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Campanulaceae: a family with small seeds that require light for germination

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• *Background and Aims* The Campanulaceae is a large cosmopolitan family, but is understudied in terms of germination, and seed biology in general. Small seed mass (usually in the range 10–200 μg) is a noteworthy trait of the family, and having small seeds is commonly associated with a light requirement. Thus, the purpose of this study was to investigate the effect of light on germination in 131 taxa of the Campanulaceae family, from all five continents of its distribution.

• *Methods* For all taxa, seed germination was tested in light (8 or 12 h photoperiod) and continuous darkness under constant and alternating temperatures. For four taxa, the effect of light on germination was examined over a wide range of temperatures on a thermogradient plate, and the possible substitution of the light requirement by gibberellic acid and nitrate was examined in ten taxa.

• *Key Results* For all 131 taxa, seed germination was higher in light than in darkness for every temperature tested. Across species, the light requirement decreased significantly with increasing seed mass. For larger seeded species, germination in the dark reached higher levels under alternating than under constant temperatures. Gibberellic acid promoted germination in darkness whereas nitrates partially substituted for a light requirement only in species showing some dark germination.

• *Conclusions* A light requirement for germination, observed in virtually all taxa examined, constitutes a collective characteristic of the family. It is postulated that smaller seeded taxa might germinate only on the soil surface or at shallow depths, while larger seeded species might additionally germinate when buried in the soil if cued to do so by fluctuating temperatures.

Key words: Campanulaceae, germination, light requirement, seed mass, constant vs. alternating temperatures, gibberellic acid, nitrate.

INTRODUCTION

Seed responses to light can control the timing of germination in the field, impacting seedling survival, as well as growth and fitness in subsequent life stages (Pons, 2000). Seeds requiring light for germination are usually small in size (Pons, 2000; Fenner and Thompson, 2005). Milberg *et al.* (2000) suggested that a light response and seed mass coevolved as an adaptation to ensure germination of small-seeded species only when close to the soil surface. On the other hand, a phylogenetic component of light-promoted germination – regardless of seed size – has been postulated (Fenner and Thompson, 2005).

Temperature is a major factor modulating seed responses to light: a seed may require light to germinate at a given temperature but not at other temperatures (Pons, 2000). Moreover, for some species, temperature fluctuations can fully or partially substitute for the light requirement (Baskin and Baskin, 1998). The amplitude of soil temperature fluctuations is highest on or close to the surface of bare soil and in vegetation gaps (Probert, 2000; Daws *et al.*, 2002).

Phytochromes are well known to mediate light-promoted germination; they are also known to increase the amount of bioactive gibberellins in seeds (Bewley *et al.*, 2013). Thus, exogenously applied gibberellins promote germination of photorequiring seeds in darkness. Conversely, nitrates which are naturally

occurring in the soil, can also substitute for the light requirement in some cases (Hilhorst and Karssen, 2000; Daws *et al.*, 2002).

The Campanulaceae *sensu lato* (APG III, 2009) is the 26th largest plant family (Stevens, 2001 onwards) and comprises 85 genera (including *Halacsyella*; Stefanović *et al.*, 2008) and approx. 2300 species (Lammers, 2007a). It is divided into five sub-families following morphological data: Lobelioideae, Campanuloideae, Cyphioideae, Nemacladoideae and Cyphocarpoideae (Brummitt, 2007; Lammers, 2007a). Campanulaceae is cosmopolitan in distribution; taxa are most often herbaceous perennials that form capsular fruits with usually numerous small seeds (Lammers, 2007b). Ecologically, the family is extremely diverse, occurring in a variety of habitats except the major deserts. Though exceptions are numerous, there is a discernible trend among Campanuloideae for more open habitats, while many Lobelioideae tend to be associated with forested areas (Lammers, 2007b).

The Campanulaceae is highly understudied in terms of germination. The effect of light on the induction of seed germination has been studied for just 20 species, spanning ten genera (Brightmore, 1968; Linhart, 1976; Baskin and Baskin, 1979, 1984; Grime *et al.*, 1981; Farmer and Spence, 1987; Lesica, 1992; Mariko and Kachi, 1995; Teketay and Granström, 1997; Morgan, 1998; Teketay, 1998; Bachmann *et al.*, 2005; Baskin *et al.*, 2005; Jankowska-Blaszczuk and Daws, 2007; Carta

et al., 2013). For all species tested to date, exposure to light promotes germination, except for *Howellia aquatilis* (Lesica, 1992).

In this study, we aim to determine the effect of light on germination for 131 taxa in the Campanulaceae. Specifically we (1) tested germination in light and darkness at constant and alternating temperatures; (2) associated the response to light with seed mass and alternating vs. constant temperatures; and (3) examined whether gibberellic acid and nitrate can substitute for the light requirement. Although the Campanulaceae representatives studied here were collected across a broad range of habitats globally, the majority of seed samples were collected from Europe and predominantly Greece (50 taxa). From the biodiversity conservation point of view, it is noteworthy to mention that Campanulaceae is the family with the highest degree of endemism (54 %, approx. 63 taxa) in Greece (Georghiou and Delipetrou, 2010).

MATERIALS AND METHODS

Seed material

Seeds of 131 taxa of Campanulaceae were either collected specifically for this study or provided from seed banks and collaborators. Seed mass ranged between 5 and 1060 μg. The taxa are classified to 27 genera and three subfamilies, and are native to all five continents of distribution: Africa (four taxa), America (26 taxa), Asia (13 taxa), Europe (75 taxa), Oceania (13 taxa). The vast majority of seed collections (121 out of 131) derive from wild-growing populations and the remainder from botanical gardens. Information on these 131 taxa is presented in Table 1. Germination experiments were carried out at least 2 months after seed collection, which would have satisfied any after-ripening requirements. Seeds were stored initially at room temperature and then, after a few months, at 10 °C with silica gel. Seed samples obtained from seed banks were also stored at 10 °C with silica gel.

Effect of light on germination

The role of light was examined at constant and alternating temperatures with daily illumination of 8 or 12 h per day (light treatment) and in continuous darkness (dark treatment). The temperatures presented in Table 1 correspond either to the single temperature applied or to the optimal temperature (among several tested); the experiments were planned depending on seed availability. Only one constant or alternating temperature was tested in 19 and 37 taxa, respectively. Light exposure in alternating temperatures regimes coincided with the period with the elevated temperature. Seeds were placed in Petri dishes containing either 1 % agar or filter paper wetted with distilled water. To ensure there was no effect of germination media on germination results, a preliminary experiment was conducted on both media using *Campanula andrewsii* subsp. *andrewsii* and *C. drabifolia*. The results of these tests were not statistically different. There were five samples of 20 seeds each for the light treatment and one sample with 100 seeds for the dark treatment. The single sample used in the dark was due to experimental space limitations and, mainly, to the difficulties in handling these tiny seeds in complete darkness. Seeds germinated in the light treatment were counted and removed regularly. For incubation in darkness, seeds were sown in the dark, then Petri dishes were wrapped in double aluminium foil and placed in metal boxes or black plastic bags; seed germination was scored with a single final measurement, after no further germination had been observed for at least 1 week in the concurrent experiment with seeds in the light. Germination percentages were corrected for viable seeds, based on post-experiment cut-tests and morphological examinations of the embryo. For the majority of the taxa studied, empty seeds constituted less than approx. 10 % of each seed sample, except for three taxa (4, 13 and 41; Table 1), probably a result of poor seed quality.

For each species, we derived an index of light requirement for germination at constant temperatures (photo-requirement germination index; PGI) such that:

$$\text{PGI}_{\text{const}} = 1 - \left(\text{FG}_{\text{Dconst}}/\text{FG}_{\text{Lconst}}\right) \tag{1}$$

where $\text{FG}_{\text{Dconst}}$ is the percentage germination at constant temperatures in the dark and $\text{FG}_{\text{Lconst}}$ is the percentage germination at constant temperatures in the light.

The PGI results in values between 0 and 1: a value of 0 corresponds to the germination percentage being equal in both light and darkness, while a value of 1 corresponds to germination only occurring in the light. A relative light germination (RLG) index has also been used previously (e.g. Milberg *et al.*, 2000; Jankowska-Blaszczuk and Daws, 2007), and also results in values of 0–1. However, RLG index values from 0 to <0.5 refer to the situation where germination in the dark is greater than in the light. This situation did not occur for any of the species tested in the current study. The PGI had the advantage of resulting in a larger spread of values. However, RLG values were also calculated and compared with the PGI, and resulted in quantitatively similar overall results (data not shown).

The PGI was also calculated using germination at alternating temperatures in the dark and constant temperatures in the light. This enabled the hypothesis that alternating temperatures are able to substitute for a light requirement in dark conditions to be tested. Thus:

$$\text{PGI}_{\text{alt}} = 1 - \left(\text{FG}_{\text{Dalt}}/\text{FG}_{\text{Lconst}}\right) \tag{2}$$

where FG_{Dalt} is the percentage germination at alternating temperatures in the dark and $\text{FG}_{\text{Lconst}}$ is the percentage germination in constant temperatures in the light.

Germination experiments on the two-way thermogradient plate

The effect of light at a wide range of constant and alternating temperatures was examined in seeds of four taxa (16, 22, 49 and 52; Table 1) on two thermogradient plates (Model GRD1, Grant Instruments, Cambridge, UK; Murdoch *et al.*, 1989). One thermogradient plate was set with a 12 h/12 h photoperiod and one in continuous darkness. Thermoperiod was set to 14 h day/10 h night, ranging from 5 to 35 °C, and seeds were illuminated 1 h after the onset of the day temperature. Seeds were placed in Petri dishes with 1 % agar (a total of 36 dishes per taxon). The dishes were arranged on the surface of the thermogradient plate in a 12 × 12 array. Thus, the 144 individual Petri dishes were arranged uniformly for the four taxa such that there were 36 different temperature combinations for each taxon. Twenty

TABLE 1. *Germination data, seed mass and country of provenance for the 131 taxa studied*

ID no.	Taxon	Constant			Alternating			PGI$_{const}$	PGI$_{alt}$	Seed mass (µg)	Country
		T	FG$_L$	FG$_D$	T	FG$_L$	FG$_D$				
1	*Adenophora remotiflora*	20	76	4				0·95		209	China
2	*Asyneuma chinense*	25	88	0				1·00		48	China
3	*Asyneuma giganteum*	10	93	77	20/10	92	53	0·17	0·43	330	Greece
4	*Asyneuma limonifolium* subsp. *limonifolium*	10	90	86	20/10	87	54	0·04	0·40	185	Greece
5	*Asyneuma pilcheri*	15	59	0	20/10	91	0	1·00	1·00	14	Greece
6	*Campanula aizoides*	5	19	0				1·00		203	Greece
7	*Campanula aizoon*	5	20	0				1·00		275	Greece
8	*Campanula albanica* subsp. *albanica*	5	48	0	20/10	1	0	1·00	1·00	87	Greece
9	*Campanula americana*	25	5	0	25/15	56	0	1·00	1·00	149	U.S.A.
10	*Campanula andrewsii* subsp. *andrewsii*	15	93	26	20/10	96	31	0·72	0·67	28	Greece
11	*Campanula asperuloides*	5	86	0	20/10	30	0	1·00	1·00	19	Greece
12	*Campanula barbata*	25	80	0	25/15	87	0	1·00	1·00	61	France
13	*Campanula calaminthifolia*	15	63	0	20/10	59	0	1·00	1·00	75	Greece
14	*Campanula camptoclada*	20	56	0	25/15	96	1	1·00	0·98	32	Jordan
15	*Campanula celsii* subsp. *carystea*	15	97	0	25/15	87	0	1·00	1·00	26	Greece
16	*Campanula celsii* subsp. *celsii*	15	87	0	25/15	87	1	1·00	0·99	24	Greece
17	*Campanula celsii* subsp. *parnesia*	10	100	0	20/10	87	0	1·00	1·00	26	Greece
18	*Campanula celsii* subsp. *spathulifolia*	15	99	0	20/10	99	0	1·00	1·00	27	Greece
19	*Campanula cervicaria*	15	100	59	20/10	100	75	0·41	0·25	55	France
20	*Campanula chalcidica*	25	67	0	25/15	16	0	1·00	1·00	21	Greece
21	*Campanula cochleariifolia*	15	67	0	25/15	86	0	1·00	1·00	63	France
22	*Campanula cretica*	15	99	24	20/10	97	7	0·76	0·93	104	Greece
23	*Campanula creutzburgii*	15	45	0	20/10	88	0	1·00	1·00	18	Greece
24	*Campanula cymaea*	15	85	1	25/15	85	4	0·99	0·95	65	Greece
25	*Campanula drabifolia*	15	90	0	30/20	91	0	1·00	1·00	9	Greece
26	*Campanula dulcis*	15	93	0	20/10	98	1	1·00	0·99	15	Saudi Arabia
27	*Campanula elatinoides*	15	80	0	20/10	71	0	1·00	1·00	51	Italy
28	*Campanula erinus*	15	97	0	20/10	99	0	1·00	1·00	11	Morocco
29	*Campanula formanekiana*	15	94	2				0·98		138	Greece
30	*Campanula garganica* subsp. *cephallenica*	15	44	0	25/15	33	0	1·00	1·00	27	Greece
31	*Campanula glomerata* subsp. *glomerata*	25	62	0	25/15	47	0	1·00	1·00	114	England
32	*Campanula goulimyi*	15	96	0	20/10	97	0	1·00	1·00	30	Greece
33	*Campanula hagielia*	15	97	0	20/10	98	0	1·00	1·00	25	Greece
34	*Campanula incurva*	15	98	0	20/10	85	3	1·00	0·97	58	Greece
35	*Campanula lanata*	15	84	0	20/10	86	0	1·00	1·00	21	Bulgaria
36	*Campanula latifolia* subsp. *latifolia*	10	96	10	20/10	94	25	0·90	0·74	201	Italy
37	*Campanula lingulata*	15	91	8	20/10	91	0	0·91	1·00	136	Greece
38	*Campanula lyrata* subsp. *lyrata*	15	97	0	25/15	84	0	1·00	1·00	27	Greece
39	*Campanula merxmuelleri*	25	99	0	25/15	99	0	1·00	1·00	33	Greece
40	*Campanula mollis*	15	98	0	25/15	94	0	1·00	1·00	12	Spain
41	*Campanula oreadum*	5	7	0				1·00		160	Greece
42	*Campanula pangea*	25	91	0	25/15	85	0	1·00	1·00	47	Greece
43	*Campanula patula*	20	3	0	25/15	46	2	1·00	0·33	20	Bulgaria
44	*Campanula pelviformis*	15	98	11	20/10	97	12	0·89	0·88	35	Greece
45	*Campanula peregrina*	20	74	0	25/15	82	0	1·00	1·00	23	Lebanon
46	*Campanula persicifolia* subsp. *persicifolia*	15	97	0	20/10	83	0	1·00	1·00	30	Greece
47	*Campanula prenanthoides*	5	53	0				1·00		77	USA
48	*Campanula punctata*	20	79	0	25/15	67	0	1·00	1·00	67	South Korea
49	*Campanula pyramidalis*	20	72	9	20/10	94	92	0·88	−0·28	136	Croatia
50	*Campanula raineri*	5	12	0				1·00		51	Italy
51	*Campanula ramosissima*	10	87	37	25/15	74	2	0·57	0·98	10	Greece
52	*Campanula rapunculoides*	15	100	0	20/10	100	46	1·00	0·54	207	Bulgaria
53	*Campanula rapunculus*	20	83	8	20/10	87	23	0·90	0·72	25	Jordan
54	*Campanula rhodensis*	20	47	0	20/10	74	1	1·00	0·98	15	Greece
55	*Campanula rhomboidalis*	5	19	0				1·00		72	France
56	*Campanula rotundifolia*	15	90	1	20/10	80	11	0·99	0·88	53	England
57	*Campanula sartorii*	15	63	0	20/10	62	0	1·00	1·00	56	Greece
58	*Campanula saxatilis* subsp. *saxatilis*	15	74	6	25/15	91	9	0·92	0·88	56	Greece
59	*Campanula scheuchzeri*	15	15	0	20/10	28	0	1·00	1·00	63	France
60	*Campanula sparsa*	15	96	12	20/10	95	3	0·88	0·97	19	Greece
61	*Campanula spatulata* subsp. *filicaulis*	15	75	8	20/10	89	9	0·89	0·88	30	Greece
62	*Campanula spatulata* subsp. *spatulata*	10	92	63	20/10	92	37	0·32	0·60	18	Greece
63	*Campanula spatulata* subsp. *spruneriana*	15	90	60	25/15	60	52	0·33	0·42	22	Greece
64	*Campanula spicata*	15	76	0	20/10	73	0	1·00	1·00	36	Italy
65	*Campanula strigosa*	5	12	0				1·00		59	Jordan
66	*Campanula thessala*	20	100	0				1·00		45	Greece
67	*Campanula thyrsoides* subsp. *thyrsoides*	20	97	0	25/15	92	1	1·00	0·99	114	France
68	*Campanula topaliana* subsp. *cordifolia*	15	100	0	25/15	94	1	1·00	0·99	22	Greece

TABLE 1. *Continued*

ID no.	Taxon	Constant			Alternating			PGI$_{const}$	PGI$_{alt}$	Seed mass (µg)	Country
		T	FG$_L$	FG$_D$	T	FG$_L$	FG$_D$				
69	*Campanula topaliana* subsp. *topaliana*	15	100	2	20/10	91	0	0·98	1·00	19	Greece
70	*Campanula trachelium* subsp. *athoa*	15	96	0	25/15	96	24	1·00	0·75	80	Bulgaria
71	*Campanula tubulosa*	15	92	1	25/15	96	4	0·99	0·96	30	Greece
72	*Campanula versicolor*	15	85	38	25/15	87	72	0·55	0·15	48	Greece
73	*Canarina canariensis*	15	98	58	20/10	98	61	0·41	0·38	1060	Spain
74	*Clermontia hawaiiensis*	20	99	0	20/10	99	0	1·00	1·00	116	USA
75	*Clermontia kakeana*	20	98	0	20/10	93	0	1·00	1·00	69	USA
76	*Clermontia oblongifolia*	20	83	0	20/10	79	0	1·00	1·00	39	USA
77	*Clermontia parviflora*	20	95	0	20/10	99	0	1·00	1·00	36	USA
78	*Codonopsis clematidea*	15	61	11	20/10	62	22	0·82	0·64	699	Kyrgyzstan
79	*Cyananthus inflatus*	25	85	2	25/15	91	0	0·98	1·00	47	China
80	*Cyanea angustifolia*	15	95	0	20/10	76	0	1·00	1·00	98	USA
81	*Delissea rhytidosperma*	20	96	5	25/15	95	80	0·95	0·17	158	USA
82	*Delissea subcordata*	15	74	0	20/10	96	6	1·00	0·92	268	USA
83	*Downingia bacigalupii*	15	52	0	15/5	81	0	1·00	1·00	55	USA
84	*Downingia bicornuta*	5	80	0	15/5	94	0	1·00	1·00	28	USA
85	*Downingia cuspidata*	15	72	0	15/5	68	0	1·00	1·00	22	USA
86	*Downingia elegans*	15	98	0	20/10	96	3	1·00	0·97	40	USA
87	*Edraianthus graminifolius* subsp. *graminifolius*	5	84	1				0·99		397	Greece
88	*Halacsyella parnassica*	5	22	4				0·82		563	Greece
89	*Hippobroma longiflora*	25	93	0	30/20	93	0	1·00	1·00	60	Mexico
90	*Isotoma axillaris*	25	56	0				1·00		74	Australia
91	*Isotoma hypocrateriformis*	10	6	0	20/10	7	0	1·00	1·00	12	Australia
92	*Isotoma luticola*	20	70	0				1·00		5	Australia
93	*Isotoma scapigera*	20	5	0				1·00		8	Australia
94	*Jasione heldreichii*	20	87	19	20/10	80	14	0·78	0·84	29	Greece
95	*Jasione montana* subsp. *montana*	15	98	0	20/10	99	0	1·00	1·00	12	Italy
96	*Jasione orbiculata*	15	87	4				0·95		31	Greece
97	*Legousia falcata*	5	100	0	20/10	9	2	1·00	0·98	149	Greece
98	*Legousia pentagonia*	5	42	0				1·00		274	Jordan
99	*Lobelia anceps*	15	1	0	20/10	96	0	1·00	1·00	15	Australia
100	*Lobelia appendiculata*	15	26	0	35/20	2	0	1·00	1·00	12	USA
101	*Lobelia cardinalis*	10	78	0	25/15	97	0	1·00	1·00	37	USA
102	*Lobelia djurensis*	20	97	0	20/10	85	0	1·00	1·00	10	Mali
103	*Lobelia fenestralis*	15	13	0	30/15	39	0	1·00	1·00	10	USA
104	*Lobelia grayana*	15	78	0	25/15	64	0	1·00	1·00	20	USA
105	*Lobelia hypoleuca*	25	90	0	25/15	94	0	1·00	1·00	20	USA
106	*Lobelia inflata*	25	59	0	20/10	40	0	1·00	1·00	27	USA
107	*Lobelia oahuense*	25	95	0	25/15	96	0	1·00	1·00	12	USA
108	*Lobelia physaloides*	15	93	0	20/10	93	0	1·00	1·00	89	New Zealand
109	*Lobelia seguinii*	20	98	0	25/15	100	0	1·00	1·00	16	China
110	*Lobelia simplicicaulis*	20	82	0				1·00		28	Australia
111	*Lobelia siphilitica*	20	4	0	20/10	85	0	1·00	1·00	30	USA
112	*Lobelia spicata*	25	3	0	20/10	17	0	1·00	1·00	21	USA
113	*Michauxia campanuloides*	15	100	6	20/10	100	2	0·94	0·98	96	Lebanon
114	*Monopsis debilis*	15	89	0	20/10	87	0	1·00	1·00	12	Australia
115	*Musschia aurea*	20	92	0	25/15	91	1	1·00	0·99	16	Portugal
116	*Nemacladus glanduliferus*	10	4	0	30/15	2	0	1·00	1·00	53	USA
117	*Petromarula pinnata*	20	76	3	25/15	61	22	0·96	0·71	17	Greece
118	*Phyteuma betonicifolium*	15	87	0	20/10	94	0	1·00	1·00	41	Italy
119	*Phyteuma hemisphaericum*	5	6	0				1·00		44	Italy
120	*Solenopsis minuta* subsp. *annua*	15	91	16	20/10	92	34	0·82	0·63	7	Greece
121	*Trachelium caeruleum*	20	90	0	25/15	93	0	1·00	1·00	11	Spain
122	*Triodanis perfoliata*	15	58	4				0·93		19	USA
123	*Wahlenbergia capillaris*	15	4	0				1·00		12	Australia
124	*Wahlenbergia ceracea*	20	2	0				1·00		22	Australia
125	*Wahlenbergia gracilis*	30	64	0				1·00		26	Australia
126	*Wahlenbergia hederacea*	20	20	0				1·00		26	England
127	*Wahlenbergia linarioides*	15	97	6	20/10	89	3	0·94	0·97	40	Chile
128	*Wahlenbergia luteola*	20	55	0	20/10	47	0	1·00	1·00	19	Australia
129	*Wahlenbergia perrottetii*	30	80	0	35/20	60	0	1·00	1·00	17	Burkina Faso
130	*Wahlenbergia preissii*	15	81	0	20/10	85	0	1·00	1·00	12	Australia
131	*Wahlenbergia undulata*	25	80	0	35/20	90	0	1·00	1·00	14	Swaziland

T, temperature (°C); FG$_L$ and FG$_D$, final germination percentage in light and darkness, respectively; PGI, photorequirement germination index at constant temperatures (PGI$_{const}$) and alternating temperatures in the dark and constant temperatures in the light (PGI$_{alt}$).

seeds were used per Petri dish; seeds remained on the thermogradient plates for 30 d.

Substitution for light requirement

To determine if gibberellic acid and nitrates can substitute for the light requirement, seed germination of ten taxa (12, 28, 38, 42, 60, 62, 81, 94, 97 and 120; Table 1) was tested in continuous darkness with GA_3 1000 ppm or KNO_3 10 mM. Two controls with distilled water were used, one under daily illuminations (12 h per day) and one in continuous darkness. One Petri dish of 100 seeds was placed under each test condition. All experiments were conducted at 15 °C, except for taxa numbered 62 (20/10 °C), 81 (20 °C) and 97 (10 °C).

Statistical analysis

Across species, a generalized linear model implemented in Minitab 16 (Minitab Inc., Pennsylvania, USA) was used to test for an effect of alternating vs. constant temperatures on PGI (i.e. do PGI_{alt} and PGI_{const} differ?). A total of 124 out of 131 taxa with percentage germination >10 % were included in the model. The model included $\log_{10}$ seed mass as a covariate and the seed mass × temperature regime interaction term. The inclusion of the interaction term allowed the test of the hypothesis that in larger seeded species, alternating temperatures can substitute for light requirement (i.e. across species does PGI_{alt} decrease more rapidly with increasing seed mass than PGI_{const}?). The analyses were also run with a smaller data set, including only taxa with final germination >70 %. This gave quantitatively similar results to the analyses with the larger data set; therefore, the results are not presented separately.

For each one of the four *Campanula* species (16, 22, 49 and 52; Table 1) tested on the thermogradient plate, germination data were analysed using binary logistic regression implemented in Minitab 16. Data were examined with respect to (1) 'day' temperature; (2) 'night' temperature; (3) light; (4) the magnitude of the diurnal temperature fluctuation (i.e. |day−night temperature|); and (5) the light × alternating temperature interaction term. The inclusion of the light × alternating temperature interaction term allowed us to test whether the effect of alternating temperatures depended on the light regime, i.e. whether alternating temperatures can substitute for the light requirement. This approach assumes that each individual seed in the population is a statistically independent unit (since each individual seed can either germinate or not) and the goodness of fit of these models was assessed using Wald tests (Tabachnick and Fidell, 2001).

RESULTS

Light requirement in incubators

For all 131 taxa, seed germination was higher in light than in darkness at both constant and alternating temperatures. Successful germination (considered as such when final germination exceeded 70 %) was achieved for the majority of the taxa studied (97 taxa) while final germination in light was >30 % in 114 taxa (Fig. 1). The PGI_{const} values varied between 0·04 and 1·00, with 118 taxa having $PGI_{const} >0·8$ and 95 taxa with an absolute light requirement ($PGI_{const} = 1·00$).
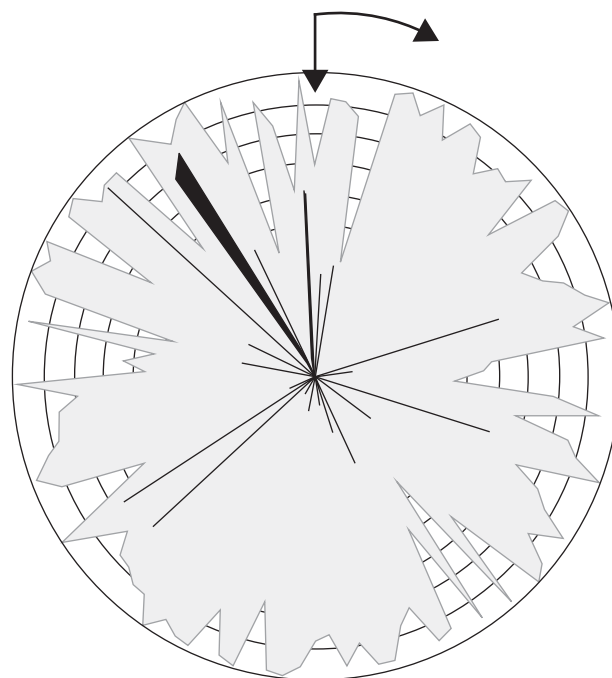

FIG. 1. Final germination percentage in the light (white) and dark (black) in the temperature regime with the highest germination for 114 taxa ($F_G \geq 30$ %). The outer circle corresponds to 100 % and the circle interval is set at 10 %. Taxa are arranged in order of increasing seed mass (clockwise from the starting point, vertical arrow).
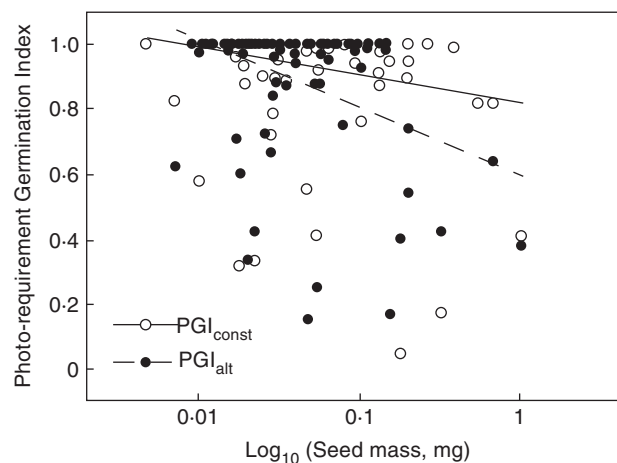

FIG. 2. Effect of seed mass on the photorequirement germination index in constant (PGI_{const}) and alternating temperatures (PGI_{alt}) for 124 and 104 Campanulaceae taxa, respectively, with $F_G >10$ %.

Across species, PGI decreased significantly with increasing seed mass: larger seeded species were less dependent on light for germination (generalized linear model, $F_{1,224} = 24·10$, $P = 0·000$; Fig. 2). In addition, across species, there was a significant difference between PGI calculated using constant temperatures in the dark (PGI_{const}) and alternating temperatures in the dark (PGI_{alt}) ($F_{1,224} = 6·04$, $P = 0·015$) and a significant interaction between seed mass and the effect of alternating vs. constant temperatures in the dark (generalized linear model,
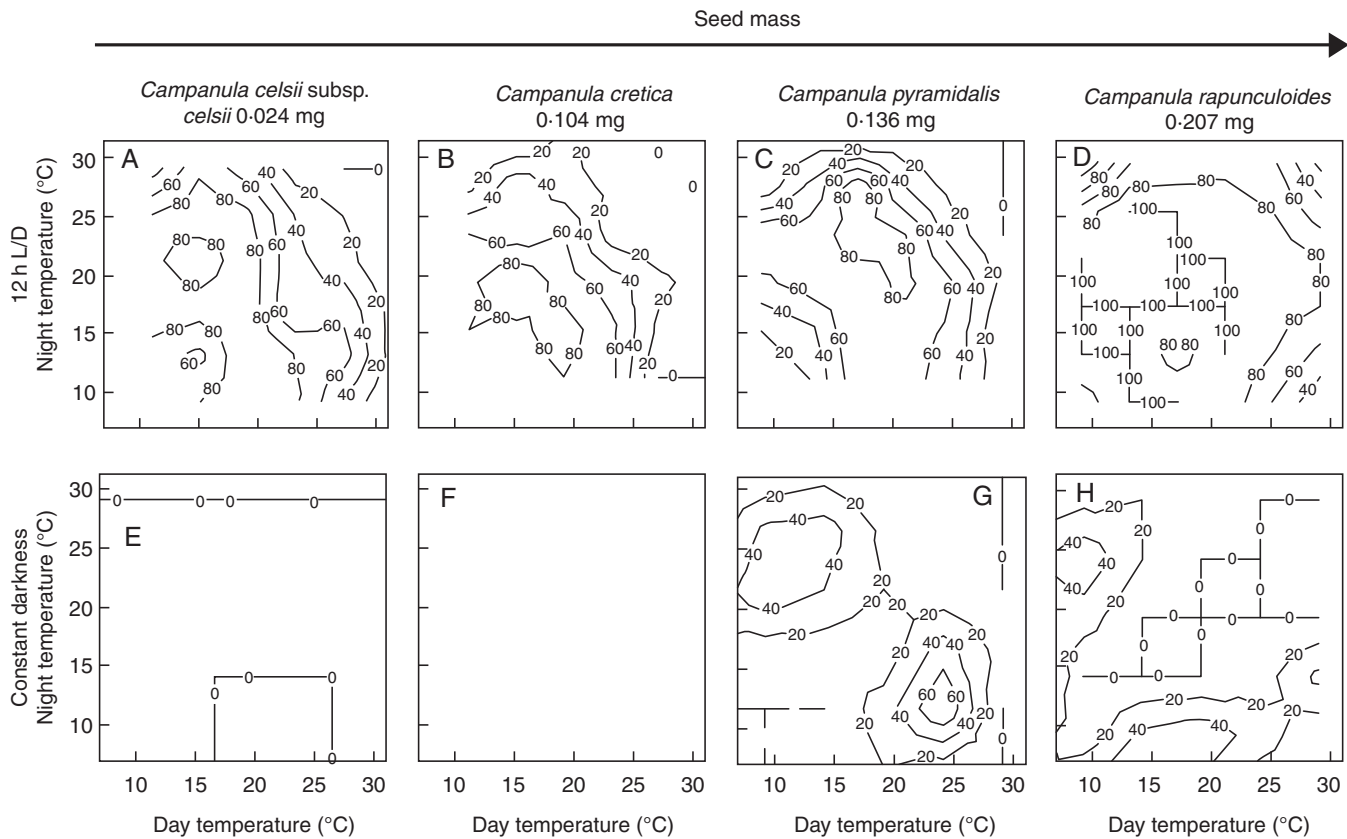
FIG. 3. Contour plots of the final germination percentage in the light (12 h photoperiod, A–D) and in continuous darkness (E–H) for four species of *Campanula* exposed to a wide range of combinations of day and night temperature on a two-way thermogradient plate. Species are arranged from left to right in order of increasing seed mass. The contour interval is set at 20 %.

$F_{1,224} = 3.95$, $P = 0.048$). This is manifest in Fig. 2 by the more negative slope for PGI_{alt} [$y = -0.089\ln(x) + 0.603$, $R^2 = 0.135$, d.f. = 102, $P < 0.001$] than for PGI_{const} [$y = -0.038\ln(x) + 0.8174$, $R^2 = 0.052$, d.f. = 122, $P < 0.02$]. This result and Fig. 2 suggest that especially for larger seeded species, germination in the dark reached higher levels under alternating rather than under constant temperatures.

Light requirement in the thermogradient plate

Germination for all four species reached ≥ 95 % in the 12 h light regime, with germination decreasing at the extreme low and high day and night temperatures. Germination was generally highest at approx. 20 °C (Fig. 3A–D). For the two smallest seeded species (*C. cretica* and *C. celsii* subsp. *celsii*), germination was ≤ 5 % at all temperatures in the constant dark treatment (Fig. 3E, F). For *C. celsii* subsp. *celsii*, there was a significant effect of light on germination (binary logistic regression, Wald test statistic = 5.25, $P < 0.001$), and neither an effect of alternating temperatures (binary logistic regression, Wald test statistic = 0.89, $P > 0.05$) nor an interaction between alternating temperatures and light was found (binary logistic regression, Wald test statistic = −1.28, $P > 0.05$). While light was clearly required for germination of *C. cretica*, this could not be tested in the logistic model due to a failure for model convergence resulting from a total

absence of germination in the dark. For the two larger seeded species, there was also a significant light effect on germination (binary logistic regression, Wald test statistic = 16.01 and 9.26 for *C. rapunculoides* and *C. pyramidalis*, respectively, $P < 0.001$; Fig. 3). However, for these two species, up to 85 % germination was also recorded in the dark treatments, but only at temperature regimes with a difference between day and night temperature of approx. 10 °C (Fig. 3G, H): for both species there was a significant interaction between light and alternating temperatures (binary logistic regression, Wald test statistic = −8.74 and −4.15 for *C. rapunculoides* and *C. pyramidalis*, respectively, $P < 0.001$). The effect of alternating temperatures on germination was significant for *C. rapunculoides* (binary logistic regression, Wald test statistic = 4.63, $P < 0.001$) but not for *C. pyramidalis* (binary logistic regression, Wald test statistic = 1.72, $P > 0.05$).

Substitution for light requirement

The gibberellin GA_3 substituted for the light requirement in all ten species examined; moreover, its application resulted in even higher values of final germination compared with the light treatment (Fig. 4). On the other hand, KNO_3 only partially promoted dark germination in three species (62, 94 and 97; Table 1), in which the percentage germination in darkness without KNO_3 was >5 %.
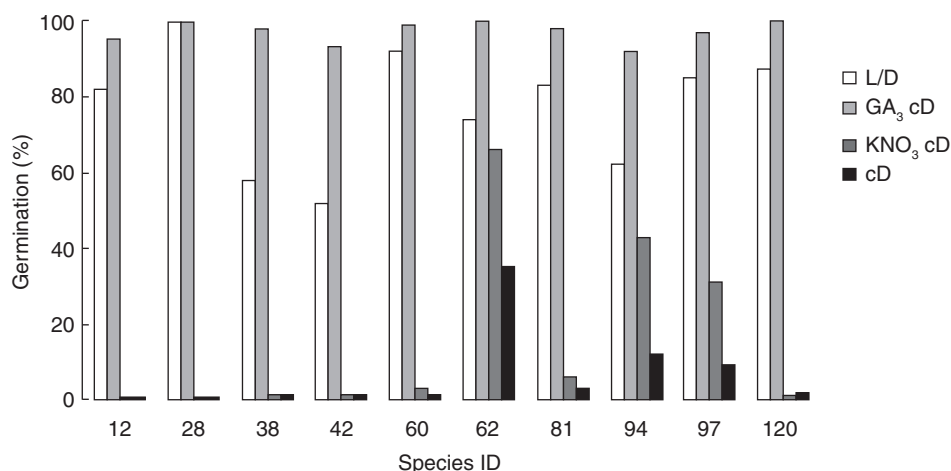
FIG. 4. Final germination percentage in the light (12 h photoperiod, L/D) and in continuous darkness with GA$_3$ 1000 ppm (GA$_3$ cD), KNO$_3$ 10 mm (KNO$_3$ cD) or distilled water (cD) for ten species (see Table 1 for species' ID numbers).

DISCUSSION

The effect of light on Campanulaceae seed germination has been studied in representatives of all five continents and for three out of five subfamilies. In this work, 19 previously unstudied genera and 123 unstudied taxa of the Campanulaceae were shown to have seeds with germination promoted by light. A light requirement for germination, observed in virtually all taxa examined, constitutes a collective characteristic of the family, apparently regardless of habitat type (sand dunes, wetlands, shrublands, forests, cliffs and rock crevices, montane and alpine meadows), life form (phanerophyte, nanophanerophyte, chamaephyte, hemicryptophyte, geophyte, therophyte) and climatic conditions (tropical– sub-tropical, mediterranean, temperate, continental, alpine). Statistical analysis aiming to discriminate variations of light requirement among different ecological factors have however proven unsuccessful (data not shown).

A light requirement for germination was stronger in smaller than in larger seeded species of the Campanulaceae: PGI decreased with increasing seed mass. Final germination of 27 of the taxa included in the analysis was <70 %. Since a portion of seeds in these seed samples may have failed to germinate due to dormancy, our data indicate that, at least for non-dormant seeds, small-seeded taxa are more likely to require light for germination. This relationship has been reported previously for local flora species (Grime *et al.*, 1981; Pons, 1991), desert species (Hammouda and Bakr, 1969; Jiménez-Aguilar and Flores, 2010), temperate herbaceous species (Milberg *et al.*, 2000), neotropical pioneer species (Pearson *et al.*, 2002), herbaceous species of northern temperate deciduous forests (Jankowska-Blaszczuk and Daws, 2007) and pioneer tree species from the Central Amazon (Aud and Ferraz, 2012). In a study of 136 taxa of the Cactaceae family (Flores *et al.*, 2011) seed mass was associated with the RLG index only when all taxa were included in the analysis and not when only taxa with germination >60 or 70 % were included in the analysis. However, for 28 temperate grassland species, seed mass and percentage germination in light or dark were not correlated (Morgan, 1998).

The low R^2 values observed in this current study indicate a significant variation in light responses in relation to seed mass. One limitation of using light requirement indices (either the RLG index or PGI) is that data sets typically contain a preponderance of taxa with either an absolute light requirement (RLG index or PGI of 1) or no light requirement (RLG index of 0·5 or PGI of 0) (e.g. Milberg *et al.*, 2000; Flores *et al.*, 2011; this study); few taxa have intermediate requirements. Consequently, data are unlikely to fit along a single fitted line and low R^2 values are likely, as found in this and other studies (e.g. Milberg *et al.*, 2000; Flores *et al.*, 2011). Nonetheless, the key point is that these analyses indicate a statistically significant, negative slope for the relationship between PGI and seed mass, i.e. a trend towards larger seeded species being less likely to require light for germination. This limitation could be addressed by simplifying the taxon level response to light to a binomial variable, i.e. seeds either do or do not respond to light. However, this would risk losing significant amounts of information present in the data.

Indifference to light or higher germination in dark conditions has only previously been reported in two Campanulaceae species. Willis and Groves (1991) reported higher germination of *Wahlenbergia stricta* in the dark than in the light. However, this contrasts with both Hitchmough *et al.* (1989) and McIntyre (1990) who reported light-stimulated germination in *W. stricta*, and our current study, where all nine *Wahlenbergia* species studied required light for germination (Table 1). Willis and Groves (1991) reported that, for the purpose of scoring germination, seeds were exposed to light every 2–3 d, which may have been sufficient to stimulate germination in their 'dark' treatment. For 'dark' treatments this highlights the importance of only scoring seeds at the end of the concurrent light treatment. For *Howellia aquatilis*, seed germination has been reported to be indifferent to light (Lesica, 1992). Although seed mass is not mentioned for this species, calculations based on seed dimensions (approx. 1 × 3 mm long) suggest a seed mass >300 μg. Consequently, *H. aquatilis* seems to be one of the largest seeded species in the Campanulaceae and its indifference to light is consistent with the results of the present study.

Light penetration falls below 0·01 % at a depth of 4 mm in sandy soil or 1 mm in dark soil, while increasing soil depth also leads to a gradual decline of the red:far red ratio (Benvenuti, 1995). Thus a light requirement for germination

acts as a surface-sensing mechanism, enabling small seeds with limited nutrient reserves to germinate when found near the soil surface rather than from depths from which they are unable to emerge physically (Bond *et al.*, 1999; Daws *et al.*, 2007). In addition, species with light-requiring seeds can form soil seed banks and germinate at a subsequent stage, after soil disturbance (Grime *et al.*, 1981).

Alternating temperatures can fully or partially substitute for light requirement in the Campanulaceae. This has been reported previously in various taxa, e.g. *Rumex obtusifolius* (Taylorson and Hendricks, 1972), *Polygonum persicaria* (Vincent and Roberts, 1977) and *Chenopodium album* (Tang *et al.*, 2008). Substitution of light requirement by alternating temperatures in the Campanulaceae is mostly associated with larger seeded species. Fluctuating temperatures can act as a sensing mechanism for both soil depth and vegetation cover, due to greater fluctuation caused by direct sun exposure. Since soil temperature fluctuations can be experienced by seeds buried at depths greater than those from which small seeds can physically emerge (Jankowska-Blaszczuk and Daws, 2007), our data suggest that substitution of a light requirement by alternating temperatures is a characteristic of larger, light-promoted seeds, i.e. those with enough nutrients to emerge from greater soil depths, as has been previously reported by Thompson and Grime (1983). For neotropical tree species, Pearson *et al.* (2002) reported that while small seeds are generally light requiring, larger seeded non-light-promoted species were likely to germinate in response to fluctuating temperatures.

Substitution of the light requirement by nitrates, observed in three out of nine Campanulaceae, can be explained in terms of plant competition. Covering vegetation decreases the concentration of nitrates in the soil due to acquisition by plants; thus, an increase in soil nitrate concentration is typically the result of some kind of disturbance (Fenner and Thompson, 2005). Promotion of germination in darkness by nitrates in photorequiring *Helichrysum stoechas* subsp. *barrelieri* seeds is particularly important in fire-prone Mediterranean ecosystems (Doussi and Thanos, 1997). The active form of phytochrome is transferred to the cell nucleus where it is known to activate gibberellin biosynthesis (Oh *et al.*, 2009); this fact may explain why exogenously applied gibberellins (such as those used here) can substitute for a light requirement in seed germination. Light requirement for germination was entirely substituted by gibberellic acid for all Campanulaceae taxa examined.

To conclude, most species of the Campanulaceae family have a light requirement for germination, substituted by gibberellic acid (although it is certainly not part of the natural environment of seeds) and partially also by nitrate (only in species that germinated to some extent in darkness). The influence of light on germination was much stronger in smaller than in larger seeded species; thus germination is prevented when seeds are buried deep in the soil. Larger seeded species can germinate in deeper soil depths in the presence of fluctuating temperatures.

ACKNOWLEDGEMENTS

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