POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 32(4): 681–693, Y. 2017

TEMPERATURE PRETREATMENT EFFECTS ON *TRIFOLIUM PRATENSE* L. SEED DORMANCY AND GERMINATION

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Key words: hard seeds, hydrotime modelling, red clover, seed physiological parameters.

Abstract

The cause of seed dormancy relief may be various external factors, however the most data suggest particular role of temperature, especially it is seasonally changing environmental cue. The impact of temperature on hydrotime model parameters of red clover seeds has not been studied up to date. The aim of the study was to determine the water relations of red clover seeds during germination after different constant or fluctuating temperature pretreatment in a dry and moist seedbed, on the basis of the hydrotime model. The highest germination was obtained as a result of temperatures in a moist seedbed thanks to a shift of the mean base water potential towards negative values. Alternating positive temperatures broke the dormancy of red clover seeds to the greatest extent. The use of the hydrotime model to characterise and predict relief of combinational dormancy may be a very effective approach, especially for cultivars, which contains a small percentage of hard seeds. Red clover seeds do not need extreme temperatures or large amplitudes of temperatures alternation to break dormancy in temperate climates. Our results acknowledged the advisability of sowing red clover in autumn because exposition to winter and early spring conditions allow seeds to reach a high vigour and successfully emerge in spring.

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WPŁYW TEMPERATURY WSTĘPNEGO PRZECHOWYWANIA NA KIEŁKOWANIE I SPOCZYNEK NASION *TRIFOLIUM PRATENSE* L.

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Słowa kluczowe: nasiona twarde, modelowanie hydroczasowe, koniczyna czerwona, parametry fizjologiczne nasion.

Abstrakt

Przyczyną ustępowania spoczynku fizycznego nasion mogą być różne czynniki zewnętrzne, jednak najwięcej danych sugeruje, że szczególną rolę może odgrywać temperatura, zwłaszcza że jest zmieniającym się sezonowo sygnałem środowiskowym. Dotychczas nie badano wpływu temperatury na parametry modelu hydroczasowego nasion koniczyny czerwonej.

Celem badań było określenie stosunków wodnych podczas kiełkowania nasion *Trifolium pretense,* wstępnie przechowywanych w stałych lub zmiennych temperaturach w suchych lub wilgotnych warunkach, w oparciu o model hydroczasowy. Najwyższe kiełkowanie otrzymano w wyniku działania różnych temperatur w środowisku wilgotnym, dzięki przesunięciu średniego progowego potencjału wodnego ku wartościom ujemnym. W największym zakresie spoczynek nasion koniczyny czerwonej został przełamany przez zmienne dodatnie temperatury. Zastosowanie modelu hydroczasowego do charakterystyki i przewidywania ustępowania mieszanego spoczynku okazało się bardzo obiecującym podejściem, szczególnie dla odmiany zawierającej niewielki procent nasion twardych. Nasiona koniczyny czerwonej do przełamania spoczynku w klimacie umiarkowanym nie wymagają ani ekstremalnych temperatur, ani zmiennych temperatur o bardzo dużej amplitudzie.

Wyniki badań potwierdziły celowość siewu koniczyny czerwonej jesienią, ponieważ ekspozycja na zimowe i wczesnowiosenne warunki pogodowe pozwala nasionom osiągnąć wysoki wigor i z sukcesem wschodzić wiosną.

Introduction

Trifolium pratense L. is a widely grown and important forage legume in many countries. However, its usefulness can be reduced due to low persistence. Physiologically, red clover is a perennial, but depending on field and weather conditions, it may behave like an annual, a biennial, or a short-lived perennial. For permanent use, seeds of this species have to be sown for several years. Although there are few studies on importance of red clover reproduction and seedling regeneration in this species perenniality. The data suggest that the red clover population can persist by natural reseeding in permanent meadows (SAKANOUE 2004).

The seed population of this species is usually comprised of a high proportion of hard seeds. Physical dormancy can be broken artificially by different treatments such as acid or mechanical scarification, or exposure to very high or very low temperatures (HERRANZ et al. 1998, MARTÍN, GUERRERO 2014, ŻUK-

-GOŁASZEWSKA et al. 2007). However, the softening of hard seeds in natural conditions is only partly understood. In many tropical and Mediterranean ecosystems, fire or very large daily temperature fluctuations are important factors (MORENO-CASOLA et al. 1994). Many authors suggest that passage through the animal's digestive tract, mechanical abrasion by soil particles or seed coat decomposition by microbial action are factors which cause hard seed permeability. However, direct evidence for it is lacking. Moreover, these factors are not dependent on the seasons of the year and many hardseed species from the *Fabaceae* family, such as red clover, exhibit seedling emergence seasonal pattern (BASKIN et al. 2000, ASSCHE VAN et al. 2003). Hard seeds need specific duration of time to become permeable and capable of germination. That is why many seeds that fall in late summer have to pass through winter before germination. There is some evidence that temperature can also be a factor in breaking the physical dormancy of the seeds of certain species in temperate climates, despite the fact that extreme temperatures do not occur there and temperature fluctuation amplitudes are not very large. It can be supposed that temperature acts as an environmental cue for the germination of hard seeds in temperate climates (ASSCHE VAN et al. 2003). It is known that in years with high temperatures and with low precipitation levels a red clover emergence is reduced (ŻUK-GOŁASZEWSKA et al. 2006).

The hydrotime model is a valuable approach to describing the phenomenon of seed germination in relation to available soil water and to analyse germination rates at different water potentials in a population. This model has explained the impact of different treatments on the seed germination of numerous species (BRADFORD 1990, BATLLA and BENECH-ARNOLD 2004, WANG et al. 2005, WINDAUER et al. 2012, BOCHENEK et al. 2009, 2010, 2016). BOCHENEK et al. (2007) have presented the influence of environmental conditions on field buried seed parameters derived from the hydrotime model. ŻUK-GOŁASZEWSKA et al. (2007) used this model to describe the physical dormancy break by acid scarification. The influence of different temperature pretreatment on red clover seed parameters derived from the hydrotime model has not yet been studied.

The aim of the study was to determine the water relations of red clover seeds during germination after different constant or fluctuating temperature pretreatment in a dry and moist seedbed, on the basis of the hydrotime model.

Material and Methods

Plant material

Red clover seeds (diploid cultivar Krynia) were obtained from field cultivation at the Experimental Station in Bałcyny, Poland (53°40’N, 19°50’E) and stored dry at room temperature (22–23°C) for one year. The initial germination was 70%, and after storage for one year, 73%. The mean value and the standard error of the mean of the calculated moisture content for all red clover seed samples after one year storage was 9.52% ± 0.14.

Seed treatment

After one year storage, four seed lots were packed separately in fine mesh nylon envelopes. Each envelope with seeds was buried in wet light loam (24% water) in a closed plastic pot and stored at constant temperatures of 3 or -10°C or fluctuating temperatures of -5/5, or 2/12°C, 12/12 h for 14 days. Other 4 seed portions were packed separately in paper bags and dry stored at the same temperatures for 14 days.

Germination test

Before the experiment and after storage of 14 days, the seeds representing each temperature and experimental variant were tested for germination at water and reduced water potentials (0, -0.2, -0.4, -0.6 and -0.8 MPa), which were determined utilizing polyethylene glycol (PEG 8000) solutions prepared as in MICHEL’S paper (1983). The water potentials were verified using a vapor pressure osmometer (Wescor model Vapro 5520) calibrated against NaCl standards. Germination tests were performed in glass 9 cm Petri dishes (3 replications of 50 seeds), on two layers of filter paper moistened with 5 ml of water or a PEG solution at the indicated ψ. The dishes were placed in plastic bags to prevent evaporation and were subsequently incubated in low-temperature incubators at a constant temperature of 19°C, for 5 days, except for brief periods when germination was scored. Seeds which did not germinate were transferred to fresh solutions after 48 h, to maintain a constant water potential in the dishes. Germination was recorded at 4, 12 or 24 h intervals depending on the rate of germination. The germination criterion was a visible radicle protrusion. Data were transformed into germination percentages and means were calculated.

Data analysis

The hydrotime model, initially proposed by GUMMERSON (1986) and developed by Bradford (1990), describes the relation between the germination rate of a given percentage g (GR_g) and the value of the difference between the seed water potential (ψ) and the physiological threshold water potential for germination of a given fraction g ($\psi_b(g)$). The form of the hydrotime model is:

$$\theta_H = (\psi - \psi_b(g)) \cdot t_g \tag{1}$$

$$GR_g = 1/t_g = (\psi - \psi_b(g))/\theta_H \tag{2}$$

where:
θ_H is the assumed hydrotime constant in seed population
t_g is the germination time for a specific fraction g.

The model supposes that ψ_b distinguishes among seed population fractions. The values of ψ_b are close to a normal distribution which can be characterized by its mean, $\psi_b(50)$ and standard deviation, σ_{ψ_b} (BRADFORD 2002).

Seed germination time courses in different water potential solutions were analyzed by probit regression according to the threshold population hydrotime model (Eqs 1, 2) and the computational procedure proposed by BRADFORD (2002) and GOŁASZEWSKI and BOCHENEK (2008):

$$probit_{(g)} = [\psi - (\theta_H/t_g) - \psi_b(50)]/\sigma_{\psi_b} \tag{3}$$

This procedure allowed the calculation of seed population hydrotime parameters, θ_H, $\psi_b(50)$ and σ_{ψ_b} and enabled the germination courses predicted for the model to be obtained. The coefficient of variation (CV) was expressed as a percentage of the mean.

Results

Red clover seed pretreatment in constant low positive and negative temperatures, both in wet and dry environments, caused an increase in final seed germination. The improvement of germination was slightly better for seed stored in humidity state (Table 1). Constant low temperatures caused a small increase of the $\psi_b(50)$ value (CV of 8% after wet storage and of 15% after dry storage) and decrease of the σ_{ψ_b} value (Table 1, Figure 1, Figure 2). The greatest changes were observed in the θ_H value, which distinctly decreased, particularly in wet environments (CV of 61%). In most cases, the model fit well with the experimental data with values of R^2 from 0.74 to 0.84, so the parameters can be used to compare the effect of the treatment on seed germination performance (Table 1).

Table 1

Effect of constant temperatures on seed germination of red clover and hydrotime model parameters

Treatment	$\psi_b(50)$ [MPa]	$\sigma_{\psi b}$ [MPa]	θ_H [MPa h]	R^2	FG [%]
Before treatment	-0.423	0.396	20.1	0.809	72.7 ± 1.4
3°C, dry	-0.391	0.228	16.07	0.836	79.9 ± 4.9
-10°C, dry	-0.312	0.204	8.37	0.827	78.5 ± 4.6
Mean (CV)	-0.375 (15)	0.276 (36)	14.85 (38)	–	77.0 (5)
3°C,wet	-0.387	0.249	6.68	0.740	80.7 ± 4.6
-10°C, wet	-0.361	0.202	8.61	0.818	83.6 ± 4.9
Mean (CV)	-0.387 (8)	0.282 (36)	11.80 (61)	–	79.0 (7)

Explanation: CV – coefficient of variation expressed as a percentage of the mean; FG – final germination in water ± standard error; $\psi_b(50)$ – mean base water potential; $\sigma_{\psi b}$ – standard deviation of base water potential; θ_H – hydrotime constant; R^2 – coefficient of determination

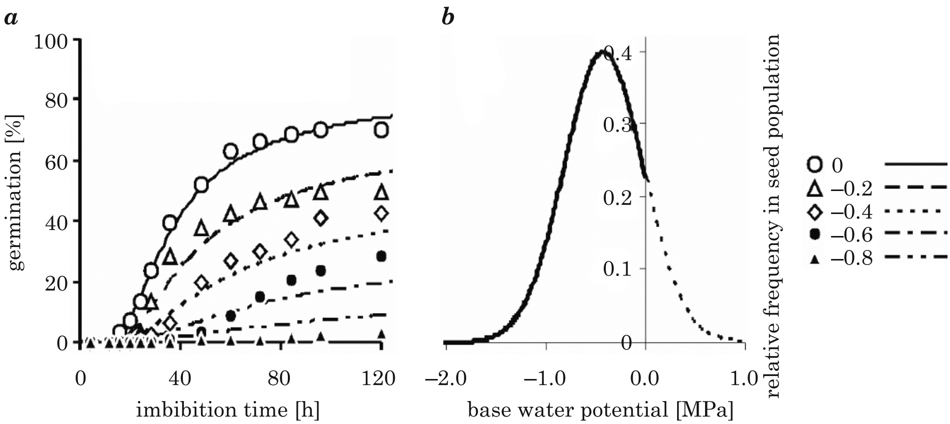

Fig. 1. Germination time courses of red clover seeds before experiment. The symbols are the actual data, and the lines are the time courses predicted by the hydrotime model using values shown in Table 1 and Table 2: *a* – germination time courses at 0(○), -0.2(△), -0.4(◊), -0.6(●) and -0.8(▲) MPa of seeds; *b* – normal distribution showing the relative frequencies of $\psi_b(g)$ values of seeds

Storage of *T. pratense* seeds for 14 days in alternating temperatures caused more variation in results. A seed treatment of alternating 12 h of -5°C and 12 h of 5°C in a dry environment caused a decrease in final germination. The same treatment in a moist environment produced a slight germination

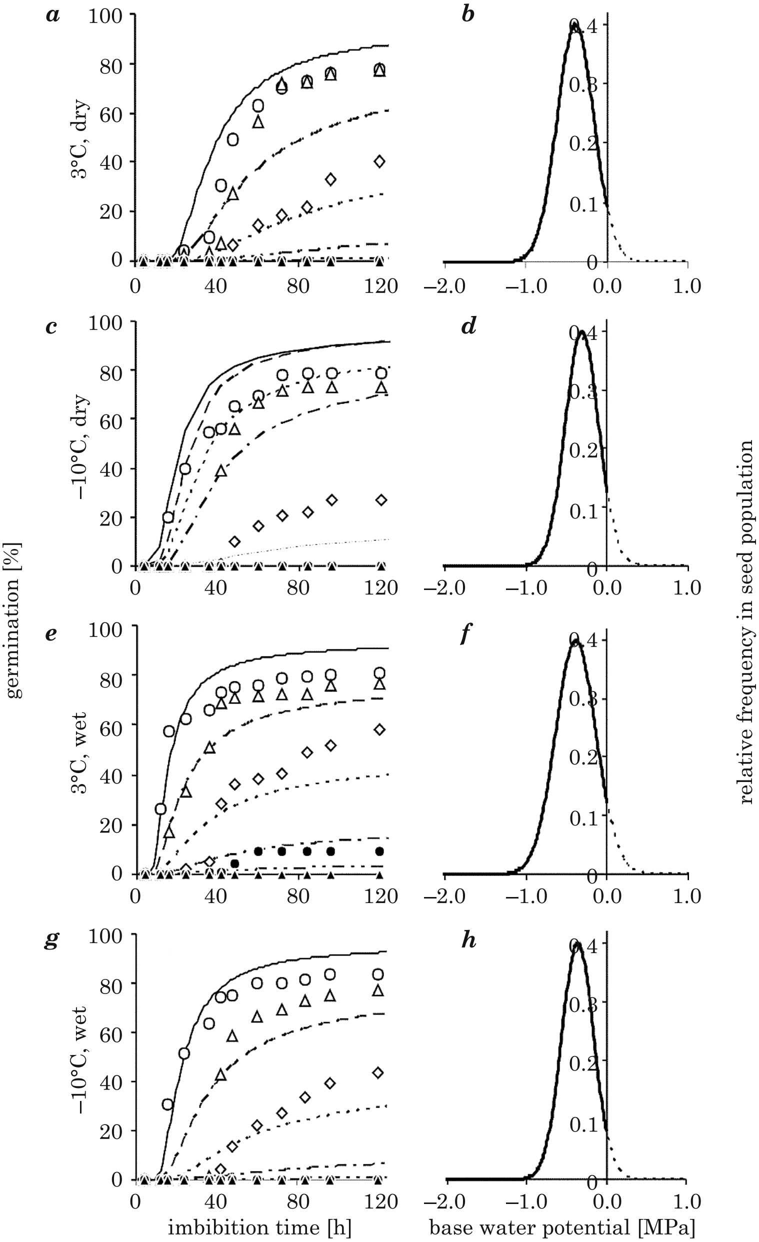

Fig. 2. Germination time courses of red clover seeds after constant low temperature pretreatment. The symbols are the actual data, and the lines are the time courses predicted by the hydrotime model using values shown in Table 1: *a, c, e, g* – germination time courses at 0(○), -0.2(△), -0.4(◊), -0.6(●) and -0.8(▲) MPa of seeds after storage at constant temperatures; *b, d, f, h* – normal distribution showing the relative frequencies of $\psi_b(g)$ values of seeds

increase (Table 2). Pretreatment in both environments brought about similar changes in hydrotime model parameters: a small shift to the right and also a small narrowing of the base water potential distribution (Figure 3). However, the hydrotime constant value was much lower in a wet than a dry seedbed (Table 1).

Table 2

Effect of fluctuating temperatures on seed germination of red clover and hydrotime model parameters

Treatment	$\psi_b(50)$ [MPa]	σ_{ψ_b} [MPa]	θ_H [MPa h]	R^2	FG [%]
Before treatment	-0.423	0.396	20.1	0.809	72.7 ± 1.4
-5/5°C, dry	-0.324	0.267	17.75	0.835	67.6 ± 3.4
2/12°C, dry	-0.318	0.169	7.71	0.859	87.9 ± 6.1
Mean (CV)	-0.355 (17)	0.277 (40)	15.19 (43)	–	76.1 (14)
-5/5°C, wet	-0.352	0.253	7.48	0.768	76.3 ± 2.2
2/12°C, wet	-0.627	0.440	18.28	0.640	90.8 ± 2
Mean (CV)	-0.467 (31)	0.363 (22)	15.29 (45)	–	79.9 (12)

Explanation: CV – coefficient of variation expressed as a percentage of the mean; FG – final germination in water ± standard error; $\psi_b(50)$ – mean base water potential; σ_{ψ_b} – standard deviation of base water potential; θ_H – hydrotime constant; R^2 – coefficient of determination

Alternating temperatures of 2/12°C distinctly increased final seed germination, although by several percent more in a wet than in a dry environment. Relatively high germination of dry stored seeds was connected with very low values of σ_{ψ_b} and θ_H, despite a less negative value of $\psi_b(50)$ than in the control sample. Wet stored seeds at 2/12°C germinated the best (90.8%), because a distinct shift of the mean base water potential value to the left, toward more negative values, although the distribution width of this parameter increased (Figure 3). The fit of experimental data for dry stored seeds at various temperatures was also good, with R^2 ranging from 0.84 to 0.90. The model fitted some worse with the experimental data for wet stored seeds with values of R^2 from 0.64 to 0.77 (Table 2).

Discussion

The occurrence of hard seeds in agricultural crop seed lots is considered undesirable because they contribute to non-uniform seedling emergence, potentially reducing yields, retarding harvest and diminishing the ability to compete with weeds. However, hardseededness may be regarded as desirable in certain situations, such as strong winter conditions, extended drought,

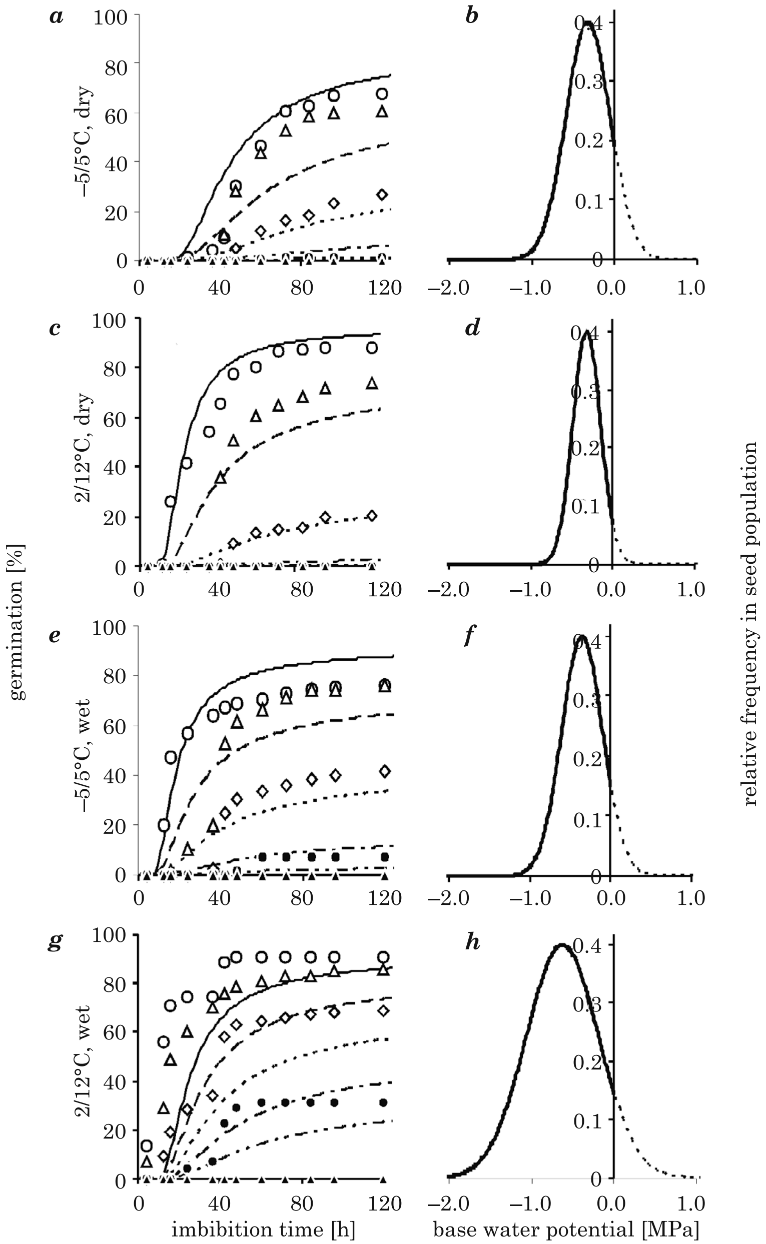

Fig. 3. Germination time courses of red clover seeds after alternating low temperature pretreatment. The symbols are the actual data, and the lines are the time courses predicted by the hydrotime model using values shown in Table 2: *a, c, e, g* – germination time courses at 0(○), -0.2(Δ), -0.4(◊), -0.6(●) and -0.8(▲) MPa of seeds after storage at fluctuating temperatures; *b, d, f, h* – normal distribution showing the relative frequencies of $\psi_b(g)$ values of seeds

or animal digestive tracts, where this feature permits a part of the seed population to survive (DEGREEF et al. 2002, ASSCHE VAN et al. 2003).

The hydrotime model fitted our experimental data quite well, so the parameters can be used to compare the effect of the treatment on seed germination performance. The values of R^2 showed that the model worked well to characterize germination time courses of red clover seeds at reduced water potentials and was comparable with the values obtained for other seed species (BATLLA and BENECH-ARNOLD 2004, HUARTE and BENECH-ARNOLD 2005, BOCHENEK et al. 2010, 2016).

The analysis of variation of hydrotime constant value was particularly significant in the case of seeds in physical or combinational dormancy. The decrease of θ_H value indicates germination acceleration of the examined seed population, which is connected with shortening of germination phase II in seeds with physiological dormancy (BRADFORD 2002). The dormancy mechanism is completely different in hard seeds. They cannot germinate, as the impermeable seed coat does not make water uptake possible. Softening the seed coat by scarification enables imbibition. If there are more seeds with damaged testae in a population, it germinates more rapidly and synchronically, because the hydrotime constant value is diminished (ŻUK-GOŁASZEWSKA et al. 2007).

The considerable decrease θ_H value after seed treatment by constant low temperatures suggested that such conditions, especially in a wet environment, caused an increase in the water permeability of the testa. Simultaneously, a small increase in germination percentage was connected with decreased vigour and sensitivity to reduced water potential (an increase in mean base water potential value). Seeds stored in fluctuating temperatures (5/-5°C) behaved similarly.

Alternating positive temperatures had a stimulating effect on red clover seed germination. The increase in germination percentage in dry conditions was connected with increased damage to the seed coat (a decrease in hydrotime constant value). There is large probability that the dry alternating temperature treatment resulted in the breaking or opening of specified structures in the testa e.g. lens or hilar fissure (HU et al. 2009). In the Krynia cultivar of clover seeds treated with concentrated sulphuric acid, the effect of which is mainly to increase seed coat permeability, the hydrotime constant value reduced most of all (ŻUK-GOŁASZEWSKA et al. 2007).

In alternating, low, above zero temperatures in a wet environment the water permeability of testae probably increased to a lesser extent; these conditions could also affect the physiological component of seed dormancy connected with the base water potential value (BASKIN and BASKIN 2004, FINCH-SAVAGE and LEUBNER-METZGER 2006). However, in such conditions this physiological element of dormancy did not decrease uniformly throughout the entire seed population (giving a high value for σ_{ψ_b}). It was the reason for incomplete germination, which other authors also observed (RIDAY 2008). A similar pattern of hydrotime model parameter variations has been observed in several non-cultivated species whose seeds are characterized by physiological dormancy (HUARTE and BENECH-ARNOLD 2005, BATLLA and BENECH-ARNOLD 2004, HU et al. 2013). ALVARADO and BRADFORD (2005) suggest that if the processes mimic one another in terms of the pattern of variations in hydrotime model parameters, their mechanisms could also be similar. However, there is no doubt that alternating positive temperatures broke the combinational dormancy (P+Y) of red clover seeds to the greatest extent (BASKIN and BASKIN 2004, FINCH-SAVAGE and LEUBNER-METZGER 2006).

Temperatures, especially fluctuating temperatures, could be an environmental cues partly responsible for breaking the combinational dormancy of red clover seeds. Our results confirmed earlier data showing that red clover seed dormancy may be broken in relatively low temperatures leading to consistently better seedling emergence (ASSCHE VAN et al. 2003, ŻUK-GOŁASZEWSKA et al. 2006). Our results acknowledged the advisability of sowing red clover in autumn. The seeds that plants shed over a given area in late summer or autumn, are then exposed to winter and early spring conditions, which allow them to reach a high vigour and successfully emerge in spring. We have confirmed that seeds do not need extreme temperatures or large amplitudes of temperatures alternation to break combinational dormancy in temperate climates. However, such conditions cause partial distribution of germination over time, as a certain proportion of seeds remain dormant and there is possibility that they will germinate in the next autumn or after the next winter. From point of view of species regeneration, this is a beneficial effect because it is more probable that part of the seedling population will survive and the next generation will give new seeds, unlike a case in which all the population germinates uniformly.

Conclusions

1. Fluctuating positive temperatures in a wet seedbed broke the combinational dormancy of red clover seeds to the greatest extent.
2. *T. pretense* seeds do not need alternating temperatures of large amplitudes or extreme temperatures to break dormancy in temperate climates.
3. The hydrotime model turned out to be a very effective approach to characterize and predict relief of combinational dormancy.

4. Red clover sowing in autumn is advisable because exposition to winter and early spring conditions allow seeds to reach high vigour and successfully emerge in spring.

Accepted for print 24.07.2017

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