Subterranean clover (*Trifolium subterraneum* L.) seed germination responses to temperature and water potential

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Abstract
We investigated sub clover germination responses to different combinations of constant temperature (*T*) and water potential (*WP*) under laboratory conditions. Seeds of sub clover 'Napier' were incubated in the dark in combinations of *T* = 5, 10, 15, 20, 25, 30 and 35°C; and *WP* = 0, -0.18, -0.37, -0.63 and -0.95 MPa. Seed germination was monitored every 8 hours for the first 4 days, then inspected every 24 hours for 14 days. Results were analysed in graphical form. Maximum or near-maximum percent germination (*GP*) occurred over a broad range of *T* (15-25°C) under moist conditions. However, the optimal temperature range for *GP* was markedly narrowed when *WP* was -0.63 MPa and -0.95MPa. In contrast to *GP*, optimum median germination rate (*GR*(50)) occurred over a much narrower range for both *T* and *WP*, reaching a maximum at 30°C and 0 MPa, and declining more-or-less linearly with decreasing *T* and *WP*. The optimal temperature for *GR*(50) appeared to interact with *WP* so that it occurred at 30°C for *WP*= 0, -0.18 and -0.37 MPa, but at 25°C for -0.63 MPa. At the maximum *T* in this experiment (35°C), *GR*(50) declined from the optimal rate but was still quite rapid under zero or mild *WP* stress. Therefore it was not possible to identify a ceiling temperature where *GR*(50) was zero, under these moist conditions. Implications of these results for field germination of sub clover are discussed.

Keywords
Seed germination, subterranean clover, *Trifolium subterraneum* L., thermoinhibition.

Introduction
Sub clover is widely established in New Zealand dryland pastures (Smetham 2003; Boswell et al. 2003). Understanding its germination and how this impacts subsequent re-establishment and regeneration under different soil moisture and temperature regimes would be useful in both ecological and agronomic management (Monks et al. 2009). Laboratory experiments under controlled conditions can assess seed germination responses to constant or varying ambient temperature and water potential. However, while a number of authors have studied thermal requirements for sub clover germination using this approach (Moot et al. 2000; Monks et al. 2009; Lonati et al. 2009), few studies have investigated effects of factorial combinations of ambient temperature (*T*) and water potential (*WP*) on sub clover germination. The objective of this study was seed germination analysis of subterranean (or sub) clover (*Trifolium subterraneum* L.) under controlled conditions. Here we report sub clover germination responses to different combinations of constant water potentials and temperatures under laboratory conditions.

Materials and Methods
Seeds of sub clover 'Napier' (supplied by Seed Force Ltd) were screened to remove empty or broken seeds from each population. Then they were lightly dusted with fungicide (Thiram). Seeds were germinated on filter papers (Whatman No.2, Advantech MFS, Inc. NO232) within plastic containers previously sterilised by ethanol 80%.

The filter papers had been soaked in polyethylene glycol (PEG) (Aldrich, mol.wt.8000) solutions of known water potential for 24 hours prior to the placement of seeds. PEG solutions were made up to a specific water potential using Equation (1) (Hardegree and Emmerich 1990, Equation 4):

$$\Psi = 0.130\,[PEG]^2\,T - 13.7\,[PEG]^2 \quad (1)$$

Where Ψ is the water potential of the solution, [PEG] is the concentration of PEG (g/g) in solution, and T is the temperature of the solution.

Water potentials of the PEG solutions and of germination media were checked twice weekly. Three replicates of 50 seeds were placed on the filter papers and 10 ml of relevant solutions added to each plastic container. Containers were then sealed and incubated in the dark in a randomised complete block design with factorial combinations of T = 5, 10, 15, 20, 25, 30 and 35°C; and WP = 0, -0.18, -0.37, -0.63 and -0.95 MPa. Seed germination was monitored every 8 hours for the first 4 days, then inspected every 24 hours for 14 days. Seeds were considered germinated when a visible radicle protrusion (≥ 2 mm) occurred, at which point they were removed (Soltani et al. 2002). Results are analysed in graphical form.

Germination percentage (GP) was defined as the final accumulated seed germination (%) at the end of each experiment. Germination rate (GR) of individual seeds or percentiles in a seed population is defined as the inverse of time taken by that seed or percentile to complete germination. Since there were 50 seeds per replicate, each germinated seed counted as 2%. The 50th seed percentile (25th seed in the replicate) to germinate was considered the median percentile, as this would be the median seed to germinate under optimum conditions.

Data analysis

Both *GP* and germination rate of the 50th percentile to germinate (*GR*(50)) were plotted against ambient *T* and *WP* using 3-D surface graphs to show the interacting effect of *T* and *WP* on germination behaviour. The *GR* of the 50th percentile is an appropriate indicator, in that it is the median *GR* for the population, and also an indicator of the speed at which an acceptable level of germination is reached from an agronomic perspective. To investigate the effects of *WP* and *T* on *GP*, analyses of variance were conducted in Genstat 16.1.

Results

Germination percentage

GP was affected by *T* and *WP* ($P<0.001$). Responses of *GP* to *T* indicates that, under zero or mild *WP* stress (0, -0.18, -0.37 MPa), the average *GP* was similar (88% ± 2%) over a broad range of *T* (15-25°C). *GP* was still above 80% at the minimum (5°C) and maximum (35°C) temperatures for this experiment (Figure 1a). However, the optimal temperature range for *GP* was markedly narrowed when *WP* was -0.63 MPa, and *GP* reduced to <10% when *WP* was -0.95MPa, for all *T* except 20ºC.

Median (GR(50)) germination rates

Germination rates of the 50th percentile (*GR*(50)) at different *T* and *WP* are shown in Fig 1b. In contrast to *GP*, optimum *GR*(50) occurred over a much narrower range for both *T* and *WP*, reaching a maximum at 30°C and 0 MPa, and declining more-or-less linearly with decreasing *T* and *WP*. Note that when *GR*(50) reaches zero, this is equivalent to *GP* not reaching the 50th percentile (GP<50%). This occurred above and below the *T* range 15-25°C when *WP* = -0.63 or -0.95 MPa. The optimum temperature for *GR*(50) appeared to interact with *WP* so that it occurred at 30°C for *WP*= 0, -0.18 and -0.37 MPa, but at 25°C for -0.63 MPa. At the maximum *T* in this experiment (35°C), *GR*(50) declined from the optimal rate but was still quite rapid under zero or mild *WP* stress. Therefore it was not possible to identify a ceiling temperature where *GR*(50) = 0, under these moist conditions.

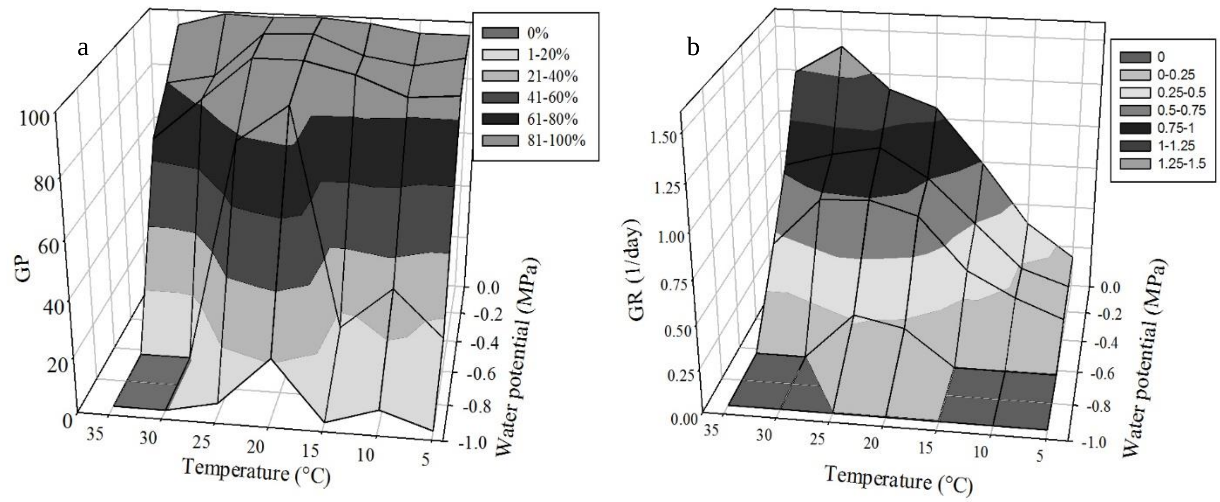

Figure 1. Surface plots of (a) germination percentage (*GP*) and (b) *GR*(50) against temperatures (ºC) and water potential (MPa) for sub clover. The key shows the different shades of grey representing different ranges for *GP* and *GR*(50), with 20% intervals (0%, 1-20%, 21-40%, 41-60%, 61%-80% and 81-100%).

Discussion and Conclusions

The results of this experiment show that sub clover germinates very rapidly compared with other commonly-used pasture species in New Zealand, and maintains high *GP* over a relatively wide range of *T* and *WP*. Both the *GP* and *GR* of sub clover 'Napier' seeds were quite resilient to changes in *T* and *WP*. Germination percentages at *WP*=0 MPa were high across the range of temperatures used in this study and only began to significantly decline when *WP* was -0.63MPa or less.

Compared with *GP*, *GR* for sub clover did show greater sensitivity to *T* and *WP*, but both *GP* and *GR* for sub clover are still much more resilient to varying *T* and *WP* conditions than for white clover (Shyanfar et al. 2017). *GR* for sub clover is also more rapid and resilient to varying *T* conditions than for perennial ryegrass (1), although ryegrass *GR* is markedly more resilient to *WP* stress than either sub clover or white clover (Shyanfar et al. 2017; McWilliam et al. 1970).

GR is a critical parameter in field germination, because early emergence may confer a competitive advantage over other species when sown in mixtures or when germinating from a seedbank in a mixed pasture. Under warm moist conditions, sub clover is likely to germinate more rapidly than white clover or perennial ryegrass, but under dry conditions this advantage will be lost (Shyanfar et al. 2017). Jansen and Ison (1994) discuss the germination of sub clover under warm conditions, terming the failure of seed to germinate at high temperatures, even with adequate moisture, as "high-temperature dormancy." This phenomenon is common to many seed plants and is also called "thermoinhibition" (Watt and Bloomberg 2012). Norman et al. (1998) state that the ecological significance of thermoinhibition for clovers is that when temperatures are >30°C after false breaks, even soft-seeded clovers will not germinate. In the absence of further rain, soils and imbibed seeds will dry rapidly under warm conditions, once again precluding germination. Thermoinhibition will, therefore, substitute for hardseededness as an adaptation that prevents germination during false breaks, and "probably does so in *T. subterraneum*."

In this experiment, the maximum *T* treatment was 35°C , and under conditions of low *WP* stress this did not seem to be warm enough to result in significant reduction in either *GP* or *GR* for sub clover 'Napier, DM32Ø45RC'. This contrasts with the results for sub clover reported in Jansen and Isons (1994) and requires further investigation. Thermoinhibition of sub clover seeds should be investigated over the range 30-45°C and for a range of hard-seeded and soft-seeded sub clover seed lines.

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