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Seed germination of calendula in response to temperature

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ABSTRACT

Calendula (*Calendula officinalis* L.) seeds contain high concentrations of calendic acid (C18:3) which can be used as tung and linseed oil substitutes. Calendula is adapted to temperate climate, but field studies in western Minnesota indicated that stand establishment was susceptible to high soil temperatures immediately after planting in spring. Consequently, understanding the temperature conditions that govern germination of calendula is necessary to incorporate the crop into crop rotations of the Upper Midwest, U.S. Temperature gradient bar and heat-shock experiments were used to characterize calendula (cv. 'Carola') sensitivity before and during germination. Seed germinated between 2 and 32 °C with the optimum germination temperature at 16–17 °C. Heat shock temperatures (35–40 °C) of less than 50 h duration reduced germination (at 16 °C) below 50%. At 45 °C, 100% seed lethality was induced within 24 h of heat treatment. Accordingly, calendula seed should be sown in the field only if forecasted soil conditions are expected to be below 30 °C during seed germination.

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1. Introduction

Calendula (*Calendula officinalis*), or pot marigold, is an annual plant that has been used historically for ornamental and medicinal purposes. Calendula typically grows 20–50 cm tall with yellow and orange flowers 4–7 cm in diameter (Tucker, 2007). Calendula is in the *Asteraceae*, and has composite-type flowers (i.e., arranged in capitula). The seed of calendula comes in three primary shapes: nuggets, winged, and hooked (Froment et al., 2003; Joly et al., 2013; De Clavijo, 2005). For agronomic production the nuggets are preferred because their shape is more compatible with seeding equipment. In the last 20 years calendula has been evaluated as a specialty oilseed crop due to its high calendic acid content (Meier zu Beerentrup and Röbbelen, 1987; Biermann et al., 2010; Cromack and Smith, 1998). Seed oil from calendula contains 59–65% calendic acid (C18:3), which has value in cosmetic, paint, and coating industries (Biermann et al., 2010). The cultivar 'Carola' was released in 2005 and is one of only a few commercial oilseed varieties (Gesch, 2013). The production and management practices for this oilseed crop have not been described fully for the Upper Midwest region of the U.S. Agronomic production of calendula has been evaluated primarily in Europe (Froment et al., 2003). Developing the best management practices for the production of calendula is necessary for optimized yield and profit for growers. Recent studies reported that calendula seed should be seeded at a depth of 1–2 cm by early May in central Minnesota fields (Joly et al., 2013; Gesch, 2013).

Observations during field trials suggested that high temperatures at planting time were associated with reduced calendula stand establishment. A germination study by Harrington (1921) concluded that at constant temperature calendula germination reached a maximum (65%) between 15 and 25 °C within 6 d. Germination of the medicinal cultivar 'Bonina Sortida' was rapid, reaching near maximum germination levels within one week in incubators (Koefender et al., 2009). For this cultivar, optimum temperature was 20 °C, at which over 80% germination was achieved, suggesting that calendula is adapted to germination during cool seasons. Lower levels of germination (52–76%) occurred at 15 and 25 °C, and only scant germination (11–28%) occurred at the higher temperatures of 30 and 35 °C. Seedling elongation and dry weight gain of this same cultivar were highest at 20 °C, in both light and dark conditions, whereas both variables were nil at 30 and 35 °C (Koefender et al., 2009). The related wild species, field marigold (*Calendula arvensis*), had similar germination responses to temperature as domesticated calendula: (a) a broad high-germination peak from 15 to 25 °C, (b) delayed germination at temperatures exceeding 30 °C, and (c) a near absence of germination at temperatures greater than 35 °C (De Clavijo, 2005). Similarly, an ornamental cultivar of calendula, 'Calypso Orange', produced only half as much dry matter and flower buds at 32 °C compared to 20 °C (Warner and Erwin, 2005). Moreover, high temperature hastened anthesis and decreased inflorescence size by one-third in this cultivar. Clearly, calendula appears intolerant of temperatures that easily could be reached in shallow soil layers after late spring planting or by air temperatures during summer in temperate zones. We selected the

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variety 'Carola' as the focus of this germination study because of its high seed oil content, commercial availability, and lack of reported germination requirements in the literature.

Here we determined the critical temperatures (T_b, T_o, and T_c) for calendula seed germination by examining germination rates over 9 d across temperatures ranging from 1 to 40 °C. Base temperature (T_b) is the temperature below which germination is 0%, optimum temperature (T_o) is the temperature where germination is maximal, and the ceiling temperature (T_c) is the temperature above which germination is 0%. Joly et al. (2013) reported the T_b for calendula to be 5.5 °C based on soil hydrothermal time to 50% field emergence. Hydrothermal time is a well documented measure where soil temperature and water potential are used to predict germination rates for different species. Using controlled temperature studies we sought to confirm the T_b for calendula and to determine the T_o and T_c, which can be used to facilitate seed sowing and agronomic production of calendula. Additionally, we determined the time period over which T_c is exerted by a series of heat-shock experiments.

2. Methods

2.1. Plant material

Nugget-type (annular) seeds of 'Carola' were used in all studies. Seeds were germinated on pre-moistened germination paper that was placed either on solid aluminum gradient bars or in glass Petri plates.

2.2. Gradient bar temperature incubations

Seeds were germinated on a temperature-gradient bar lined with filter paper as described by Berti and Johnson (2008) for 9 d with nine thermal couple probes evenly spread along the bar in the range of 1.1–39.0 °C. The thermocouples (copper-constantan) were attached flush to the aluminum bar with high temperature conductivity epoxy resin. The thermocouples were monitored with a CR-10X (Campbell Scientific, Logan, UT) data logger every 15 s and 1 h averages recorded. Seeds were placed in 3 rows of 10 at each thermal couple, each row served as an experimental replicate. Seeds were checked daily for germination and filter paper was remoistened as needed. Radical emergence of greater than 0.5 cm was considered germination, and the germinated seed was removed from the gradient bar. The entire experiment was performed three times.

Cumulative germination percent was calculated for each seed row after 9 d. Results from the experimental trials varied significantly and, consequently, different quadratic and cubic regression models were fit to each experimental trial. Critical temperatures were calculated from the different models.

Germination rate was calculated as the inverse of the number of days (d^{-1}) to reach 50% germination for each row of 10 seeds. If the seeds for a given treatment never reached 50% germination, the rate was entered as 0.0 d^{-1}. Germination rate was separated into sub- and supra-optimal ranges by visual inspection of the data. Linear regression models were fit to the sub- and supra-optimal data sets. The intersection of the sub- and supra-optimal regressions within each experimental trial denoted the T_o for germination rate. Both T_b and T_c were determined from the intercept of the sub- and supra-optimal regressions, respectively.

2.3. Heat shock incubations

Calendula seeds were incubated at three temperatures above the predicted T_c (35, 40, and 45 °C) expected to induce heat shock. Exposure times (treatments) were 0, 2, 8, 24, 48, 96, and 192 h, except trial 1 at 40 °C wherein seeds were incubated for 1, 2, 4, 8, 24, 48, and 192 h. All seeds were incubated at 16 °C following heat shock treatments for a total cumulative incubation period of 14 d. There were 20 seeds per plate and 3 plates per treatment. Petri plates were sealed with parafilm to prevent drying. Seeds were allowed to imbibe for 1 h at room temperature in standard Petri plates on germination paper wetted with 10 ml water before heat shock incubation. Dry seed treatments had 10 ml of water added to the Petri plate after heat shock incubation and before 16 °C incubation. Each experiment was performed at least twice. Cumulative germination percent was measured at incubation day 14 for each plate.

2.4. Tetrazolium chloride viability staining

Wet calendula seed was incubated at 45 °C for 0, 2, 8, or 24 h followed by incubation at 16 °C for a total cumulative incubation period of 24 h. Following modified procedures from the Tetrazolium Testing Handbook (Peters, 2000), seeds were bisected longitudinally, and the largest portion of the embryo was excised. There were 20 seeds per plate and 3 plates per treatment. The experiment was repeated twice. All 20 embryos from each plate were placed in 1 ml of 0.1% tetrazolium chloride solution and stored in the dark at room temperature for 4 h. Seeds were rinsed in 2 volumes of MiliQ water and scored for viability. Seeds only were considered viable if the entire embryo stained bright pink. All embryos with partial staining were recorded as dead. Percent viable seed was calculated for each treatment.

2.5. Statistical analysis

All statistical analyses were performed using SAS for Windows 9.3 (SAS Institute, Cary, NC). Gradient bar and heat-shock data were analyzed using nonlinear regression techniques. Based on general linear model (GLM) analyses, the percent viability for the two trials for tetrozolium staining did not differ significantly and the trials were pooled for each treatment. A Tukey's HSD mean separation ($\alpha = 0.05$) was used to determine treatment differences.

3. Results and discussion

3.1. Germination of calendula at constant temperature

3.1.1. Cumulative germination percent

Calendula seeds that were germinated at different temperatures had a reduction in cumulative germination as gradient bar temperature increased and decreased around T_o (Fig. 1). Temperatures from 13.6 to 21.1 °C supported an average cumulative germination percent of 87 ± 8.3% SD after 9 d. The three experimental trials (1–3) were significantly different from each other, and quadratic and cubic regression models were fit to germination data within each experiment (Table 1). Critical temperatures (T_b, T_o, and T_c) were calculated from each model (Table 1). The predicted T_b was in the range of 0.0–3.2 °C. For five of the models the T_o was in the range of 15.6–16.8 °C, with the cubic model for experiment 1 giving a T_o of 11.7 °C, much lower than the others. This same pattern was observed for the T_c, with the cubic model for experiment 1 predicting the T_c as 23.7 °C, and the other five models ranging from 29.3 to 32.6 °C. Because of these discrepancies, the cubic model for experiment 1 was not used for critical temperature estimates. From these models we concluded that the T_o for calendula is 15.6–16.8 °C, T_b is 0–3.2 °C and T_c is 29.3–32.6 °C. Joly et al. (2013) reported a T_b for calendula of 5.5 °C for soil hydrothermal time based on field germination studies and simulated soil temperatures. Our models predict a lower T_b although the values are similar.

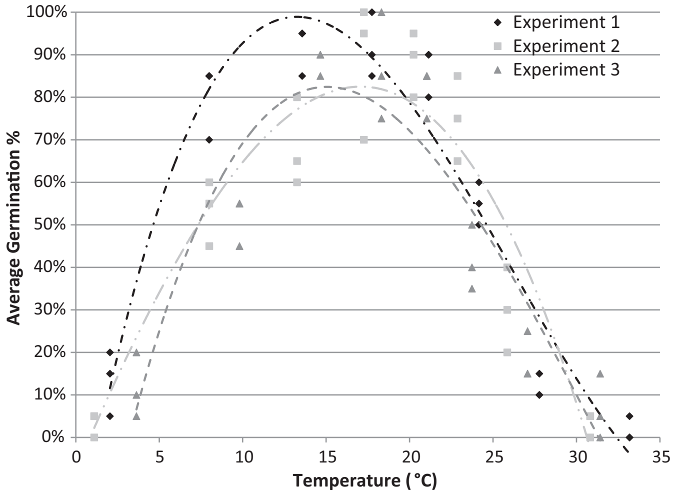

Fig. 1. Cumulative calendula germination across temperature after 9 d. Each data point represents mean germination percent of a single row of 10 seeds. Model curves are fit to the cubic regression (Table 1) for each experimental trial. The data and model for the three experimental trials (experiments 1–3) are shown separately.

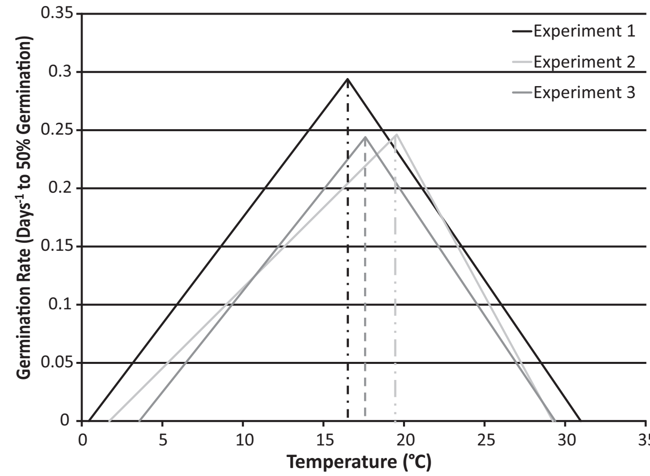

Fig. 2. Sub- and supra-optimal linear regression of calendula germination rate (d^{-1}) to 50% cumulative germination across temperature. Dashed vertical lines mark the intercept corresponding to where the sub- and supra-optimal regressions intersect indicating T_o for each experimental trial.

3.1.2. *Germination rate*

Germination rate to 50% germination (GR50) was regressed for sub- and supra-optimal germination rates for each experimental trial (Fig. 2). T_o was calculated at the intersection of the sub- and supra-optimal linear regressions (Table 2). The values of T_b and T_c were calculated where the sub- and supra-optimal regressions were equal to zero (Table 2). T_o for GR50 was 16.5–19.6 °C, which is higher than predicted by the quadratic and cubic regression models for cumulative germination. T_b and T_c for GR50 were 0.4–3.6 °C and 29.2–31 °C, respectively. These values were very similar to those predicted by the cubic and quadratic models for each experiment. Within each experiment

Table 1
Quadratic and cubic models of cumulative germination % for each experimental trial. Predicted base temperature (T_b), optimum temperature (T_o), and ceiling temperature (T_c) are given for each model.

Experimental trial	Regression equations	r^2	T_b	T_o	T_c
1	$y = 0.0267 + 0.1094\,x - 0.004^2\,x$	0.85	0	15.6	31.4
2	$y = -0.1366 + 0.1203x - 0.004^2\,x$	0.87	1.2	16.1	31.3
3	$y = -0.3036 + 0.1312x - 0.004^2\,x$	0.83	2.5	16.8	31.1
1	$y = -0.2829 + 0.2166x - 0.011^2\,x + 1E{-}04^3\,x$	0.93	1.5	11.7	23.7
2	$y = -0.0778 + 0.0926x - 0.002^2\,x - 5E{-}05^3\,x$	0.88	0.9	16.4	29.3
3	$y = -0.5744 + 0.2081x - 0.009^2\,x + 1E{-}04^3\,x$	0.85	3.2	15.3	32.6

Table 2
Sub and supra linear regression of germination rate for each experimental trial. Predicted base temperature (T_b), optimum temperature (T_o), and ceiling temperature (T_c) are given for each model.

Experimental trial	Model	Linear equation	r^2	T_b	T_o	T_c
1	Sub-optimal	$y = 0.0183\,x - 0.008$	0.85	0.4	16.5	
	Supra-optimal	$y = -0.0203\,x + 0.6288$	0.86		16.5	31.0
2	Sub-optimal	$y = 0.0138\,x - 0.0236$	0.91	1.7	19.6	
	Supra-optimal	$y = -0.0254\,x + 0.7428$	0.79		19.6	29.2
3	Sub-optimal	$y = 0.0174\,x - 0.0622$	0.78	3.6	17.6	
	Supra-optimal	$y = -0.0207\,x + 0.6083$	0.68		17.6	29.4

Table 3
Significance of trial, treatment (trt), temperature (temp), and interactions on calendula seed germination response to heat shock treatment. Bold *p* values indicate significance.

	trial	trt	trial × trt	temp	temp × trt	trial × temp	trial × temp × trt
All seed	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.007**	0.0849
Dry seed	**0.0040**	0.3140	0.1012	0.2105	0.0858	0.3898	0.3659
	0.0054	0.3648	0.1293				
Wet seed	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0052**	0.0940
	0.0001	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0069**	
35 wet	0.0699	**<.0001**	0.8488				
	0.0552	**<.0001**					
40 wet	**0.0001**	**<.0001**	**0.0001**				
Trial 1&2 only	0.1555	**<.0001**					
45 wet	0.5180	**<.0001**	0.8536				
	0.4946	**<.0001**					

the slope for the supra-optimal regression was steeper than the sub-optimal regression slope: 11%, 85%, and 19% steeper, respectively (Table 2 supra-optimal slope/sub-optimal slope). This trend demonstrates that calendula germination rate is reduced more rapidly at temperatures above T_o then at temperatures below. The flatter slopes of the sub-optimal regression correlate with the distribution of calendula culture. That is, it is grown in areas with cool climates, like the United Kingdom, Holland, and the upper Midwest. The broader range of germination at sub- compared to supra-optimal temperatures demonstrates an adaptation to low temperatures.

The T_o estimates from cumulative germination regression models and germination rate models overlap in the range from 16.5 to 16.8 °C and cover a 4 °C range of temperatures from 15.6 to 19.6 °C. Therefore, we estimate that the T_o for calendula germination, where both germination percent and rate are optimized, is 16.5–16.8 °C. Although this is a narrow temperature range, the data also indicate a 4 °C range where temperatures and germination rate also are optimized and a 30 °C range where germination occurs.

3.2. Germination response to heat shock temperatures and duration

The empirical data that calendula seed germination is more susceptible to high temperatures prompted further investigation of the response. Calendula seed was incubated at heat shock temperatures, above the predicted T_c, of 35, 40, and 45 °C (temp) for variable times (trt) to determine how long seed can withstand heat shock before germination is impacted (Fig. 3). For dry seed, time of heat shock and temperature did not significantly affect germination (Table 3). The only significant term in the dry seed GLM was "Trial." Pre-imbibed (wet) seed germination decreased when heat shock incubation time increased at all temperatures (Fig. 3). Temperature was a significant term in the GLM for wet seeds (Table 3) showing that germination responded differently to the three temperatures. Within each temperature, the treatment time was significant for wet seed germination (Table 3). At 40 °C wet seed germination for Trial 3 was significantly different from Trial 1 and Trial 2 (Table 3). Trial 3 had a higher germination percent at 48 and 96 h than the other two trials, but the germination percent still decreased as the incubation time increased (Fig. 3). As heat shock temperature increased from 35 to 45 °C, the time required for germination to drop to 0% was shortened. After 196 h at 35 °C, wet seed germination stayed above 10% germination. While after 192 h at 40 °C, all three trials had 0% germination. After only 48 h at 45 °C, the germination was 0% (Fig. 3). Additionally, after only 48 h exposure to 35, 40, or 45 °C, seed germination was less than 50% even after seeds were transferred to T_o (16 °C).

To elucidate if calendula germination at heat shock temperatures was reduced because of embryo death or thermodormancy we stained embryos excised from seeds with tetrazolium chloride (TZ) and measured viability (Fig. 4). Pre-imbibed seeds were incubated at 45 °C for 0, 2, 8, or 24 h followed by 16 °C for a cumulative incubation time of 24 h. After TZ treatment embryos that stained completely pink were counted as viable (Fig. 4a; V) and embryos that had partial pink staining or no staining were counted as dead (Fig. 4a; D). Seeds incubated at 0/24 h and 2/22 h at 45/16 °C had 70% viable embryos and the two treatments were not significantly different (Fig. 4b). At 8/16 h embryos were 37% viable, and at 24/0 h there were 0% viable embryos (Fig. 4b). The percent viable seed decreased with increased incubation time at 45 °C showing that with heat-shocked calendula seeds were inhibited irreversibly. The exact cause of seed death was not investigated, but thermal death of seeds has been shown to be moisture sensitive (Couture and Sutton, 1980). The cause of thermal death has been attributed to membrane fluidity and function as well as protein breakdown (Hendricks and Taylorson, 1976; Murphy and Noland, 1981). Active oxygen species are known to increase during seed imbibition and are scavenged by the cells to prevent damage (Bailly, 2004). They possibly remain in high concentration at these temperatures and cause seed death.

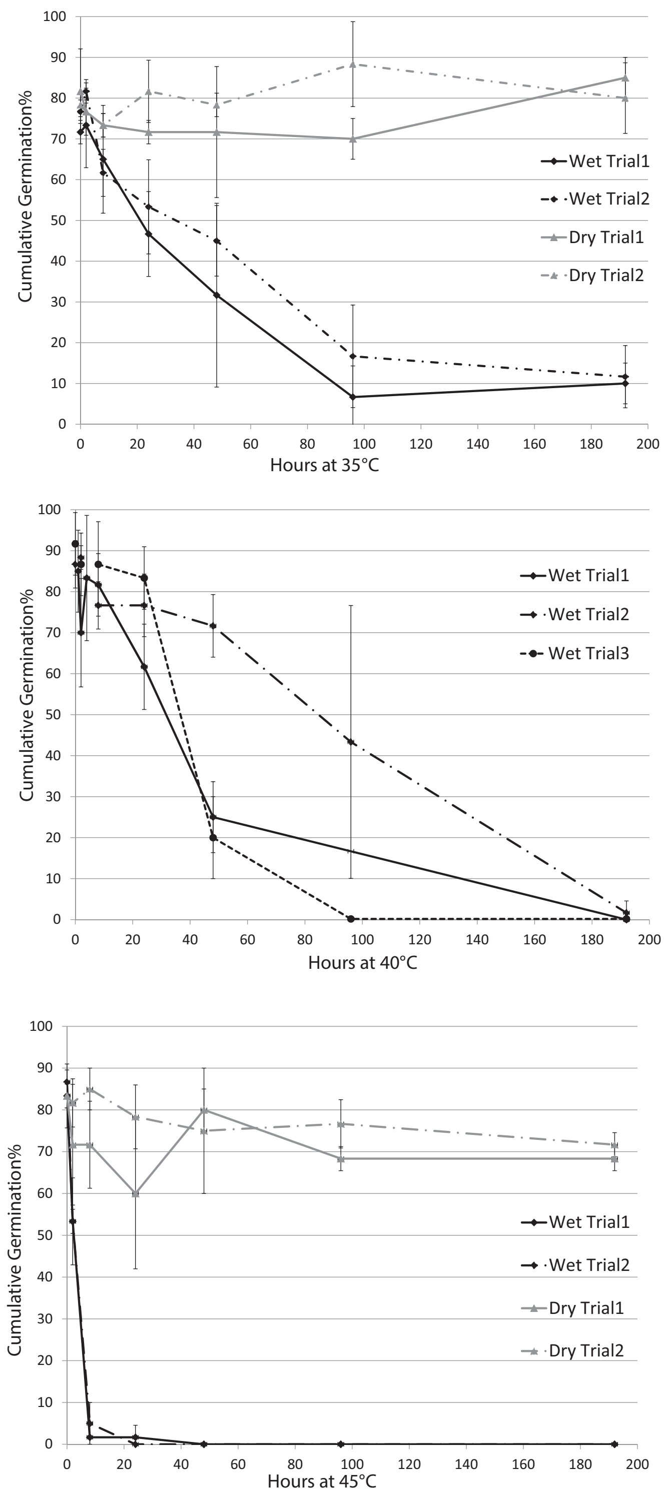

Fig. 3. Calendula germination response to heat shock temperature and duration. Error bars are StDev. Following heat shock, seed was incubated at 16 °C for a cumulative incubation time of 14 d when percent germination was measured.

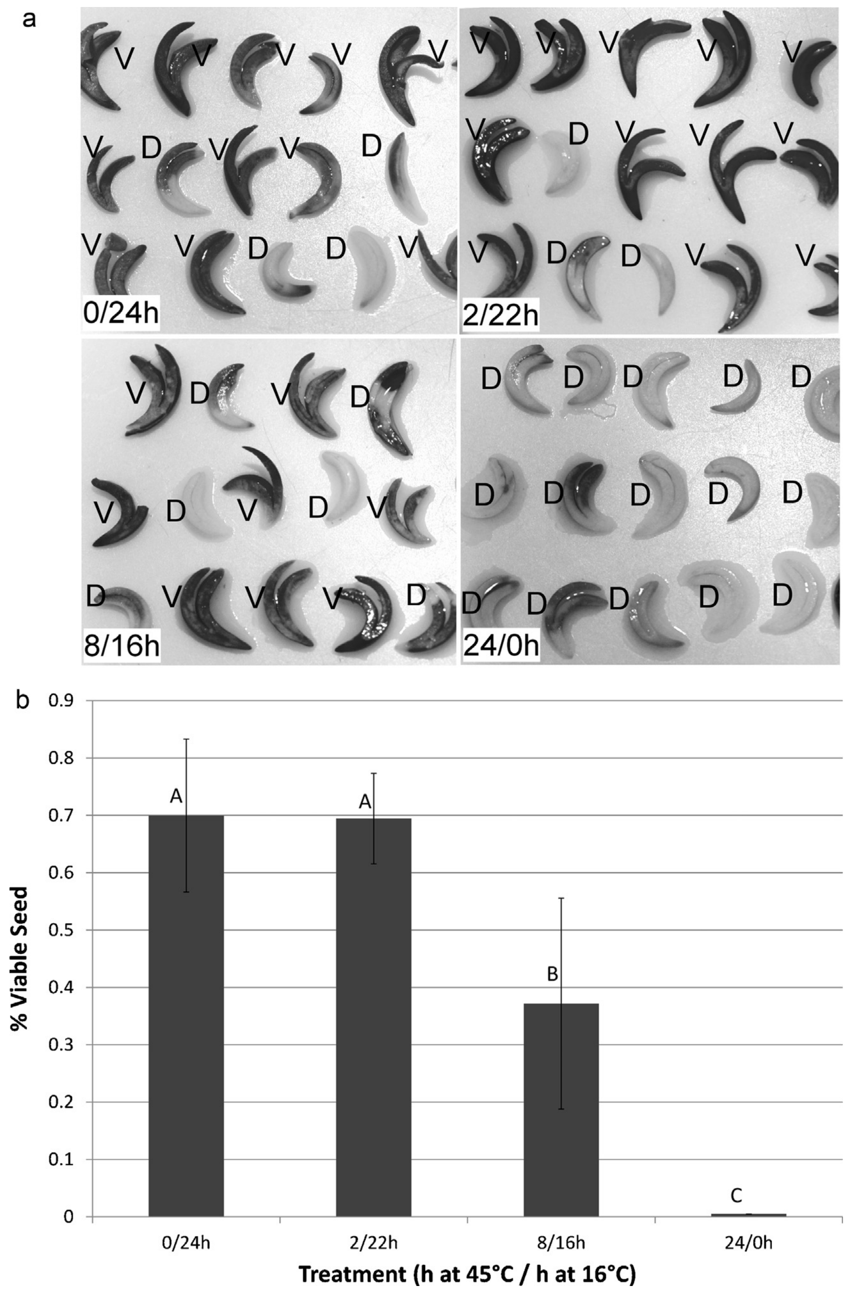

Fig. 4. Viability of calendula embryos following four different 45 °C incubation treatments. Seeds were incubated at 45 °C for 0, 2, 8, or 24 h followed by 16 °C for 24, 22, 16, or 0 h (0/24 h, 2/22 h, 8/16 h, 24/0 h, respectively) for a total treatment time of 24 h. (a) Excised calendula embryos stained with tetrazolium chloride. Black embryos have high staining which marks live tissue. Viable embryos are marked with a 'V' and dead embryos are marked with a 'D'. Panels show representative embryos for the 4 seed treatments. (b) Percent of embryos that were viable in each treatment. Averages were calculated from three replicates of 20 seeds per trial with two trials in time. Error bars are SD and letters above bars are Tukey's HSD mean separation classification at $\alpha = 0.05$.

4. Conclusion

Calendula germination is optimized both in rate and cumulative percent at temperatures of 16.5–16.8 °C. As temperature increased above 17 °C, germination decreased. Furthermore, as imbibed calendula seed was exposed to heat shock temperatures, germination was unable to recover even after seed was placed at T_o. These results indicate that if calendula seed is planted at a relatively low soil depth as is currently recommended, followed by a period of high temperature, stand establishment likely will be affected negatively. While germination percent and rate also were reduced by temperatures below T_o, it was previously demonstrated that calendula requires a cumulative soil hydrothermal time (duration in degree-days that soil was above T_b and base water potential) of 89 °C d to emerge; therefore, the reduced germination under low temperatures is caused most reasonably by a delay in germination and not seed lethality (Joly et al., 2013).

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