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THE EFFECT OF TEMPERATURE, LIGHT AND CALCIUM CARBONATE ON SEED GERMINATION AND RADICLE GROWTH OF THE POLYCARPIC PERENNIAL *GALIUM CRACOVIENSE* (RUBIACEAE), A NARROW ENDEMIC SPECIES FROM SOUTHERN POLAND

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Germination responses of *Galium cracoviense* Ehrend. (Rubiaceae), a narrow endemic species from southern Poland, were tested in light and dark conditions at three constant temperatures (5, 10, or 22°C), before and after cold-wet stratification. Additionally, seeds were germinated under different calcium carbonate ($CaCO_3$) concentrations (1, 5, 10, 15, 20, or 25 mM/L $CaCO_3$) at 22°C in light. The high germination capacity of seeds incubated at different temperatures, shortly after collection, already suggested the absence of dormancy in this species. Thus, the seeds are ready to germinate immediately in the field when water resources are available and the temperature is adequate. Light was a significant factor for *G. cracoviense*; more seeds germinated in light than in darkness at all temperatures tested. Cold stratification decreased germination especially at higher temperatures. The light requirement for *G. cracoviense* germination ensures their successful germination on or near the soil surface, and in cracks and crevices in limestone, when temperature and edaphic conditions are favourable. Seeds of this species show temperature enforced dormancy throughout the winter. Germination was significantly affected by calcium carbonate. Non-germinated seeds germinated well after being transferred from higher $CaCO_3$ concentrations to distilled water. The results indicate that the seeds of this species can endure $CaCO_3$ stress without losing their viability and start germination once $CaCO_3$ concentration is reduced. It can be concluded that the seeds of this species require lower Ca^{2+} ion concentration, moderate temperatures and the presence of light to germinate.

Key words: Calcium carbonate, dormancy, rare species, recovery.

INTRODUCTION

Germination is one of the most important stages in the life-cycle of plants (Baskin and Baskin, 1989). Seed germination is affected by many environmental factors, such as temperature, salt, light, soil moisture, oxygen concentration, and Ca^{2+} ions (Harper, 1977; Woolley and Stoller, 1978; Baskin and Baskin, 1988; Benvenuti et al., 2001; Koyuncu, 2005; Travlos, 2009; Arslan et al., 2011). The temperature, which influences dormancy and germination, is the primary environmental factor regulating the latter process, and light and soil moisture are of secondary importance (Baskin and Baskin, 1988).

Saline soils contain different salt components that affect seed germination differently (Tobe et al., 2003). Most studies examining the effects of salinity on seed germination were carried out with individual salts, especially NaCl solution (Bliss et al., 1986; Tobe et al., 1999, 2000; Huang et al., 2003; Zia and Khan, 2004; Li, 2008; Qu et al., 2008; Ahmed and Khan, 2010), but little information exists concerning the effect of Ca^{2+} ions on seed germination (see Shaikh et al., 2007 and references therein). Calcium has been shown to inhibit Na^+ uptake and thereby reduce its adverse effect on seed germination (Marcar, 1986; Rengel, 1992; Ebert et al., 2002; Bonilla et al., 2004; Yang et al., 2007; Zehra et al.

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2012), increase plant growth (Colmer et al., 1996; Kinraide, 1999; Tobe et al., 2003; Hirschi, 2004; Joshi et al., 2012) and alleviate the toxic effects of Na^+, Mg^{2+} (Tobe et al., 2002, 2004; Probert, 2000; Bonilla et al., 2004; Zhang and Mu, 2009) and various sulphate salts on germination or seedling growth (Tobe et al., 2002, 2004). Alkaline salts have a more severe effect on plant growth than neutral salts (Shi and Yin, 1993; Tang and Turner, 1999). Prolonged elevated Ca^{2+} concentration may also pose a stress to seed germination although this has received less attention in the literature (Guan et al., 2009; Parida and Das, 2005; Shi et al., 1998).

The germination requirements of *Galium cracoviense* Ehrend. have not been studied so far. In the present research we investigated the germination responses of *G. cracoviense* seeds to Ca^{2+} ions, light and temperature. *Galium cracoviense*, a species that is endemic to Poland, is included in Annex II of the European Habitats Directive (92/43/EEC – Council of the European Communities, 1992) and in Annex I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention – Council of Europe, 1992). As a species of limited extent and numbers, it is listed as Rare in the national Red List (Mirek et al., 2006), as Vulnerable in the Polish Red Data Book (Kaźmierczakowa and Zarzycki, 2001) and is strictly protected at the national level (Piękoś-Mirkowa and Mirek, 2006).

The preservation and rescue of rare and threatened plants requires considerable research and adequate knowledge about their biology (Planta Europa, 2008; see also Kadis et al., 2009). Germination requirements for rare and/or endemic species are often unknown, particularly of those whose material is difficult to obtain (Cerabolini et al., 2004). The knowledge of seed germination behaviour is considered vital in developing effective procedures for promoting ex situ conservation for rare and threatened species in seed banks (Kirmizi et al., 2010). In a seed bank, it is important to know the viability of seeds that are being stored (Smith et al., 2003; Fenner and Thompson, 2005). However, we do not have such basic information for *G. cracoviense*.

The main aim of this study was to investigate the germination variability of *G. cracoviense* seeds and their ability to germinate under diverse environmental conditions. The specific objectives of our study were to (1) determine if freshly matured seeds are dormant, and (2) determine what temperatures are suitable for the germination of freshly matured and cold stratified seeds, (3) elucidate the effects of different concentrations of calcium (in the form of $CaCO_3$) on the germination and growth of plants.

MATERIALS AND METHODS

SPECIES STUDIED

The following description is based on information from Piotrowicz (1958), Kucowa (1962), Ehrendorfer et al. (1976), Mirek and Piękoś-Mirkowa (2009), Cieślak and Szeląg (2009, 2010) and observations of the authors. *Galium cracoviense* is a polycarpic, dicotyledonous perennial herb, 5–9 cm tall, that reproduces both vegetatively and by seed. Leaves are obovate, 3–8 mm long and 0.75–1.9 mm wide, entire. Flowers are bisexual, white in colour. The fruit is a schizocarp 1.0–2.0 mm thick, split into two carpels, each containing one seed measuring 0.5–0.75 × 1.0–1.25 mm. Seeds are subglobose, or ellipsoid-oblong, brown or dark brown in colour. Seed sculpturing under scanning electron microscopy (SEM) shows a tuberculate pattern (Fig. 1). The mean (± S.D.) mass of four groups of 1000 seeds each was 226.34 mg ± 12.26 mg (our measurements). Flowering begins in May and early June, and fruit ripening is completed in July. The diploid number of chromosomes (2n = 22) has been reported.

SEED COLLECTION AND FIELD SITE DESCRIPTION

The total number of *G. cracoviense* individuals is approximately 20 000, distributed in seven populations over a 9-km^2 area in the central part of the Jura Krakowsko-Wieluńska (close to the village of Olsztyn near Częstochowa). It is found in association with other species such as *Allium montanum* F.W. Schmitd (Alliaceae), *Jovibarba sobolifera* (Sims) Opiz (Crassulaceae), *Potentilla arenaria* Borkh., *P. verna* L. (Rosaceae) and *Saxifraga paniculata* Mill. (Saxifragaceae). The shallow soils derived from calcareous rocks have a high rhizosphere pH of 7.0–8.0. The root system of this species was found to be confined to the surface layers in calcareous habitats.

Freshly matured seeds of *G. cracoviense* were collected on the 25th of June, 2013, from a single population in the vicinity of the village of Olsztyn (Zamkowa hill, 50°45'N, 19°16'E, alt. 350 m a.s.l.), 10 km east of Częstochowa, southern Poland. We harvested seeds from the largest population of *G. cracoviense*, which is representative of the associated habitat type and the altitude gradient of the species. Plants were haphazardly chosen throughout the population to maximize the collection of seeds from different genetic individuals. Local government representatives were informed of the research, and no destructive sampling was conducted.

The research area is located in a temperate climatic zone with a transitional type of climate resulting from the interaction of both maritime and conti-

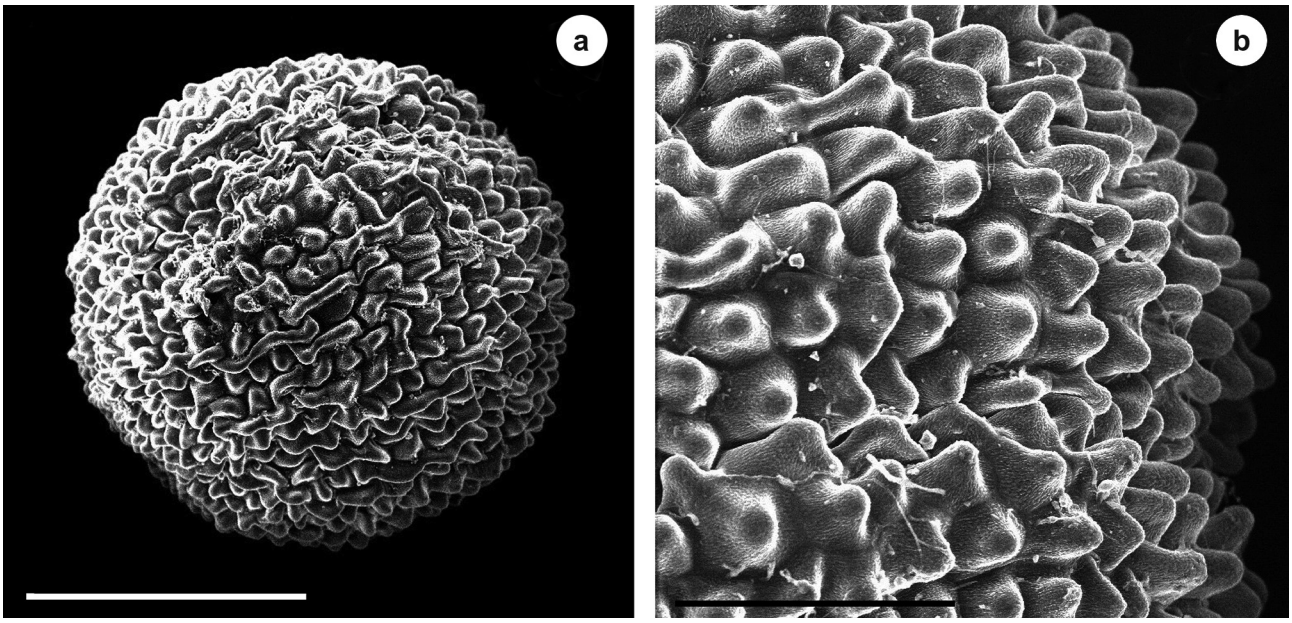

Fig. 1. Scanning electron micrographs of a *Galium cracoviense* seed. (**a**) entire mericarp (Bar = 500 μm), (**b**) enlargement of mericarp coat (Bar = 200 μm).

nental air masses. The seasons are clearly differentiated. Meteorological data (obtained from a meteorology station 15 km away, the Institute of Meteorology and Water Management, Poland), based on 39 years of records (1961–2000), indicated that the mean annual temperature was 8°C, the highest summer temperature 35.5°C, the lowest winter temperature – 26.7°C, the mean temperature of the warmest month (July) 17.8°C and the mean temperature of the coldest month (January) – 2.5°C. Annual precipitation (rain and snow) was 637 mm, and the annual number of frost-free days was 284 days. The mean monthly (1961–2000) maximum and minimum temperatures at the nearest meteorological station Częstochowa are shown in Figure 2.

OVERVIEW OF THE EXPERIMENTS

The seeds were dried for 10 days and after cleaning stored in paper bags [relative humidity (RH) 4060%] at 22.5 ± 1°C in the laboratory prior to the start of the germination experiment or of the stratification treatment. Three laboratory experiments were conducted to assess the effect of (1) the incubation temperature and light conditions (2) cold stratification and (3) the concentration of Ca^{2+} ions on germination.

THE EFFECT OF INCUBATION TEMPERATURE AND LIGHT CONDITIONS ON GERMINATION OF FRESH SEEDS

This germination experiment was performed using fresh seeds (14 days after harvest). Four 25-seed replicates were surface-sterilised with 1% sodium hypochlorite and then placed in 5-cm diameter plastic Petri dishes with four filter paper discs moistened with distilled water until saturated. To find out whether *G. cracoviense* seeds are dormant, a batch of seeds was sown 14 days after harvesting (fresh seeds). To assess the effect of light and temperature on germination, four Petri dishes were wrapped in aluminium foil (dark treatment) and four dishes were sealed with parafilm (light treatment). Light treated dishes were placed on top of dark treated ones and placed in a room with one of three constant temperature regimes: 5, 10, or 22°C

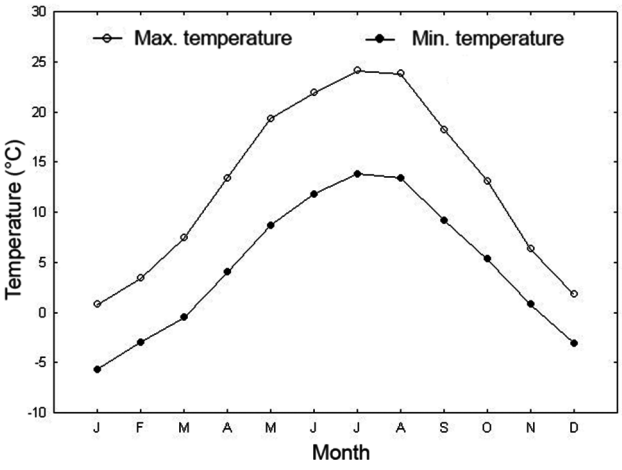

Fig. 2. Mean (1980–2000) minimum and maximum temperatures at the Częstochowa meteorological station.

TABLE 1. Germination percentages (mean ± S.D.) of the non-treated (fresh) and 12-weeks cold-stratified seeds of *Galium cracoviense* at three temperatures (5, 10 and 22°C) in light or in darkness. Each value is a mean ± S.D. of four replicates of 25 seeds.

Light condition	Temperature	Non-treated seeds*	12 week cold-stratified*
Light	5°C	56 ± 4^{Ab}	2 ± 2^{Bc}
	10°C	67 ± 5^{Ab}	44 ± 4^{Bb}
	22°C	84 ± 6^{As}	69 ± 3^{Ba}
Dark	5°C	32 ± 4^{Ac}	1 ± 1^{Bc}
	10°C	39 ± 3^{Ac}	24 ± 4^{Bb}
	22°C	80 ± 7^{Aa}	61 ± 2^{Ba}

*different upper-case letters indicate significant differences by Student's t-test in the germination percentages between the non-treated (fresh) and 12-weeks cold-stratified seeds at the same temperature and different lower-case letters in each column with the same light condition indicate significant differences by ANOVA followed by Tukey's test at $P < 0.05$ in the germination percentages of the seeds among different temperatures

TABLE 2. Index of germination velocity of the non-treated and after 12-weeks cold-stratified seeds of *Galium cracoviense* at three temperatures (5, 10 and 22°C) in light or in darkness. Each value is a mean ± S.D. of four replicates of 25 seeds.

Light condition	Temperature	Non-treated*	12 weeks cold-stratified*
Light	5°C	23 ± 1^{Ac}	1 ± 1^{Bc}
	10°C	33 ± 1^{Ab}	17 ± 2^{Bb}
	22°C	54 ± 2^{Aa}	49 ± 3^{Ba}
Dark	5°C	8 ± 1^{Ad}	0 ± 0^{Bc}
	10°C	19 ± 1^{Ac}	9 ± 2^{Bb}
	22°C	44 ± 5^{Aa}	41 ± 1^{Ba}

*different upper-case letters indicate significant differences by Student's t-test between the germination rate of the non-treated and 12-weeks cold-stratified seeds at the same temperature and different lower-case letters in each column with the same light condition indicate significant differences by ANOVA followed by Tukey's test at $P < 0.05$ in the germination percentages of seeds among different temperatures

in light (with alternating 12 h light/dark regimes; 3.000 lux, Philips 35 W/33 lamps). These thermoperiods represent the mean daily maximum and minimum monthly temperatures at the Częstochowa Weather Station during the growing season: 4.8°C (early April and October), 10.3°C (late May), 21.5°C (June and early July), when most seeds germinate in the natural habitat (Figure 1). Germinated seeds were counted daily, in the ease of dark treatments dim green safe light was used (Amaral-Baroli and Takaki, 2001; see also Simao et al., 2010). Germination was defined as radicle or hypocotyls emergence visible without magnification. After twenty three days, the seeds were no longer germinating, so all germinated seedlings were removed.

The rate of germination was estimated using a modified Timson's index of germination velocity G/t, where G is seed germination percentage each day and t the total germination period (Khan and Ungar, 1997). Therefore if all of the seeds germinated in one day, the Timson's index would be 100 (i.e. 2300/23). A higher value indicates more rapid germination.

THE EFFECTS OF TEMPERATURE AND LIGHT ON THE GERMINATION OF 12-WEEK COLD STRATIFIED SEEDS

Four 25-seed replicates were placed on filter paper, wrapped in aluminium foil and placed in a refrigerator in the dark for 12 weeks at 5°C. This temperature is near-optimal for many seeds requiring low moisture and low temperature to break dormancy (Stokes, 1965). After treatment, the seeds were surface-sterilised with 1% sodium hypochlorite and then transferred under green light to 5-cm diameter plastic Petri dishes with four filter paper discs moistened with distilled water until saturated. The germination conditions were the same as those described in Experiment 1 with fresh seeds. Germination was monitored daily and recorded as before for 23 more days. The rate of germination was also determined using Timson's index of germination velocity as described in Experiment 1.

THE EFFECTS OF CALCIUM CARBONATE ON GERMINATION AND RECOVERY

This germination experiment commenced 14 days after harvesting. To test the effects of Ca^{2+} ions on germination, four replicates of 25 surface-sterilised seeds were incubated in 0 (distilled water control), 1, 5, 10, 15, 20, or 25 (mM/L) $CaCO_3$ concentration at 22°C in light (16 h-photoperiod, 3.000 lux, Philips 35 W/33 lamps) for 23 days. Preliminary experiments showed that a constant 22°C was found to be optimal for germination of fresh seeds in both light and dark conditions. After twenty three days, the seeds were no longer germinating, so all the germinated seedlings were removed and their hypocotyl and root lengths measured. Non-germinated seeds from all the treatments were then transferred to distilled water and exposed to 22°C again to measure the recovery of seed germination, which was recorded daily for ten days. The recovery percentage was calculated using the following formula: $[(A - B)/(C - B)] \times 100$, where A is the number of seeds that germinated in salt solution plus those that recovered to

TABLE 3. Three-way ANOVA of effects of temperature (T), light condition (L, light vs. darkness), cold stratification (CS, non-treated vs. 12-week cold-stratified) seeds of *Galium cracoviense* and their interactions on seed germination and rate of germination.

Dependent variable	Factor	d.f.	SS	MS	F-value	P-value
Germination (%)	Light (L)	1	1.55	1.55	69.67	< 0.001
	Temperature (T)	2	64.29	32.14	1443.41	< 0.001
	Cold stratification (CS)	1	16.01	16.01	719.17	< 0.001
	L × T	2	4.013	2.00	90.11	< 0.001
	L × CS	1	0.02	0.02	1.01	0.320
	T × CS	2	15.00	7.50	336.91	< 0.001
	L × T × CS	2	0.41	0.20	9.37	< 0.001
Rate of germination	Light (L)	1	3.19	3.19	64.30	< 0.001
	Temperature (T)	2	60.89	30.44	613.18	< 0.001
	Cold stratification (CS)	1	19.35	19.35	389.74	< 0.001
	L × T	1	0.75	0.37	7.64	< 0.001
	L × CS	1	0.05	0.05	1.04	0.315
	T × CS	2	19.17	9.58	193.13	< 0.001
	L × T × CS	2	0.12	0.06	1.22	0.308

germinate in distilled water (pH 5.7). B is the number of seeds germinated in salt solution and C is the total number of seeds tested (Gul and Weber, 1999). Final germination was recorded as (A/C) × 100 (Wang et al., 2008).

The non-germinated seeds were tested for viability using the tetrazolium test (Grabe 1970). The seeds were kept in water for 16 h at 25°C and then they were submerged in a 1% aqueous solution of 2,3,5-triphenyl-tetrazolium chloride, pH 7, in darkness for 24 h at 25°C. Subsequently, the seeds were dissected and embryos were analysed under a magnifying glass (Bradbeer, 1998). Seed viability was expressed as [(A + D)/C] × 100, where D is the number of embryos that stained pink in the TTC solution following the seed germination test (Wang et al., 2008).

DATA ANALYSIS

All data were expressed as mean ± S.D. Prior to data analysis, the percentage of germination, germination velocity, hypocotyl and root lengths data were arcsine square root transformed to the correct sample heterogeneity (non-transformed data appear in all figures). The data were tested for normality with the Kolmogorov-Smirnov test with the Lilliefors correction and homogeneity of variance with the Brown-Forsythe test. When significant differences were found among means, a Tukey's multiple comparison post hoc test (HSD-test) was carried out to determine if significant ($P < 0.05$) differences occurred between individual treatments. Light and dark treatments and each incubation temperature (4, 10, or 22°C) were analyzed separately. A Student's t-test was carried out to determine the differences among treatment group means for percent germination and rate of germination. Three-way ANOVA of effects of incubation temperature (T), 'light-level' (L, light and darkness), stratification (S, before and after stratification) and their interaction on seed germination and rate of germination were conducted. Statistical analysis was carried out using Statistica v. 10 (Statsoft. Inc., 2011).

RESULTS

GERMINATION RESPONSE TO LIGHT AND TEMPERATURE

Fresh seeds of *G. cracoviense* have fully developed embryos and are non-dormant; they can germinate directly after dispersal. Among the seeds incubated in light the highest germination percentage (84 ± 6%) was observed when they were germinated at 22°C followed by 10 and 5°C when the germination percentage was 67 ± 5% and 56 ± 4%, respectively. A similar tendency was observed among the seeds incubated in darkness although low temperature inhibited germination to a greater extent, namely 22°C – 80 ± 7%, 10°C – 39 ± 3%, 5°C – 32 ± 4% (Tab. 1, Figs. 3a–c). Germination velocity (Timson's Index) was significantly higher when more light and higher temperatures were given ($P < 0.01$) (Tab. 2).

A three-way ANOVA showed that the cumulative germination percentage and germination velocity were significantly affected by the light conditions ($P < 0.001$), temperature ($P < 0.001$) and their interaction ($P < 0.001$) (Tab. 3).

GERMINATION AFTER THE STRATIFICATION TREATMENT

Overall, germination was found in both light and dark conditions to be significantly higher before stratification ($P < 0.01$). The germination in light was considerably higher at low temperatures compared with that in darkness, while at 22°C it was slightly higher ($P > 0.05$) (Tab. 1).

The germination percentage of the seeds after stratification treatment was significantly different to that of the from the non-treated ones at the same temperature ($P < 0.01$) (Tab. 1, Figs. 4a–c). The seeds showed a clear reduction of germination in response to cold stratification when tested at 5, 10, or 22°C. For example, the cumulative germination of the non-treated seeds was 56 ± 4% at 5°C in light conditions, whereas only 2 ± 2% in the seeds after 12 weeks stratification at this regime (Fig. 4A). In addition, differences between germination in light and darkness were larger after stratification than in the seeds tested after two weeks of dry-storage (Tab. 1, Figs. 4a–c).

At 5 and 10°C, the germination velocity of the non-treated seeds in both light and darkness was significantly higher than that of cold-stratified ones, but at 22°C, their parameter values were similar (Tab. 2). For example, at 5 or 10°C in light, the germination index of the non-treatment seeds was 23 ± 1 and 33 ± 1, respectively, whereas for the cold-stratified ones it was only 1 ± 1 and 17 ± 2, respectively (Tab. 2).

A three-way ANOVA showed that germination was significantly affected by light ($P < 0.001$), temperature ($P < 0.001$), cold stratification ($P < 0.001$) and their interactions ($P < 0.001$) because the cumulative germination percentage of the non-treated seeds was significantly higher than that of 12-week cold-stratified ones in the same light conditions and temperature. In addition, a three-way ANOVA showed that the germination velocity was significantly affected by light ($P < 0.001$), temperature ($P < 0.001$), cold stratification ($P < 0.001$) and the interaction between light and temperature ($P < 0.001$) as well as by the interaction between temperature and cold stratification. However, the interaction of light and cold stratification was non-significant ($P > 0.05$) (Tab. 3).

EFFECTS OF CALCIUM CARBONATE ON GERMINATION AND RECOVERY

Calcium carbonate showed a significant inhibitory effect on seed germination ($P < 0.05$) (Tab. 4). Maximum seed germination occurred in the non-saline control treatment. The seeds treated with a lower concentration of $CaCO_3$ (1 mM) also showed a high percentage of germination, slightly lower than that of the distilled water control. Four lower-salt-concentration-treatment groups (1, 5, 10, or 15 mM $CaCO_3$) did not differ from one another, but did dif-

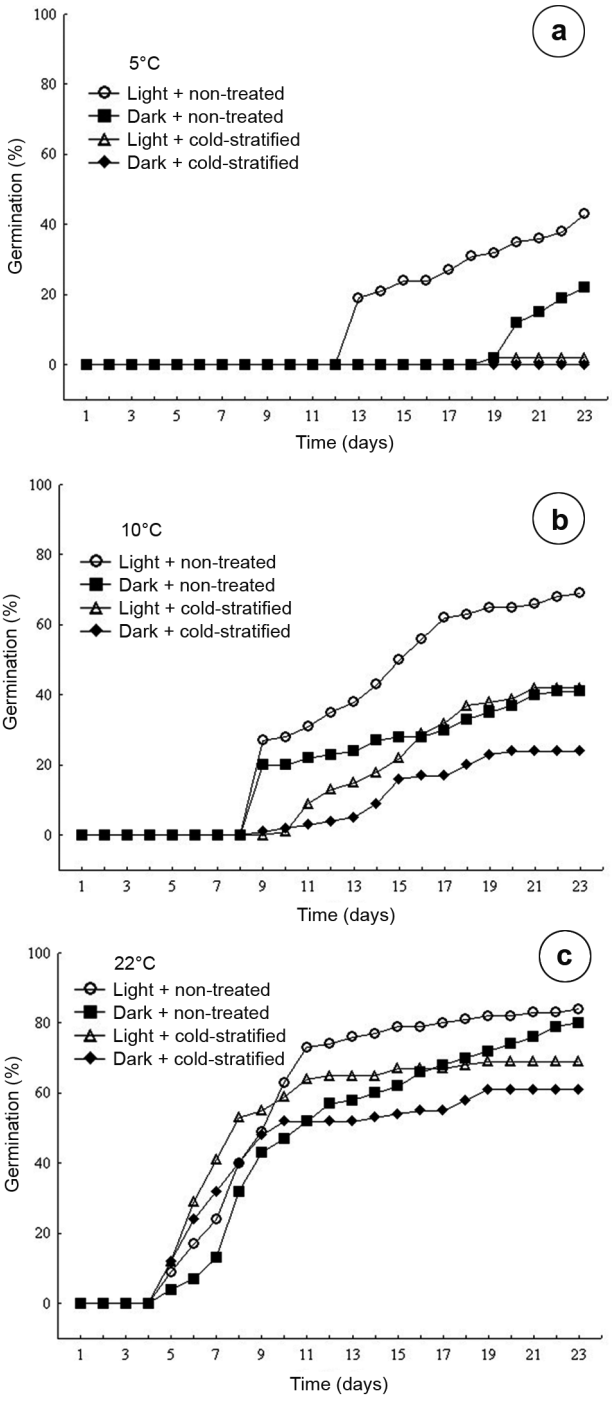

Fig. 3. The cumulative germination percentages of the non-treated (fresh) and 12-week cold-stratified seeds of *Galium cracoviense* incubated at 5°C (**a**), 10°C (**b**) and 22°C (**c**) underdifferent light conditions for 23 days. Each value is a mean of four replicates of 25 seeds.

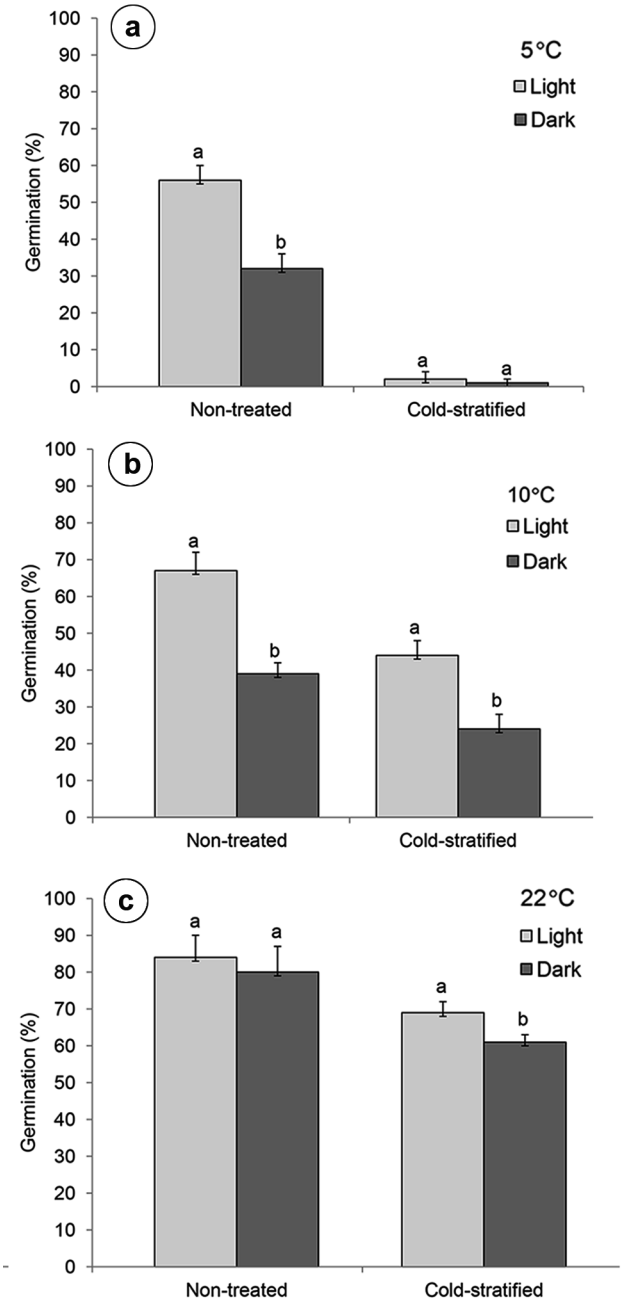

Fig. 4. The effects of temperature and light on the germination of non-treated and 12-week cold-stratified seeds of *Galium cracoviense*. Different lower-case letters indicate significant differences in germination percentages of the same treatment between light and dark at 5°C (**a**), 10°C (**b**) and 22°C (**c**) for 23 days (Student's t-test, $P < 0.05$). Each value is a mean of four replicates of 25 seeds.

fer from the highest salt concentrations (20 and 25 mM $CaCO_3$). Under the highest salt concentration, 25 mM $CaCO_3$, only 51% of the seeds germinated. The highest rate of germination was observed at 1 mM $CaCO_3$, whereas the lowest at 25 mM $CaCO_3$ concentration.

Germination recovery differed significantly among the seeds transferred from 1-25 mM $CaCO_3$ concentrations to deionized water ($P < 0.05$). Final germination percentages were higher in distilled water than at any $CaCO_3$ concentration. However, seed viability was not affected by salt concentration (Tab. 4).

Overall, root growth (radicle length) in the distilled water control was usually lower than that at all $CaCO_3$ concentrations. The best root growth was obtained at the 10 mM $CaCO_3$ concentration. However, above 10 mM $CaCO_3$ concentration no significant changes ($P > 0.05$) in radicle lengths were observed. There was no significant effect of $CaCO_3$ concentration on hypocotyl length ($P > 0.05$). Average hypocotyl length after 23 days varied between 2.5 mm in distilled water and 2.8 mm at 10 mM $CaCO_3$ concentration (Tab. 5).

DISCUSSION

Our results show that fresh seeds of *G. cracoviense* are highly permeable to water, have fully developed embryos and do not pass through seed dormancy immediately after dispersion in July. They can germinate in the field with the first rains of summer.

According to the classification system proposed by Mayer and Poljakoff-Mayber (1989), seeds can be divided into three broad groups based on their response to light: (1) those that require light for germination; (2) those in which germination is inhibited by light; and (3) those in which light has no effect on germination. It was shown that the germination percentage and rate of germination of *G. cracoviense* seeds were significantly higher in light than in darkness at all temperatures tested, indicating that a considerable portion of the seed population is light sensitive. This characteristic of *G. cracoviense* is similar to that of *G. mollugo* (Mersereau and DiTomasso, 2003). The light requirement for the germination of *G. cracoviense* seeds ensures that they will germinate successfully on or near the soil surface and in cracks and crevices in limestone, when temperature and edaphic conditions are favourable. Baskin and Baskin (1998) suggested that on the soil surface seeds were exposed to light and improved oxygen levels, and that promoted germination. A light requirement for germination is frequently associated with small seeds (Taylorson, 1987) which are considered to contain rather small amounts of reserve materials (Wang et al., 2008) and to germinate close to the soil surface in vegetation gaps (Probert, 2000), and this is also true for the species studied. A tendency to retard germination in darkness is observed in species with small

TABLE 4. The effect of $CaCO_3$ concentration on germination and germination recovery in distilled water of *Galium cracoviense* seeds. Each value is a mean ± S.D. of four replicates of 25 seeds.

$CaCO_3$ (mM/L)	Initial germination (%)*	Germination rate (%)*	Recovery percentage (%)*	Final germination (%)*	Total viable seeds (%)*	Viable non-germinated seeds (%)*
0	85 ± 3^{a}	54 ± 2^{b}	25 ± 25^{d}	85 ± 3^{a}	100 ± 0^{a}	15 ± 1^{a}
1	77 ± 2^{b}	67 ± 1^{a}	22 ± 7^{d}	82 ± 2^{a}	100 ± 0^{a}	18 ± 2^{a}
5	73 ± 2^{bc}	60 ± 4^{b}	26 ± 11^{d}	79 ± 3^{a}	100 ± 0^{a}	21 ± 2^{a}
10	71 ± 2^{bc}	59 ± 1^{b}	28 ± 10^{d}	84 ± 3^{a}	100 ± 0^{a}	16 ± 2^{a}
15	70 ± 2^{c}	58 ± 1^{b}	36 ± 9^{c}	81 ± 2^{a}	100 ± 0^{a}	19 ± 2^{a}
20	62 ± 2^{c}	54 ± 1^{c}	52 ± 1^{b}	80 ± 2^{a}	100 ± 0^{a}	20 ± 2^{a}
25	51 ± 2^{d}	44 ± 1^{d}	65 ± 7^{a}	83 ± 3^{a}	100 ± 0^{a}	17 ± 3^{a}

*the values followed by the same letter within a column do not differ significantly by ANOVA followed by Tukey's test at $P < 0.05$

seeds, which is explained as a mechanism to avoid fatal germination at a depth too great for the seedling to reach the surface (Milberg et al., 2000). Because *G. cracoviense* seeds in complete darkness exhibit enhanced germination after exposure to moderate temperatures (at 22°C) this could explain why germination occurs in shaded areas such as holes, cracks or crevices in the rock face accumulating water and sediments that allow seed germination and seedling development.

There is no data available on seed banks or the seed viability of this species. It was shown in this study that cold stratification reduced germination and limited the rate of the germination of its seeds. Thus, it seems that this species may be expected to form a transient seed bank lasting only until spring, with all viable seeds germinating at that time.

In our experiments the temperatures were, on average, equal to the air temperature recorded close-by at a meteorological station; the monthly averages for the daily maximum air temperatures (early April and October 4.8°C; late May 10.3°C; June and early July 21.5°C) give a useful indication of the temperature which will be reached at the ground surface in a sward on most days. The present study showed that temperature was an important factor regulating the germination of *G. cracoviense* seeds. As the temperature rose from 5 to 22°C, the germination percentage and germination velocity of fresh seeds increased, which means that temperature is not a limiting factor for germination from spring until autumn. We did not test storage temperatures higher than 22°C. As the temperature near Olsztyn (in southern Poland) often exceeds 30°C (higher than in the experiment) it can accelerate germination. On the other hand, at this time of year (early summer), soil temperatures are significantly higher than the maximum temperature favouring germination and therefore seeds do not germinate. Thus, the germination of seeds mainly depends on moisture conditions in the field. Amen (1966) indicated that seeds of alpine species have a high germination capacity immediately after dispersal at high temperatures. This agrees with the results of Shimono and Kudo (2005), Giménez-Benavides et al. (2005), Estrelles et al. (2010) and with our own results for *G. cracoviense*. In both light and dark conditions, seeds of *G. cracoviense* germinated > 50% only at 22°C.

Cold-stratification, generally in the range of 1–10°C, is effective for breaking seed dormancy in a number of species (Bewley and Black, 1994). Cold-stratification at 5°C is known to break dormancy in *G. aparine* L. (Slade and Causton, 1979), *G. tricornutum* Dandy (Chauhan et al., 2006), *G. spurium* (L.) Simonkai (Royo-Esnal, 2010) and in *G. spurium* var. *echinospermon* (Masuda and Washitani, 1992). However, when non-stratified seeds were exposed to temperatures unfavourable for germination, they cycled back into a dormant state, in a process called 'secondary' dormancy

TABLE 5. The effect of $CaCO_3$ concentration on radicle and hypocotyl lengths of *Galium cracoviense* at 22°C in light after 23 days of incubation. Values are means ± S.D.

$CaCO_3$ (mM/L)	Radicle length (mm)*	Hypocotyl length (mm)*
0	5.8 ± 0.09^{e}	2.5 ± 0.12^{a}
1	8.2 ± 0.08^{c}	2.4 ± 0.07^{a}
5	8.8 ± 0.11^{b}	2.5 ± 0.08^{a}
10	9.7 ± 0.14^{a}	2.8 ± 0.12^{a}
15	6.8 ± 0.07^{d}	2.7 ± 0.02^{a}
20	7.0 ± 0.12^{d}	2.6 ± 0.05^{a}
25	6.9 ± 0.05^{d}	2.7 ± 0.14^{a}

*the values followed by the same letter within a column do not differ significantly by ANOVA followed by Tukey's test at $P < 0.05$

induction (Baskin and Baskin, 1998; Nordborg and Bergelson, 1999; Rubio de Casas et al., 2012) and our results showed the same tendency for *G. cracoviense*. Thus, when the seeds of *G. cracoviense* are exposed to low (5°C) winter temperatures, they enter (secondary) conditional dormancy, losing the ability to germinate at low but not at high temperatures. Winters are severe in this region, so freezing usually causes a secondary dormancy. Seeds that do not germinate in summer and autumn remain in the soil seed bank over the winter and may germinate the following spring as a second flush. Thusgermination can start at 10°C (April), depending on the combination of temperature and rainfall. Since its seeds do not germinate in early spring this species behaves as summer annuals or spring ephemerales. Other *Galium* species exhibit strong seed dormancy. Since stratified seeds germinated well at moderate (22°C) temperature, *G. cracoviense* might be expected to establish dense populations even when the seeds are dispersed in fields abandoned in early or late summer (Willemsen, 1975).

In alkaline soils Ca^{2+} is the dominant cation. Thus, the germination percentage as affected by Ca^{2+} salts is therefore of particular interest (Ryan et al., 1975). *Galium cracoviense* percentage of germination generally decreased as Ca^{2+} salt concentration increased, i.e. germination was reduced from 85% in distilled water (pH 5) to 51% in the 25 mM $CaCO_3$ solution. The decreased germination at the higher Ca^{2+} salt concentration might have been due to reduced seed water imbibition needed for germination. Kumar et al. (2009) observed the same effect in seeds of *Jatropha curcas* L. Under Ca^{2+} stress, high environmental pH is a main factor that affects root growth and development during the life cycle of a plant (Liu and Guo, 2011). As Hepler pointed out (2005), Ca^{2+} inhibited shoot growth and promoted root growth. In this study the increase in calcium concentration had no effect on hypocotyl development but promoted root growth. It is not known whether such roots are actively involved in the transport of ions into a plant. However in the present study, the root system was found to be confined to the surface layers in calcareous habitats. Although evaporation following precipitation decreases soil water potential, seedlings of *G. cracoviense* can develop in solutions with high concentrations of $CaCO_3$.

The data obtained in this investigation with *G. cracoviense* indicate that Ca^{2+} stress delays germination but neither induces dormancy nor kills the seeds. Under high saline conditions, seed survival rather than germination is a more appropriate mechanism for plants to establish successfully, because the recovery of germination occurs when high salinity is alleviated (Guan et al., 2009). Thus, for the successful germination of *G. cracoviense* seeds, a high Ca^{2+} concentration on the soil surface and at shallow soil depths needs to be diluted by precipitation or by melt water from snow.

In the present experiments, non-germinated seeds of *G. cracoviense* germinated well after they were transferred from high salt solutions to distilled water. Germination recovery increased gradually with increase in pre-treatment salt solution concentration and was quite high for the seeds pre-treatment in 25 mM $CaCO_3$ solution, which is near the molarity of a saturated solution of $CaCO_3$. Thus, the seeds of *G. cracoviense* are well adapted to alkaline habitats via their high capacity for germination recovery.

Germination characteristics of endemic species are very similar to geographically widespread members of the same genus (Baskin, Baskin 1988). This is also the case for *G. cracoviense* and more widely distributed *G. mollugo* (both polycarpic perennials). Bakker et al. (1985) demonstrated that seeds of *G. mollugo* germinated to nearly 70% at 20°C, but that germination totals were slightly lower at 10°C (65%). These results are similar to those reported by Kolk (1962) who pointed out that fresh seeds germinated best at 22°C and that the germination percentages were reduced by lower as well as higher constant temperatures.

Little is known about the mechanisms of this species' seed dispersal. One possible mechanism might be anemochory, taking advantage of strong air currents characteristic of rock faces on which this species lives. Furthermore, seeds collected by granivorous ants may remain underground in nest chambers after the colony dies or moves to a new nest site along ant trails.

CONCLUSIONS

The present study demonstrated that the ex situ propagation of *G. cracoviense* was possible from seeds. To germinate seeds of this species require moderate temperatures and the presence of light. Germination occurs from late spring to early summer when the temperature window is suitable and $CaCO_2$ content in soil is low. This species may not be tolerant to extreme $CaCO_2$ content in soil during germination but is highly tolerant during storage in soil. *Galium cracoviense* seeds are non-dormant at maturity and can be induced into dormancy by low temperatures. Hence, seeds dispersed in the previous growing season that do not germinate in autumn remain in the soil seed bank over the winter and may germinate the following spring as a second flush.

The obtained results seem to highlight the sexual reproductive ability of *G. cracoviense*. This indicates that its conservation problems are not

due to agents related to its reproductive biology, but mostly to other agents, such as the abandonment of traditional pastoral systems, recreational activities and ecological succession. The germination requirements found in this study will be useful for the future ex situ conservation of *G. cracoviense*.

AUTHORS' CONTRIBUTIONS

JK designed experiments, analyzed data, wrote the manuscript, and revised the manuscript. JP performed experiments. The authors declare that they have no conflicts of interest.

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