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NOTE

Can alternating temperature, moist chilling, and gibberellin interchangeably promote the completion of germination in *Clematis vitalba* seeds?

Rosangela Picciau, Marco Porceddu, and Gianluigi Bacchetta

Abstract: Each plant species has particular requirements for seed germination, and some of them respond differently to constant or alternating temperature regimes. In this study, the interchangeable effects of different treatments and temperatures on the completion of seed germination of *Clematis vitalba* L. were investigated. The seeds were tested with a constant (from 5 °C to 25 °C) or a fluctuating (25/10 °C) temperature regime, and the effect of gibberellic acid (GA_3), incubation at warm (W) or cold (C) temperatures while being imbibed, and drying after ripening (DAR) were evaluated. The final germination percentages and the time in days required to reach 50% of germination (T_{50}) were calculated. GA_3 and C significantly enhanced completion of seed germination at all of the temperatures tested. A strong positive effect of alternating temperature was observed, which triggered completion of seed germination regardless of treatment. Under the fluctuating temperature, the chilled seeds had the most rapid germination. Low germination rates were observed for both control and DAR treatments. Seeds of *C. vitalba* display a certain degree of dormancy, which can be broken by moist chilling and GA_3 treatments. Moreover, alternating temperature stimulates the completion of seed germination by satisfying certain physiological requirements for germination under constant temperatures.

Key words: fluctuating temperature, hormone treatment, Ranunculaceae, seed germination, cold imbibed incubation.

Résumé : Chaque espèce végétale a ses exigences propres en ce qui concerne la germination de ses semis et certaines d'entre elles répondent différemment à des régimes de températures constantes ou alternées. Dans cette étude, les effets interchangeables de différents traitements et températures sur la complétion de la germination de *Clematis vitalba* L. ont été examinés. Des semis soumis un spectre de régimes de températures constantes (de 5 à 25 °C) ou fluctuantes (25/10 °C) ont été testés, et les effets de l'acide gibbérellique (GA_3), d'un trempage à la chaleur ou au froid, ainsi que de la sécheresse après la maturation ont été évalués. Les pourcentages finaux de germination et le temps requis, en jours, pour atteindre 50 % de germination (T_{50}) ont été calculés. Le GA_3 et le trempage au froid accroissaient significativement la complétion de la germination des semis à toutes les températures testées. Un fort effet positif des températures alternées était observé, qui déclenchait la complétion de la germination, sans égard au traitement. Sous des températures fluctuantes, les graines refroidies présentaient la germination la plus rapide. De faibles taux de germination étaient observés tant chez les contrôles que chez les semis soumis à la sécheresse après maturation. Les semis de *C. vitalba* présentent un certain degré de dormance qui peut être brisée par une humidité au frais et par le traitement au GA_3. De plus, les températures alternées stimulent la complétion de la germination des semis en satisfaisant certaines exigences physiologiques requises pour la germination sous des températures constantes. [Traduit par la Rédaction]

Mots-clés : températures fluctuantes, traitement hormonal, Ranunculaceae, germination des semis, trempage au froid.

Introduction

Each plant species has particular ecological requirements for seed germination (i.e., temperature, water, light) and can show seed dormancy (Baskin and Baskin 2014). In some species for example, to break the dormancy, the seeds may require a chilling (e.g., *Rhamnus persicifolia* Moris; Porceddu et al. 2013), moist warm treatment (e.g., *Paeonia corsica* Sieber ex Tausch; Porceddu

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R. Picciau, M. Porceddu, and G. Bacchetta. Centro Conservazione Biodiversità, Dipartimento di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Viale S. Ignazio da Laconi, 13, 09123, Cagliari, Italy. Banca del Germoplasma della Sardegna (BG-SAR), Hortus Botanicus Karalitanus (HBK), Università degli Studi di Cagliari, Viale Sant'Ignazio da Laconi, 9-11, 09123, Cagliari, Italy.

Corresponding author: Marco Porceddu (email: porceddu.marco@unica.it).

et al. 2016), drying after ripening (e.g., *Arabidopsis thaliana* (L.) Heynh.; Finch-Savage et al. 2007), or treatment with gibberellins (Cuena-Lombraña et al. 2016). For many species, constant or alternating temperature regimes can affect seed germination differently (Probert 2000). Different studies have reported an increase in germination percentage of species under a fluctuating temperature regime (e.g., Thompson and Grime 1983; Schütz and Rave 1999; Liu et al. 2013). Koller (1972) and Thompson and Grime (1983) have suggested that the sensitivity of seeds to fluctuations in temperature (fluctuations that rapidly diminish with increasing soil depth) could be a depth-sensing mechanism. Therefore, seeds that require diurnal fluctuations in temperature for the completion of germination may be inhibited by the more uniform temperatures at greater depths. They would then be preserved until conditions became more favourable for the establishment of seedlings (Ghersa et al. 1992).

The genus *Clematis* (Ranunculaceae) includes more than 300 taxa of climbing vines, and erect or ascending perennial herbs (sometimes woody) that are widely distributed through the temperate regions, chiefly in the Northern Hemisphere (Rehder 1940; Wang and Li 2005). *Clematis vitalba* L. is a perennial vine with climbing, woody stems that can grow up to 30 m in height. It is originally a forest species of Europe, Asia, and northern Africa, and can be found in woodlands, borders of woods, thickets, and hedgerows (Bojňanský and Fargašová 2007). It is also possible to find *C. vitalba* in riparian areas established with willows, in waste areas, and in coastal and lowland areas. Seeds of *C. vitalba* can have a high degree of dormancy (Lhotská 1974; Rudolf 1974; Grime et al. 1981), which can be reduced by moist chilling, light, and nitrates (e.g., Henson 1970; Vincent and Roberts 1979; Hilhorst 1990*a*, 1990*b*). A moist chilling requirement for germination has obvious potential ecological benefits for species growing in seasonal climates, delaying completion of germination until after winter when temperatures are more suitable for seedling growth and survival (Probert 1992). Previous studies report that germination of *C. vitalba* seeds is stimulated by moist chilling (stratification at 1–5 °C for 2–5 months; Rudolf 1974; Grime et al. 1981). However, there are also other treatments (i.e., incubation under moist warm conditions and drying after ripening) that have not yet been investigated, and that could be used to evaluate the germination capacity of *C. vitalba* seeds.

In this study, we examined the seed germination requirements for *C. vitalba* by studying the interchangeable effects of fluctuating temperature, gibberellins (GA_3), and other treatments, to identify the temperature requirements for germination and to identify the possible ecological adaptive significance of the germination requirements for this taxon.

Materials and methods

Seed lot details

The seed collection of *C. vitalba* was carried out in October 2012, in Monte Padenteddu (Pula, Cagliari), in Southern Sardinia, Italy. The seeds were collected directly from plants during the time of natural dispersal. In this site, the species is part of *Ilici aquifolii – Salicetum arrigonii* growing in association with *Salix arrigonii*, *Ilex aquifolium*, *Arbutus unedo*, and *Rubus ulmifolius* (Angius and Bacchetta 2009).

Experimental trials

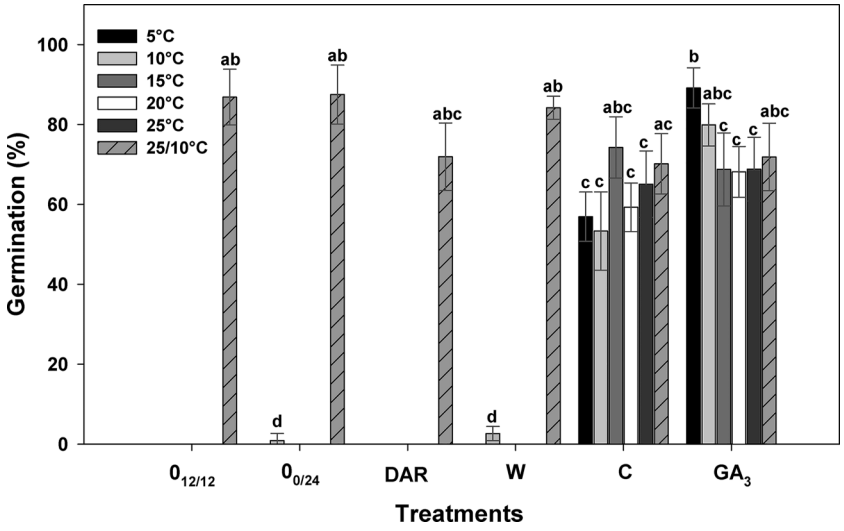
Four replicate samples of 30 seeds each were sown on the surface of 1% agar water, in 60 mm diameter plastic Petri dishes and incubated over a range of constant temperatures (5, 10, 15, 20, and 25 °C) or under an alternating temperature regime (25/10 °C), either in the light (12 h of irradiance per day), or in the dark (0 h of irradiance per day). In the alternating temperature regime, the 12 h light period coincided with the elevated temperature period. Darkness was achieved by wrapping the Petri dishes in one layer of aluminium foil. In addition, different additional treatments were also applied, specifically: moist chilling ("C", imbibed seeds were incubated for three months at 5 °C on 1% agar water); imbibed seeds were incubated in warm conditions ("W", imbibed seeds were incubated for three months at 25 °C on 1% agar water); and, drying after ripening ("DAR", seeds were stored for three months at 25 °C on silica gel), before germinating the seeds at the abovementioned temperatures. In addition, to determine the effects of GA_3 on seed germination, four replicate samples of 30 seeds each were sown on the surface of 1% agar water containing 250 $mg \cdot L^{-1}$ of GA_3 and incubated under a light–dark cycle (12 h light – 12 h dark), at the previously cited temperatures. All of the germination tests started two weeks after seed collection, and were conducted using the same environmental test chamber (Sanyo MLR-351) equipped with white fluorescent lamps (FL40SS.W/37 70-10 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Completion of germination, defined as visible radicle emergence (>1 mm), was recorded three times a week, except for the dark-incubated seeds, which were scored only once, at the end of the test, to avoid any exposure to irradiance. At the end of the germination tests, when no additional seeds completed germination over two weeks, after a minimum of one month from sowing and a maximum of 120 days, a cut-test was carried out to determine the viability of the remaining seeds and the firm seeds were considered viable.

Data and statistical analysis

The final germination percentage was calculated as the mean of the four replicates (±SD) on the basis of the total number of filled seeds. Germination rate (T_{50}) was determined as the time in days required to reach 50% completion of germination (Cuena-Lombraña et al. 2016)

Fig. 1. Cumulative germination percentage for seeds of *Clematis vitalba* at the tested temperatures after each treatment ($0_{12/12}$, control seeds in 12 h light – 12 h dark; $0_{0/24}$, control seeds kept in darkness (dishes wrapped in one layer of aluminium foil); DAR, seeds kept at 25 °C for three months on silica gel; W, seeds incubated under moist conditions at 25 °C for three months; C, seeds incubated under moist conditions at 5 °C for three months; GA_3, 250 mg·L^{-1} of GA_3 in the germination substrate); values with the same letters are not statistically significantly different ($P > 0.05$) as determined by post-hoc Tukey tests.



for those seeds that did complete germination. The generalized linear model (GLM) was used to evaluate the effects of incubation temperature within each treatment on final seed germination percentage, and to evaluate the effects of alternating temperatures after each treatment on the rate of the germination to 50% of the total germination (T_{50}) for those seeds that completed germination. A logit link function and quasibinomial error structure was performed for analysing seed germination percentages, whereas a log link function and Poisson error structure was used for analysing T_{50}. Quasibinomial error structure and F tests with an empirical scale parameter, instead of a χ^2 test, on the subsequent ANOVA were used to overcome residual overdispersion (Crawley 2007). Significant differences highlighted by GLM were then analysed by post-hoc Tukey multiple comparisons tests. All statistical analyses were carried out with R version 3.0.3 (R Core Team 2015).

Results

Seed germination

Seeds of *C. vitalba* only completed germination under constant temperatures following GA_3 application, or following moist chilling treatment. Specifically, these seeds showed germination percentages >50% at each tested temperature in both treatments; however, seeds reached highest germination percentage at 5 °C (~89%) and 10 °C (~80%) in the GA_3 treatment group and at 15 °C (~74%) in the C treatment group, without statistically significant differences between them ($P > 0.05$; Fig. 1). In addition, for all treatment groups except for C and GA_3, the seeds showed a preference for alternating temperatures (25/10 °C). In particular, seeds in the 0, W, and DAR treatment groups only achieved a high percent germination at 25/10 °C with values of ~87%, 84%, and 72%, respectively (Fig. 1). The statistical analysis showed that the values for percent germination obtained from the alternating temperatures in all treatment groups were statistically similar ($P > 0.05$; Fig. 1).

Fig. 2. Germination trends and the number of days to attain 50% completion of seed germination for those live seeds of *Clematis vitalba* kept under an alternating temperature regime of 25/10 °C after each treatment ($0_{12/12}$, control seeds in 12 h light – 12 h dark; GA_3, 250 mg·L^{-1} of GA_3 in the germination substrate; W, seeds incubated under moist conditions at 25 °C for three months; C, seeds incubated under moist conditions at 5 °C for three months; DAR, seeds kept at 25 °C for three months on silica gel). Symbols correspond to the actual data and solid lines indicate the fitted curves of the sigmoidal regression. Data are mean of four replicates (±SD).

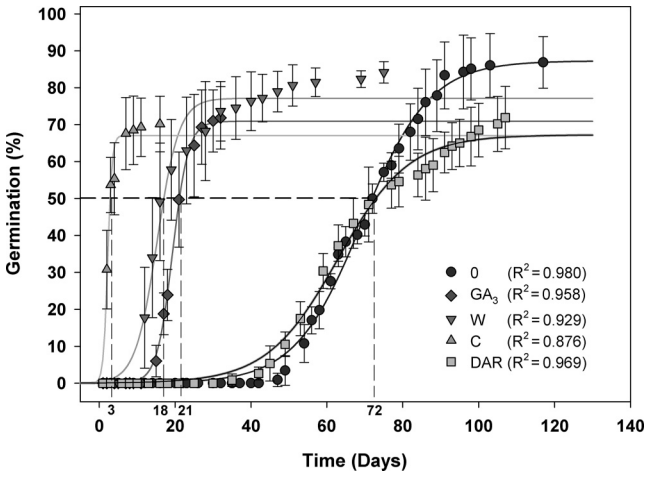


Table 1. Details of the germination responses of *Clematis vitalba* seeds for each treatment under the alternating temperature regime (25/10 °C).

	Germination response			
Treatment	Final (% ± SD)	First completion (days ± SD)	T_{50} (days ± SD)	Last completion (days ± SD)
0 (12 h light – 12 h dark)	86.86±6.99	49.75±2.99	72.4±1.06a	101.25±11.95
GA_3	71.85±8.45	15.50±1.00	21.96±2.65b	28.50±3.11
W	84.17±2.90	12.50±1.00	18.67±6.71b	62.00±15.10
C	70.16±7.54	2.00±0.00	3.18±1.01c	10.25±4.27
DAR	71.91±8.44	41.00±5.72	72.50±9.04a	102.5±4.04

Note: GA_3, gibberellic acid in the incubation medium; W, incubated under moist warm conditions (25 °C) for three months; C, incubated under moist cold conditions (5 °C) for three months; T_{50}, time required to reach 50% germination; DAR, drying after ripening. Values in the T_{50} column followed by the same letters are not significantly different ($P > 0.05$; post-hoc Tukey test).

Seed germination rate under alternating temperature regimes

Although seed germination of *C. vitalba* occurred at 25/10 °C after each treatment, seeds completed germination at statistically different rates ($P < 0.05$). In particular, untreated (0) and DAR seeds needed 72 days to reach T_{50}, but after GA_3 and W treatment, this time was reduced approximately three-fold, with a T_{50} values of ca. 22 and 19 days, respectively. Following C treatment, seeds completed germination very rapidly, reaching the T_{50} value in ca. three days (Fig. 2; Table 1).

Discussion

The study species highlighted different germination responses to imbibed incubation and temperatures. The seeds are insensitive to constant temperature (in either light/dark conditions), also when warmed while imbibed (W) and following DAR treatment. By contrast, seeds of *C. vitalba* respond positively to GA_3, which promoted the completion of germination at all tested temperatures. Similarly, cold, imbibed incubation had an important effect on completion of seed germination, albeit to a slightly lesser extent than GA_3. This indicates that a certain degree of dormancy could be found in the study species, which is consistent with previous reports (Rudolf 1974; Van Gardingen 1986; West 1992; Bungard et al. 1997). Moreover, it is widely reported that cold, moist, incubation and (or) GA_3 treatment promote the completion of germination in dormant seeds (e.g., Finch-Savage and Leubner-Metzger 2006; Baskin and Baskin 2014), especially in temperate species, for which moist chilling is considered to be an effective mechanism that delays seed germination until after winter when conditions are more favourable for plant establishment (Bewley and Black 1985). This behaviour provides a considerable ecological advantage for seeds, allowing completion of germination immediately after snowmelt in spring, under higher temperatures, with a lower probability of damage of the young seedlings because of frost (Billings and Mooney 1968; Cavieres and Arroyo 2000; Baskin and Baskin 2014).

The germination response to a range of constant and alternating temperatures can be vastly different for different species, but it is common in plant species that the completion of germination is stimulated by alternating temperatures (e.g., Thompson and Grime 1983; Probert et al. 1986; Schütz and Rave 1999; Liu et al. 2013). In agreement with this, a fluctuating temperature regime was found to be necessary for the completion of germination in untreated seeds of *C. vitalba*. Further, a fluctuating temperature regime generally improved the completion of germination or the speed with which the seeds germinate after each treatment (i.e., GA_3, C, W, and DAR). In fact, the alternating temperature regime triggered the completion of germination under all of the tested conditions, including untreated seeds, even if, in this last case and in those seeds that had experienced DAR, 72 days were needed to attain T_{50}. The variability observed in the germination characteristics of *C. vitalba* may be considered as an indicator of suitable conditions for seedling establishment and growth. Indeed, according to Liu et al. (2013), fluctuating temperature requirements for the completion of seed germination can partly explain the germination niche of a species, and thus its habitat requirements and distribution. This behaviour detected for *C. vitalba* may be interpreted as an adaptive mechanism of plants to the natural environment, where a positive response to temperature fluctuation enables seeds to complete germination only in gaps and at or near the soil surface, to avoid death by shading or germinating deep in the soil (Thompson et al. 1977; Benech Arnold et al. 1988; Ghersa et al. 1992; Liu et al. 2013). The results obtained in this work are consistent with these previous studies, confirming that, in general, species such as *C. vitalba* growing in forest margin/scrub, have a positive response to temperature fluctuation.

In conclusion, the principal aims of this study were to examine whether the fluctuating temperature, gibberellins, and dormancy release treatment have an interchangeable effect on the completion of seed germination of *C. vitalba*.

It is noteworthy that the alternate temperature regime enhanced seed germination even in the absence of dormancy release treatment (i.e., chilling) or hormone treatment (GA_3).

This aspect underlines the importance of testing the fluctuating temperatures under controlled conditions to identify and define the real temperature requirements for seed germination of a taxon.

In this research, experiments were designed to clarify the biological mechanisms of seed germination for *C. vitalba*. This species was chosen because it has seeds that have displayed a certain degree of dormancy, which can be broken by moist chilling or GA_3. Moreover, it was determined that an alternating temperature regime stimulated the completion of seed germination by satisfying the physiological requirements.

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