

EFFECT OF THERMAL SHOCK AND RUMINAL INCUBATION ON SEED GERMINATION IN *HELIANTHEMUM APENNINUM* (L.) MILL. (CISTACEAE)

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ABSTRACT. *Effect of thermal shock and ruminal incubation on seed germination in Helianthemum apenninum* (L.) Mill. (Cistaceae). Here, we analyse the effect of different treatments on seed germination in *Helianthemum apenninum* (L.) Mill. (Cistaceae), considering scarification with sandpaper, thermal shock simulating the heat from fire, and incubation in the rumen of sheep and goat simulating passage through the gut of ruminants. Mechanical scarification boosted the germination (95% vs. 6% of control treatment), indicating that the seeds have a potentially high germination rate if the coat is eroded. Thermal shock did not improve germination. Incubation in rumen increased seed germination, up to a 32% after 48h in ruminal liquid versus 12% for control seeds in the case of sheep. The results suggest that germination in *H. apenninum*, while not enhanced by heat from fires, may be enhanced by herbivore ingestion.

Key words. Dormancy, Mediterranean shrublands, ruminal digestion, thermal shock.

RESUMEN. *Efecto del choque térmico y de la incubación ruminal en la germinación de semillas de Helianthemum apenninum* (L.) Mill. (Cistaceae). En este trabajo se analiza el efecto que tienen diversos tratamientos sobre la germinación de las semillas de *Helianthemum apenninum* (L.) Mill. (Cistaceae), siendo éstos 1) la escarificación mecánica mediante abrasión con papel de lija, 2) choque térmico (que simula el efecto del fuego), y 3) incubación en el rumen de oveja y cabra (que simula el paso por el tracto digestivo del ganado). La escarificación mecánica aumentó considerablemente la germinación (95% vs 6% en el control), indicando una alta viabilidad de las semillas. El choque térmico no incrementó la germinación en ninguna de las combinaciones de tiempo y temperatura probadas. La incubación en el rumen incrementó el porcentaje de germinación, siendo 32% tras 48h de incubación frente a 12% para el control en el caso de oveja. Los resultados sugieren que el calor producido durante un fuego no promueve la germinación de las semillas de *H. apenninum*, mientras que podría ser promovida por la ingestión de semillas por herbívoros.

Palabras clave. Arbustos mediterráneos, choque térmico, dormancia, digestión ruminal.

INTRODUCTION

Cistaceae is a family of shrubs and herbs that are characteristic of dry, sunny habitats, its main diversification centre being the

Mediterranean region (Proctor, 1978). The taxa in this family present seeds with hard coats (hardseededness, Thanos *et al.*, 1992), which impede water imbibition and gas exchange and, therefore, seed germination (Mayer &

Poljakoff-Mayber, 1989; Bewley & Black, 1994; Baskin & Baskin, 1998). Once the seed coat is broken by some mechanism, seed germination is commonly a massive process in this family, reaching values approaching 100% (e.g. Thanos *et al.*, 1992; Trabaud, 1995).

Mechanisms that may break the dormancy imposed by hard coats are diverse in nature (Mayer & Poljakoff-Mayber, 1989; Bewley & Black, 1994; Baskin & Baskin, 1998). Among them, thermal shock produced during fires has been reported as a particularly important one for breaking dormancy of many Cistaceae species. In fact, fires in the Mediterranean shrublands are frequently followed by massive recruitment of Cistaceae seedlings (Trabaud & Oustric, 1989; Roy & Sonié, 1992; Ferrandis *et al.*, 1999), and several laboratory studies have demonstrated that thermal shock comparable to soil temperatures during a fire can promote germination in this family (González-Rabanal & Casal, 1995; Trabaud, 1995; Castro & Romero-García, 1999). Passage through the digestive tract of vertebrates may also promote germination of hard-coated seeds (Mayer & Poljakoff-Mayber, 1989; Baskin & Baskin, 1998), a mechanism which has been reported for some Cistaceae species (Malo & Suárez, 1996), although such information is scarce for this family.

In the present work, we investigate the impact that the heat shock and the ingestion by vertebrates may have on the germination of *Helianthemum apenninum* (L.) Mill., a species that, like the rest of the members of Cistaceae, has hard-coated seeds (Thanos *et al.*, 1992; Tébar *et al.*, 1997). For this, we expose the seeds to laboratory treatments simulating i) the effect of fire (thermal shock matching the temperatures reached in the upper layer of the soil during wildfires in Mediterranean shrublands), and ii) simulating the effect of ungulate ingestion (placing seeds in the rumen of sheep and goat, which are common consumers of this plant).

MATERIALS AND METHODS

The species

Helianthemum apenninum (L.) Mill. (Cistaceae), a dwarf shrub reaching 40 cm in height, is a common scrubland plant in the Mediterranean Basin, growing in dry, sunny environments from sea level to 2100 m. The fruit is a thin, woody capsule with 3 valves (4–8 mm in size) containing ca. 10 seeds, which are released in mid-summer (López-González, 1993; Tébar *et al.*, 1997). The seeds are small, ca. 2mm thick, and subspherical in shape. The species is palatable and heavily grazed by free-ranging livestock, mainly domestic sheep and goats (Fernández, 1995), which frequently ingest ripe fruits while browsing (personal observation; see also Tébar *et al.*, 1997).

Seed treatments and germination experiments

Ripe fruits from at least 30 *Helianthemum apenninum* plants were collected in July 1994 in a stand located at Natural Park of Sierra de Castril (SE Spain, 37°46'30''N, 2°46'24''W) at 1500 m a.s.l. Seeds were extracted from the fruits and stored in paper bags at room temperature. Germination experiments, spanning 1995, were performed in a growth chamber under a photoperiod of 16h light and 8h darkness. For technical reasons, the temperature of the chamber was different depending on the experiment, being either a constant temperature of 10°C or an alternating cycle of 15°C during dark periods and 20°C during light periods. Nevertheless, previous trials showed lack of germination differences for these two temperature regimes for control seeds (see also Martin *et al.* [1995] for similar results). In addition, each experiment was compared with its own control, and therefore differences in germination temperatures are not likely to affect the results.

Before starting germination test, seeds were individually examined under a dissecting

microscope and any damaged or empty seed was discarded. Seeds were placed in Petri dishes of 9 cm in diameter, resting on filter-paper disks and were watered as needed with sterilised distilled water. Petri dishes were randomly repositioned within the chamber every 5 days. Germination, identified as visible protrusion of the radicle, was recorded at 4-5 days intervals. The experiments were ended when all treatments registered 10 consecutive days without germination, this occurring within 5 weeks in all the cases. We performed three different experiments.

Experiment #1. Effect of mechanical scarification

Seeds were placed on a woody surface and were gently rubbed with sand paper until the seed coat was partially eroded. To prevent fungal attack after scarification, seeds were disinfected by immersion in a 1% sodium hypochlorite solution for 10 min, followed by thorough rinsing with sterile distilled water. Untreated seeds were also disinfected. We used 4 replicates per treatment containing 100 seeds each. The temperature of the chamber was 15°C during dark periods and 20°C during light periods. This treatment provides valuable information on seed viability of hard-coated seeds, being a reference to compare the effectiveness of other treatments.

Experiment #2. Effect of thermal shock

Dry seeds were placed in glass dishes and heated in an oven (precision $\pm 2^\circ\text{C}$) using two different schedules. In one, temperature was kept constant at $100 \pm 2^\circ\text{C}$ and the seeds were exposed for 10, 30 or 60 minutes (treatments Th10 to Th60). In the other procedure, exposure time was fixed at 10 min, and the seeds were heated at 80, 100, 120 or 140°C (treatments K80 to K140). These heat treatments were selected because they matched time and temperature ranges commonly reached in the upper layer of the soil (2-5 cm) during fires in

Mediterranean shrublands (Whelan, 1997), the depth where most seeds are located (Simpson *et al.*, 1989). In addition, the temperatures selected were similar to those tested in other works that have reported a sharp increase on seed germination for other Cistaceae species (e.g. Thanos *et al.*, 1992; Valbuena *et al.*, 1992; Trabaud 1995; Pérez-García & Escudero, 1997; Castro & Romero-García, 1999). We used 4 replicates per treatment containing 100 seeds each, with the chamber temperature at 15°C during dark periods and 20°C during light periods. For treatments K100 and Th10, a single set of 4 replicates was used, as they represent the same conditions (seeds heated 10 min at 100°C).

Experiment #3. Effect of ruminal incubation

The effect of sheep and goat ruminal incubation was studied following the methodology proposed by Olson & Wallander (2002). Seeds inside nylon bags (40 μm mesh) were introduced in the rumen of a fistulated sheep (Segureña race) or goat (Granadina race) and incubated for 24h, 48h and 72h (two bags containing 250 seeds each per treatment), the animal having been fed with lucerne hay at maintenance level. These are the standard times used in experiments to assay the degradability of food in ruminants (Mehrez & Osrskov, 1977), and in addition match the usual food retention times in the digestive tract of these animals (Arbiza, 1986; Gardener *et al.*, 1993a). Seed bags were inserted into the rumen by using a permanent ruminal cannula. After ruminal incubation, each bag and its content was thoroughly washed with sterile distilled water (see Gardener *et al.*, 1993b; Ibáñez & Passera, 1997 for a similar procedure). Germination was tested with 5 replicates per treatment, each containing 25 seeds randomly taken from the digested pool, and the temperature of the chamber was kept constant at 10°C (see Martin *et al.*, 1995).

For each experiment, final germination percentages were submitted to a one-way ANOVA, all data being previously arcsin-transformed. After ANOVAs, the means of the groups were compared with the mean of their control treatment by using Dunnett's test at a level of 0.05 (Zar, 1996). Analyses were performed using the computer software JMP 3.2.6 (SAS Institute, 1999). Throughout the paper, values are mean \pm 1SE.

RESULTS

Mechanical scarification boosted germination of *H. apenninum* seeds, with values of $95.0 \pm 1.1\%$ against $6.0 \pm 0.7\%$ of untreated seeds ($F=1546.91$, $df=1, 6$, $p<0.0001$).

Flash heating of the seeds at 100°C for variable times (Th treatments) reduced the germination percentage ($F=4.29$, $df=3, 12$, $p=0.0284$), with germination percentage tending to decrease as exposure times increased (tab. 1). Similarly, flash heating for 10 min at variable temperatures (K treatments) also reduced germination ($F=7.86$, $df=4, 15$, $p=0.0013$), until no germination was registered at 140°C (tab. 1).

Seed incubation in sheep rumen enhanced germination ($F=2.62$, $df=3, 16$, $p=0.0862$), with the maximum percentage being reached after 48h in the ruminal liquid (tab. 1). Incubation in goat rumen also affected germination ($F=3.74$, $df=3, 16$, $p=0.0328$), reaching the highest value after 24 h (tab. 1).

DISCUSSION

According to our results, the germination percentage of *Helianthemum apenninum* seeds was very low when no treatment was applied, with values of around 10%. This percentage is similar to that reported for several *Helianthemum* species (Gutterman & Agami,

1987; Thanos *et al.*, 1992; Pérez-García *et al.*, 1995; Escudero *et al.*, 1997), and even lower than in previous studies on this species (Thanos *et al.*, 1992; Martín *et al.*, 1995; Tébar *et al.*, 1997). However, after mechanical scarification, germination was boosted (up to 95%; see also Thanos *et al.*, 1992; Tébar *et al.*, 1997), implying that the germination of the seeds relies on some mechanisms breaking their coats.

Thermal shock simulating the heat wave in the soil during a fire in Mediterranean shrublands has been found to promote germination in many Cistaceae species, particularly in the genus *Cistus* (Thanos *et al.* 1992; Trabaud, 1995; Castro & Romero-García, 1999). Nevertheless, thermal treatments failed to improve seed germination in *H. apenninum*, as has been shown for other *Helianthemum* species in the Mediterranean region. In this sense, Thanos *et al.* (1992) found that seed-germination percentages of *H. croceum* and *H. pilosum* seeds were only slightly higher when heated. Similarly, Pérez-García *et al.* (1995) found that dry heat did not improve germination, and even killed the seeds, in *H. polygonoides* and *H. squamatum*. Therefore, germination after fires may not be a general characteristic in Cistaceae.

Seed incubation in the rumen raised germination percentages, particularly in the case of sheep, from 12.0% to 32.0%. Ruminal liquid, which has a neutral pH, contains proteolytic and cellulolytic enzymes (Prins & Van der Vorstenbosch, 1975) that may soften the seed coat and thereby increase germination percentages. Seeds having passed from the rumen into the abomasum and duodenum are exposed to an acid medium as well as to proteolytic, amilolytic and lipolytic enzymes (Gardener *et al.*, 1993a). This could further erode seed coats, as happens when Cistaceae seeds are exposed to acid scarification (Peña *et al.*, 1988; Pérez-García *et al.*, 1995; Pérez-García & Escudero, 1997; Castro & Romero-García, 1999), and could encourage even higher

Treatments	Mean \pm s.e.	Treatments	Mean \pm s.e.
A) Heated at 100 °C for:		C) Sheep Rumen for	
10 min (Th10)	7.2 \pm 2.5	24 h	20.8 \pm 3.2
30 min (Th30)	3.5 \pm 1.0	48 h	32.0 \pm 7.7*
60 min (Th60)	1.0 \pm 0.4	72 h	17.6 \pm 4.7
Untreated seeds	4.2 \pm 0.8	Untreated seeds	12.0 \pm 2.2
B) Heated 10 min at		D) Goat Rumen for	
80 °C (K80)	7.5 \pm 1.4	24 h	25.6 \pm 7.0
100 °C (K100)	7.2 \pm 2.5	48 h	17.6 \pm 3.5
120 °C (K120)	0.5 \pm 0.5*	72 h	7.2 \pm 3.2
140 °C (K140)	0.0 \pm 0.0*		
Untreated seeds	4.2 \pm 0.8	Untreated seeds	12.0 \pm 2.2

Table 1. Mean germination percentages (\pm s.e.) of *Helianthemum apenninum* seeds after different treatments: A) seeds heated at 100 °C for 10, 30 and 60 min (Th treatments); B) seeds heated at 80, 100, 120 and 140°C for 10 min (K treatments); C) seeds incubated in sheep rumen for 24h, 48h, 72h; D) seeds incubated in goat rumen for 24h, 48h, 72h. Asterisks show means that differ from the control after Dunnett's test at a-level of 0.05.

final germination percentages. In this sense, it is noticeable that Malo & Suárez (1996) found that the passage of *Cistus ladanifer* seeds through the guts of red deer increased the germination percentage respect to the control. Thus, our results suggest that, as in other Cistaceae species, herbivores may promote the germination of *H. apenninum* by virtue of scarification during the digestive processes.

Hardseededness in the Cistaceae has been linked to two processes. First, in view of the burst in germination after fires for many Cistaceae species, hardseededness has been related to the recurrence of fire in the Mediterranean region (Valbuena *et al.* 1992; Doussi and Thanos, 1993; Ojeda, 2001). Second, given that the passage of seeds through

the gut of vertebrates increases germination percentages, it has been suggested that the presence of hard seed coats in the Cistaceae could be related to endozoochorous seed dispersal (Malo and Suárez, 1996, 1998). Our results support this latter hypothesis, as germination in *H. apenninum* is not likely to be benefited by the heat of a fire, but it is promoted by incubation in ruminal liquid. Nevertheless, we should consider that hardseededness is advantageous for maintaining a persistent soil seed bank, a characteristic that is highly appropriate for the viability of plant populations, especially in unpredictable environments such as the Mediterranean (Fenner, 1985; Thompson, 1992). Provided that in nature several mechanisms can erode hard

seed coats and thus break dormancy (e.g. fungal attack, rainfall wash, mechanical abrasion by soil particles, daily fluctuations in temperature; see Mayer and Poljakoff-Mayber, 1989; Bewley and Black, 1994; Baskin and Baskin, 1998), the possible role of both fire and animals in hardseededness evolution should be taken with caution.

In summary, our results suggest that germination in *H. apenninum* may not be promoted by the heat produced during fires, whereas could be enhanced by herbivore ingestion. The study of more species of *Helianthemum* as well as of other genera would be appropriate to expand our current knowledge of the effect of these two factors in the germination ecology of this family, and to understand the evolutionary framework of hard seed coats in Cistaceae.

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