**Title:** An easy and automated calculation of the germination cardinal temperatures and thermal time using R

**Running Head:** Thermal Time & Cardinal Temperatures in R

Emma Ladouceur1,2, Hugh W. Pritchard3, Eduardo Fernández-Pascual3\*

1Museo Delle Scienze (Muse), Corso del Lavoro e Scienze, 3, Trento, Italy

2University of Pavia, Pavia, Italy

3Royal Botanic Gardens, Kew, Wellcome Trust Millennium Building, Wakehurst Place, West Sussex RH17 6TN, England

**\*Corresponding author:** [eduardofp.indurot@uniovi.es](mailto:eduardofp.indurot@uniovi.es); Tel.: +44(0)1444894184

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**Abstract (Currently 176--250 max)**

Seed germination traits depend on both environmental and biological factors, and are thus emerging as an underappreciated and powerful mechanism to understand regeneration, ecological restoration, ecosystem management actions, conservation and practical seed use. Currently, identifying some of the most applicable seed germination traits, the three cardinal temperatures; minimum (Tbase), optimum (To), and maximum germination temperature (Tceling), as well as thermal time is of great interest to these fields. However, in the past calculating these traits has been problematic as it requires user judgement which can introduce bias into the analysis, and is a lengthy process. Here, we present a method to calculate these traits in the R language and environment for statistical computing which has three main benefits; 1. The method systematically identifies the breaking point in the data (i.e. the separation between the sub-optimal and supra-optimal germination temperature ranges), avoiding personal bias; 2. The analysis is computed in seconds, in contrast to the hours or days it may take using alternative methods; and 3. The method does not require the purchase of commercial statistical software.

**Introduction**

The quantification of seed germination traits provide insights on plant and seed ecology: from niche competitiveness, to seed stress tolerance and potential impacts of climate change on the timing of seedling emergence (Fenner 2000, Grubb 1977, Jiménez-Alfaro et al 2016, Larson and Funk 2016, Walck et al 2011). Of prime interest currently is the contribution that large datasets can make to the development of predictive models that connect seed responses in the micro-environment to global datasets on the macro-environment, including climate change models. Such studies are best advanced through comparative studies, but the value of these will only be maximized if there is a consistent approach to the generation of data on the germination phenotype and robust means of analyzing the outputs.

Germination is the transition from seed to seedling, and occurs in response to a series of environmental cues including temperature, moisture, light and chemical signals (Bewley et al 2013). For each of these cues there is an optimal value at which the rate of germination is maximal. Below and above this optimum, the rate progressively decreases until the cue reaches base and ceiling thresholds beyond which germination stops. Thus, the rate of germination as a function of temperature can be described in non-dormant seeds as an accumulation of degrees-day above or below these thresholds (Garcia-Huidobro et al 1982). When a certain amount of degrees-day has been accumulated, the seed germinates. Every seed will require its own amount, but since experiments need to be done with a seed population, thermal time is expressed as the degrees-day needed to produce germination in a percentage of this population, usually the 50 %. Measuring the germination cue in degrees-day, i.e. in thermal time, has the advantage of integrating time and temperature (Romo and Eddelman 1995). Therefore, the thermal control of germination can be described in a mechanistic way using a handful of seed traits: the three cardinal temperatures, sub-optimal (base temperature Tb), optimal (To) and supra-optimal (ceiling temperature Tc); and the thermal time. Thermal time traits have two powerful applications. First, parameters calculated in a restricted set of experimental treatments can give good estimations of germination in more complex thermal environments (Hardegree et al 1999). Second, thermal time parameters can be compared in a standard way across seed individuals, populations and species (Trudgill et al 2000). As such, thermal time models provide the broadest base to conduct comparative studies of germination (Dürr et al 2015).

Here, we present a novel method to calculate the thermal time and the germination cardinal temperatures using segmented regression in the open-source software *‘R statistical computing language and platform’* (R Core Development Team (n.d.)) . This method has three advantages over methods currently in use: 1. The method systematically identifies the breaking point in the data (i.e. the separation between the sub-optimal and supra-optimal germination temperature ranges), rather than the user estimating breaking points visually and on a case-by-case basis, which can lead to personal bias; 2. The analysis is computed in seconds, in contrast to the hours or days it may take using alternative methods; and 3. The method does not require the purchase of commercial statistical software. We test the method calculating cardinal temperatures in two mock species; one in which the three cardinal temperatures can be identified, and one in which experimental results did not lead to the successful identification of all the cardinal temperatures. We present here the format in which the data should be arranged (Supplementary Information 1) the script to analyse the data (Supplementary Information 2), and basic instructions for users new to *R* (Supplementary Information 3) so that out method can be completely transparent and rigorously reproducible by all researchers (Rocchini and Neteler 2012).

**Description of the method**

*Data preparation*

The dataset used here was assembled from the data presented by Fernández-Pascual et al. (Fernandez-Pascual et al 2015) and is given as a working example in *‘Supplementary Information 1’*. We present two species in the dataset, ‘*species A’* and ‘*species ‘B*, indicated in the first column, *‘Grouping’*. The second column, *‘Treatment’* indicates the range of temperatures each species was tested by. The third column *‘Dish’* indicates the petri dish number of each *‘Treatment’*. Next, *‘Time’* is time (in days in the example, but can be in any other unit) in which each data point was recorded. *‘G’* indicates cumulative germination count, and *‘PG’* indicates the total sample size of each dish, which in this case is 25 seeds. In this example dataset we present here, there is only one *Dish* for each *Treatment*. In other cases, where an experiment may have several *Dishes*, the script should work in the same way as in this example. The two *Grouping* species used here can also be substituted for different individuals, populations or experimental treatments, depending on the data being analysed.. Although the example is presented with two species, the script is prepared to analyse larger numbers with slight amendment of the script.

*R and R packages needed*

All analyses have been performed in the *R language and environment for statistical computing* (R Core Development Team (n.d.)) and all plots created using the *ggplot2* package in R (Wickham 2009) (Supplementary Information 2). The script we have prepared (Supplementary Information 2), presents the method we have developed, and when used with the example dataset we have prepared (Supplementary Information 1) produces four tables, and the four figures we present here. For users new to *R*, we have prepared the essential basic information to get started in *Supplementary Information 3,* which can be used as a complimentary resource to understand the following*.* In total six specialist packages are required to install into your *R* library. First, we used the packages *plyr* and *dplyr* (Wickham and Francois 2016) for all activities related to management and filtering of data.The package *binom* (Dorai-Raj (n.d.)) was used to manage and analyse binomial data which is a particularity of data analysis in germination experiments. The package *drc* (Ritz et al 2015) was used to fit a dose-response model to the cumulative germination data. The package *segmented* (Vito (n.d.)) was used to set a function to fit a segmented regression and compute the intercept and the slope for each segmented relationship in the model.

*Step 1: Checking whether the data represents the full germination temperature range*

The data is first grouped by germination treatment and dish, and then filtered into a new file to only represent the results of the final scoring date (i.e., the final germination proportions). Then, a function is created to estimate the mean final germination proportions and binomial confidence intervals and this function is applied across treatments and the first new dataset is produced, *Table 1:‘FGP*’ (Final germination proportions). Then this new dataset is plotted in the first plots we create *Figure 1:* *FGPfig*, which represents the total mean germination of each treatment, plotted against temperature treatment (Figure 1). At this point, *Figure 1* needs to be visually inspected for evidence that the temperature treatments used in the experiment resulted in representing the full germination temperature range of the study species. If this is the case, the segmented model we present here can be fitted and used to calculate the three cardinal temperatures. In *species A*, we generated data representing the full range of germination temperatures (Figure 1A). When the experimental treatments only give results in either the sub- or the supra-optimal germination temperature range, the segmented model cannot be fitted and only the base or ceiling temperatures can be calculated. In *species B,* the supra-optimal temperatures were not identified, as after the best germination rate was reached at treatment ‘23.75’, higher temperatures did not produce any germination and had a total cumulative germination of zero (0) (Figure 1B).

*Step 2: Estimating germination rates from the cumulative germination curves*

A dose-response model is fitted to the cumulative germination data. This stages allows you to test and to fit different functions, and we have chosen by default the log-logistic in the example dataset because it gave the best fit in previous experiences with other dataset. The user needs to check if this function gives the best fit to his/her own data. The script automatically tests the fit of different functions, and suggests the model that best fits each treatment. The user should use this information in the table *‘FSfit’* to change the function chosen manually in the next steps (see comments within Supplementary Information 2). This is the most particular part of this analysis. When comparing any grouping or treatment against another, different models may fit particular temperatures better than others. However, it is very important that the same model must be used across all treatments for balanced comparison. If this is the case, we suggest to use the model that fits the majority of treatments. The model with the best fit is used to plot the cumulative germination of each treatment against time, and the function fit needs to be checked visually in our second figure *Figure 2:* *CGfig* (Figure 2). The user must decide if they can trust the fit of their curve to the data using both the information in table *‘FSfit’* and the curves produced in *Figure 2:* *‘CGfig’*. The models are used to calculate the time to reach successive deciles of germination (from 10 to 90 %) in each treatment. It can be possible that not good fit can be found, for example, if you have very low final germination, or if you scored once per week, and all of the seeds germinated in the first week. If this is the case, you cannot calculate cardinal temperatures, and we suggest repeating the experiment, trialling new treatments, or even trying a move-along experiment (C. Baskin and Baskin 2003), to learn more about the species before attempting another trial aimed at the specifics of cardinal temperatures. The inverse of the time is then calculated to obtain the germination rate of each temperature treatment, and we create a second new dataset, *Table 2: GR* (Germination rates).

*Step 3a: Fitting a segmented model to the full germination temperature range*

Where sub-optimal and supra-optimal temperatures are available in the data set (Figure 1a), germination rates in the suboptimal (Tb-To) and supraoptimal (To-Tc) temperature ranges and thermal time (theta-sub, theta-supra) can be calculated. In this scenario, a segmented regression model is fitted to the data (Figure 3A). A segmented model allows the identification of breaking points in the data (i.e., the optimal germination temperature in which the germination rate is maximal). Then the model fits linear regressions separately to the two segments defined by this breaking point (*i.e*., the suboptimal and supraoptimal germination temperature ranges) (Figure 3A). A function is set to fit a segmented regression and compute the intercept and the slope for each segmented relationship in the model. The function requires the user to give an estimation of the breaking point (‘psi=’) , which can be based on the visual inspection of *Figures 1 & 2*. In this example we estimate the breaking point to be ‘psi=25’. Once the model is fitted, the base temperature (Tb) is calculated by solving the suboptimal linear regression for the x-intercept (i.e., the temperature in which the value of the germination rate equals zero). An analogous procedure calculates the Tc using the supra-optimal linear regression. The inverse of the slopes of each regression gives the estimated thermal time for germination in degrees-day. The script calculates the cardinal temperatures and thermal times for each of the ten decile germination rates (10-90%). The script exports this in a third new dataset, *Table 3: CT* which compiles the estimated cardinal temperatures and thermal time. The plots with the germination rate versus temperature and the fitted models for every decile are exported in the third figure we create *Figure 3:* *BLfig*. *Figure 3A* allows to the user to visually check the fit of the model to the data.

It may be the case that the temperatures used in a given experiment fall only on the suboptimal or supraoptimal germination temperature range, and do not succeed to identify the full range of temperatures (Figure 1B). In this case, the dataset *Table 3:* *CT* cannot be produced. In the example of *species B* it is impossible to determine the Tc nor the To, and the segmented model cannot be fit to the data (Figure 3B), and it is necessary to skip forward to the next step in the script.

*Step 3b: Fitting a linear model to the sub- or supraoptimal germination temperature range*

In the case of *species B*, or any experiment which fails to identify the full range of temperatures (Figure 3B), a standard linear regression can be used to identify either the Tb or the Tc. The inverse of the slope of this regression gives the thermal time for germination in degrees-day. Here, we can produce a fourth new dataset, which indicates the suboptimal temperatures identified for *species B; Table 4: LM*. This will automatically produce information for whichever single segment was identified in each unique dataset either Tb or Tc and thermal time. We then plot the germination rate against each temperature treatment using a smooth linear model for each decile in *Figure 4B* (*LMfig*). Furthermore, it must be noted that this script can be used to calculate the germination base water potential, if the data set that is analysed represents an experiment of germination in different water potential solutions. In this case, the values of the column *Treatment* would be the water potentials instead of the temperatures. Lastly, we attach a short script to produce a smooth curve in the case of irregular data exploration in *SMTHfig*.

**Discussion**

This method is appropriate for the analysis of germination data to identify cardinal temperatures and thermal time. The method we present here offers an opportunity to calculate the analyses rapidly, and to select the breaking point in the data without user bias. When using this script, we recommend that users become familiar with the basics to using R, and seek out one of the many introductory tutorials that exist online. We provide some guidance here (Supplementary Information 2 & 3). We suggest to ensure all packages are installed, and libraries uploaded, and follow the notes in the script using the example data sets in the supplementary information, using this paper as a complimentary resource to the script.

To engage in an experiment that will set out to identify cardinal temperatures, one must plan an experimental design which will purposefully identify all the germination temperature range, with data points below the optimal temperature (sub-optimal) and above the optimal germination temperature (supra-optimal), pushing each species to its thermal thresholds. In order to fit a segmented curve, there must be a minimum of three sub-optimal temperatures, and a minimum of three supra-optimal temperatures. It is thus recommended that experimental designs include a minimum of 5-6 temperatures for each to effectively explore potential germination temperature ranges. The less that is known about a given species, the more temperatures that should be tested.

Constant temperatures are known to produce promising results for testing cardinal temperatures, particularly for agricultural varieties which have been bred for uniformity, and stable germination temperatures (Covell et al 1986, Ellis et al 1986). However many wild species may require alternating temperatures, or alternating temperatures may better represent real ambient conditions found in nature (Carol C Baskin and Baskin 2014). When testing with alternating temperatures, it is recommended for plotting that the average temperature be taken of the two alternating temperatures for each treatment (Ellis and Barret 1994). Thus, alternating temperature regimes should be carefully planned to still result in temperature gradients when averaged, and may lead to odd results when one of the two alternating steps is outside the germination temperature range. Constant temperatures should never be compared to alternating temperatures within the same analysis, but if testing both, the two temperature regimes should be set up as two experiments or *‘Groupings’*, to compare against each other. Ideally, in setting up an experimental design for wild species, a regime of both constant and alternating temperatures could be tested separately, and the results compared. Experiments are recommended to be run until cumulative germination stops or reaches a plateau. Scoring dates should be adjusted to the speed of germination of the study species, so a cumulative germination curves with good resolution are produced. Furthermore, it is important to consider that the cardinal temperatures are not fixed values but depend on the dormancy state of the seeds (Pritchard et al 1999).

We have found and confirmed that this method is useful to calculate thermal time and cardinal temperatures in germination experiments which have tested a full range of temperatures across a given species germination gradient, both rapidly and rigorously. We believe that this method will be of great value to generate cardinal temperature data for an ever increasing number of species. Physiological thresholds such as the cardinal temperatures are highly informative plant traits, and a key tool to integrate plant regeneration into multi-species community studies (Jiménez-Alfaro et al 2016, Larson and Funk 2016).

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**Figure 1:** Final Germination proportions across all temperature treatments for species A) and species B)



**Figure 2:** Cumulative Germination Curves across all temperature treatments for species A) and species B)



**Figure 3:** Time to germination across each decile (%) of total germination, across each treatment using a segmented model for species A) and species B).



**Figure 4:** Time to germination across each decile (%) of total germination across each temperature treatment, using a smooth linear model for species A) and species B)