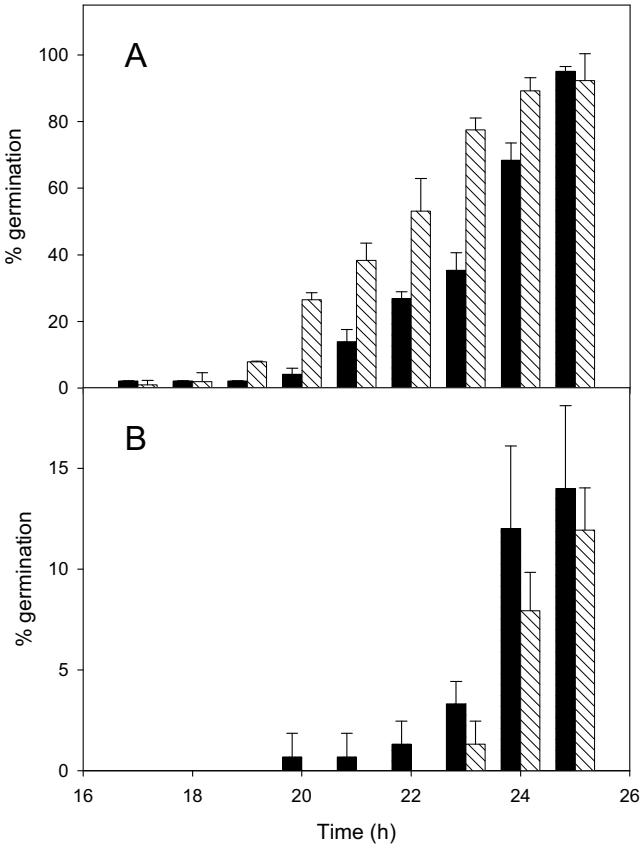

Fig. 6 Germination percentage of brown (A) and light-brown seed lot (B) of *S. officinale* seeds in presence (striped bars) or absence (black bars) of ET. Data are means ± standard error (S.E.) of 3 independent experiments. Differences between ET treated and non-treated seeds in the brown lot were significant (P<0.05) between 19-24 h; for the light-brown lot, differences were significant (P<0.05) as determined by LSD test.

also different hormonal regulation of germination. A very similar profile of germination stimulation found for brown seeds in the presence of GA_{4+7} was also found in the presence of ET (**Fig. 6A**). The fact that the kinetics in the presence of ACC (the immediate precursor of ET) and ET are similar shows that the brown seeds of *S. officinale* have the capacity of oxidizing ACC (i.e. ACC-oxidase). This also occurs in other crucifers (Matilla 2000; Rodríguez-Gacio *et al*. 2004; Matilla *et al*. 2005). On the contrary, ET was not capable of stimulating the germination in light-brown seeds, which had a far lower germination capacity than did the brown seeds (**Fig. 6B**); that is, the results appear to indicate that the ET was strongly inhibitory. Although we cannot discard that the light seeds have difficulties to oxidize their endogenous ACC, the fact that the exogenous ET did not accelerate its germination supports the assumption that this hormone is not required for germination. In heterogeneous turnip-tops seeds, the final step of the ET pathway was altered concomitantly with the changes in germinating capacity affecting the levels and expression of ACC-oxidase (Puga-Hermida *et al*. 2003; Rodríguez-Gacio *et al*. 2004). GA_{4+7} and ET together were not capable, either, of boosting the germination percentage of the light-brown seeds lot.

The addition to the germination medium of inhibitors of ET and GA synthesis gives us an idea of the participation of the two hormonal signals in the germination process of the brown seeds of *S. officinale*. Thus, the AVG (inhibitor of ACC-synthetase activity), Co_2Cl (inhibitor of ACC-oxidase activity), and NO_3Ag (inhibitor of the bonding of ET to its receptor) diminished the percentage of germination, particularly when these compounds were added jointly (**Table 2**). The presence of GA_{4+7} partially reversed the inhibition, again demonstrating the preferential action of GAs in the germination process. This last observation is supported by

Table 2 Effect of several treatments on germination percentage (23 h) of *S. officinale* brown-seeds. Data are mean values of three replicates ± S.E. Significant differences between values as assessed by LSD test ($P<0.05$) are shown as different letters.

Treatment	Germination (%)
control	40 ± 3 a
ET	75 ± 8 b
GA_{4+7}	91 ± 9 c
NO_3Ag	27 ± 2 d
AVG	25 ± 4 d
Co_2Cl	35 ± 3 e
AVG + Co_2Cl	8 ± 2 f
AVG + Co_2Cl + NO_3Ag	10 ± 3 f
AVG + Co_2Cl + NO_3Ag + GA_{4+7}	52 ± 5 g
NO_3Ag + GA_{4+7}	58 ± 6 g
AVG + Co_2Cl + GA_{4+7}	42 ± 5 a
PC	2 ± 1 h
PC + ET	3 ± 1 h

the total inhibition of germination in the presence of paclobutrazol, an inhibitor of GA synthesis (**Table 2**). However, the fact that the reversal caused by GA_{4+7} is not total, leads us to conclude that, apart from the presence of GA, other hormonal signals are needed for right radicle emergence.

CONCLUDING REMARKS

In this work, we have demonstrated several physical and physiological differences between heterogeneous lots of seeds from *Sisymbrium officinale*. The examination of the causes of this different behaviour, concerning the hormonal balance and physical requirements, complemented with a molecular approach, could shed light on the processes involved in germination for these species. Whether or not this heterogeneity reflects an adaptative strategy to control the timing and requirements for germination remains to be investigated; as discussed earlier, it could be also a matter of incomplete embryogenesis/maternal effect. In any case, both these heterogeneous lots are dispersed from wild populations of this weed, and probably this seed heterogeneity has a deep impact on the ecophysiology of this species.

ACKNOWLEDGEMENTS

This work was supported by the Dirección General de Investigación (Spain; grant no. CGL2004-01996/BOS). The English version of the text was corrected by D. Nesbitt.

REFERENCES

Barroco RM, van Poucke K, Bergervoet JHW, de Veylder L, Groot SPC, Inzé D, Engler G (2005) The role of the cell cycle machinery in resumption of postembryonic development. *Plant Physiology* **137**, 127-140

Bassel GW, Zielinska E, Mullen RT, Bewley JD (2004) Down-regulation of *DELLA* genes is not essential for germination of tomato, soybean, and Arabidopsis seeds. *Plant Physiology* **136**, 2782-2789

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between ABA and ethylene signalling cascades. *Plant Cell* **12**, 1103-1115

Berridge MV, Tan AS, McCoy KD, Wang R (1996) The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica* **4**, 15-20

Bewley JD (1997a) Seed germination and dormancy. *The Plant Cell* **9**, 1055-1066

Bewley JD (1997b) Breaking down the walls: a role for endo-β-mannanase in release from seed dormancy? *Trends in Plant Science* **2**, 464-469

Boesewinkel FD, Bouman F (1995) The seed: structure and function. In: Kigel J, Galili G (Eds) *Seed Development and Germination*, Marcel Dekker, New York, pp 1-24

Cadman CSC, Toorop PE, Hilhorst HWM, Finch-Savage WE (2006) Gene expression profiles of *Arabidopsis* Cvi seeds during dormancy cycling indicate a common underlaying dormancy control mechanism. *The Plant Journal* **46**, 805-822

da Silva EAA, Toorop PE, van Aelst AC, Hilhorst H (2004) ABA controls embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv. Rubi) seed germination. *Planta* **220**, 251-261

da Silva EAA, Toorop PE, Nijsse J, Bewley JD, Hilhorst H (2005) Exogenous gibberellins inhibit coffee (*Coffea arabica* cv. Rubi) seed germination and cause cell death in the embryo. *Journal of Experimental Botany* **413**, 1029-1038

de la Torre F, Rodríguez-Gacio MC, Matilla AJ (2006) How ethylene works in the reproductive organs of higher plants. *Plant Signaling and Behavior* **1**, 231-242

Chibani K, Ali-Rachedi S, Job C, Job D, Jullien M, Grappin P (2006) Proteomic analysis of seed dormancy in *Arabidopsis*. *Plant Physiology* **142**, 1493-1510

Debeaujon I, Koornneef M (2000) GA requirements for *A. thaliana* seed germination in determined both by testa characteristics and embryonic ABA. *Plant Physiology* **122**, 415-424

Debeaujon I, León-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. *Plant Physiology* **122**, 403-413

Derkx MPM, Karssen CM (1993) Effects of light and temperature on seed dormancy and gibberellin-stimulated germination in *Arabidopsis thaliana*: Studies with gibberellin-deficient and gibberellin-insensitive mutants. *Physiologia Plantarum* **89**, 360-368

Ellery AJ, Gallagher RS, Dudley SV (2003) Dormancy and germination ecology of annual ryegrass (*Lolium rigidum* Gaud.). In: Nicolás G, Bradford KJ, Côme D, Pritchard HW (Eds) *The Biology of Seeds: Recent Research Advances*, CABI Publishing, Wallingford, Oxon, UK, pp 389-396

Fenner M (1991) The effects of the parent environment on seed germinability. *Seed Science Research* **1**, 75-84

Fenner M (1993) Environmental influences of seed size and composition. *Horticultural Reviews* **13**, 183-213

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytologist* **171**, 501-523

Forbis TA, Floyd SK, 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

Frey A, Godin B, Bonnet M, Sotta B, Marion-Poll A (2004) Maternal synthesis of ABA controls seed development and yield in *Nicotiana plumbaginifolia*. *Planta* **218**, 958-964

Friedman WE (1998) The evolution of double fertilization and endosperm: an "historical" perspective. *Sexual Plant Reproduction* **11**, 6-16

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P (2000) Regulation of ABA signaling by the ethylene response pathway in Arabidopsis. *Plant Cell* **12**, 1117-1126

Grossmann K, Hansen H (2001) Ethylene-triggered ABA: a principle in plant growth regulation? *Physiologia Plantarum* **113**, 9-14

Gutterman Y (1992) Maternal effects on seeds during development. In: Fenner M (Ed) *Seeds. The Ecology of Regeneration in Plant Communities*, Wallingford, CAB International, UK, pp 27-59

Hilhorst HWM, Toorop PE (1997) Review on dormancy, germinability, and germination in crop and weed seeds. *Advances in Agronomy* **61**, 111-165

Hilhorst HWM, Downie B (1995) Seed dormancy in tomato (*Lycopersicon esculentum* cv. Moneymaker): Studies with the *sitiens* mutant. *Journal of Experimental Botany* **47**, 89-97

Homrichhausen TM, Hewitt JR, Nonogaki H (2003) Endo-β-mannanase activity is associated with the completion of embryogenesis in imbibed carrot (*Daucus carota* L.) seeds. *Seed Science Research* **13**, 219-227

Huang Z, Gutterman Y (1998) *Artemisia monosperma* achene germination in sand: effects of sand depth, sand/water content, cyanobacterial sand crust and temperature. *Journal of Arid Environments* **38**, 27-43

Huang Z, Huc Z, Gutterman Y (2000) Structure and function of mucilaginous achenes of *Artemisa monosperma* inhabiting the Negev desert of Israel. *Israel Journal of Plant Science* **48**, 255-266

Huang Z, Boubriak I, Osborne DJ, Dong M, Gutterman Y (2007) Possible role of pectin-containing mucilage and dew in reairing embryo DNA of seeds adapted to desert conditions. *Annals of Botany*, in press

Imbert E (2002) Ecological consequences and ontogeny of seed heteromorphism. *Perspectives in Plant Ecology, Evolution and Systematics* **5**, 13-36

Kantar F, Pilbeam CJ, Hebblethwaite PD (1996) Effect of tannin content of faba bean (*Vicia faba*) seed on seed vigour, germination and field emergence. *Annals of Applied Biology* **128**, 85-93

Kepczynski J, Kepzcynska E (1997) Ethylene in seed dormancy and germination. *Physiologia Plantarum* **101**, 720-726

Khan M, Cavers PB, Kane M, Thompson K (1996) Role of the pigmented seed coat of proso millet (*Panicum miliaceum* L.) in imbibition, germination and seed persistence. *Seed Science Research* **7**, 21-25

Kigel J (1995) Seed germination in arid and semiarid regions. In: Kigel J, Galili G (Eds) *Seed Development and Germination*, Marcel Dekker, New York, pp 645-700

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. *Current Opinion in Plant Biology* **5**, 33-36

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**, 281-307

Lee CS, Chien CT, Lin CH, Chiu YY, Yang YS (2006) Protein changes

between dormant and dormancy-broken seeds of *Prunus campanulata* Maxim. *Proteomics* **6**, 4147-4154

Legesse N, Powell AA (1992) Comparisons of water uptake and imbibition damage in eleven cowpea cultivars. *Seed Science Technology* **20**, 173-180

Leubner-Metzger G (2003) Functions and regulation of β-1,3-glucanase during seed germination, dormancy release and after-ripening. *Seed Science Research* **13**, 17-34

Leubner-Metzger G, Petruzzelli L, Waldvogel R, Vögeli-Lange R, Meins F (1998) Ethylene-responsive element binding protein (EREBP) expression and the transcriptional regulation of class I β-1,2-glucanase during tobacco seed germination. *Plant Molecular Biology* **38**, 785-795

Leubner-Metzger G, Kucera B, Müller K (2006) Emerging and established model systems for endosperm weakening. In: Navie S, Adkins S, Ashmore S (Eds) *Seeds: Biology, Development and Ecology*, CAB International, UK, pp 195-204

Liu PP, Koizuka N, Homrichhausen TM, Hewitt JR, Martin RC, Nonogaki H (2005) Large-scale screening of Arabidopsis enhancer-trap lines for seed germination-associated genes. *The Plant Journal* **41**, 936-944

Luzuriaga AL, Escudero A, Pérez-García F (2006) Environmental maternal effects on seed morphology and germination in *Sinapis arvensis* (Cruciferae). *Weed Research* **46**, 163-174

Manz B, Müller K, Kucera B, Volke F, Leubner-Metzger G (2005) Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. *Plant Physiology* **138**, 1538-1551

Matilla AJ (2000) Ethylene in seed formation and germination. *Seed Science Research* **10**, 111-126

Matilla AJ, Gallardo M, Puga-Hermida MI (2005) Structural, physiological and molecular aspects of heterogeneity in seeds: a review. *Seed Science Research* **15**, 63-76

Matilla AJ (2007) How is the silique fruit dismantled over its maturation? *Functional Plant Science and Biotechnology* **1**, 85-93

Moles AT, Westoby M (2006) Seed size and plant strategy across the whole life cycle. *Oikos* **113**, 91-105

Müller K, Tintelnot S, Leubner-Metzger G (2006) Endosperm-limited Brassicaceae seed germination: ABA inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiology* **47**, 864-877

Penfield S. Meissner RC. Shoue DA. Carpita NC. Bevan MW (2001) *MYB61* is required for mucilage deposition and extrusion in the Arabidopsis seed coat. *Plant Cell* **13**, 2777-2791

Petruzzelli L, Müller K, Hermann K, Leubner-Metzger G (2003) Distinct expression patterns of β-1,3-glucanases and chitinases during the germination of Solanaceous seeds. *Seed Science Research* **13**, 139-153

Puga-Hermida MI, Gallardo M, Rodríguez-Gacio MC, Matilla AJ (2003) The heterogeneity of turnip-tops (*Brassica rapa*) seeds incide the silique affects germination, the activity of the final step of the ethylene pathway, and ABA and polyamine content. *Functional Plant Biology* **30**, 767-775

Raghavan V (1986) Developmental Embryogenesis. In: Barlow PW, Green PB, Wylie CC (Eds) *Embryogenesis in Angiosperms*. *A Developmental and Experimental Study*, Cambridge University Press, Cambridge, UK, pp 13-45

Rodriguez-Gacio MC, Nicolás C, Matilla AM (2004) The final step of the ethylene biosynthesis pathway in turnip tops (*Brassica rapa*): molecular characterization of the 1-aminocyclopropane-1-carboxylate oxidase *BrACO1* throughout zygotic embryogenesis and germination of heterogeneous seeds. *Physiologia Plantarum* **121**, 132-140

Schwachtje J, Baldwin IT (2004) Smoke exposure alters endogenous gibberellin and abscisic acid pools and gibberellin sensitivity while eliciting germination in the post-fire annual *Nicotiana attenuata*. *Seed Science Research* **14**, 51-60

Siriwitayawan G, Geneve RL, Downie AB (2003) Seed germination of ethylene perception mutants of tomato and Arabidopsis. *Plant Cell* **14**, 3133-3147

Steel RGG, Torrie JH (1982) *Principles and Procedures of Statistics*, McGraw-Hill, Tokyo.

Toorop PE, van Aelst AC, Hilhorst H (2000) The second step of the biphasic endosperm cap weakering that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA. *Journal of Experimental Botany* **51**, 1371-1379

Torres-Ruiz RA (1998) Embryogenesis. In: Anderson M, Roberts JA (Eds) *Arabidopsis*. *Annual Plant Reviews* (Vol 1), CRC Press, Boca Raton, Florida, pp 223-261

Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the Arabidopsis seed coat. *Plant Physiology* **122**, 345-355

Western TL, Diana S, Young DS, Dean GH, Tan WL, Samuels AL, Haughn GW (2004) MUCILAGE-MODIFIED4 encodes a putative pectin biosynthetic enzyme developmentally regulated by APETALA2, TRANSPARENT TESTA GLABRA1, and GLABRA2 in the Arabidopsis seed coat. *Plant Physiology* **134**, 296-306

Wu, CT, Leubner-Metzger G, Meins F Jr., Bradford KJ (2000) Class I β-1,3-glucanase and chitinase are expressed in the micropylar endosperm of tomato seeds prior to radicle emergence. *Plant Physiology* **126**, 1299-1313

Yamauchi Y, Ogawa M, Kuwahara A, Hanada A, Kamiya Y, Yamaguchi S (2004) Activation of GA biosynthesis and response pathways by low temperature during imbibition of *Arabidopsis thaliana* seeds. *Plant Cell* **16**, 367-378