## Seed germination requirements of Hypericum scruglii, an endangered medicinal plant species of Sardinia (Italy)

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<td>Porceddu, Marco; Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente Sanna, Martina; University of Cagliari Serra, Sara; Università degli Studi di Cagliari, Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente (DISVA) Manconi, Maria; Universita degli Studi di Cagliari, Department of Life and Environmental Sciences Bacchetta, Gianluigi; Università degli Studi di Cagliari, Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente (DISVA)</td>
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Seed germination requirements of *Hypericum scruglii*, an endangered medicinal plant species of Sardinia (Italy)

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Abstract

*Hypericum scruglii* is an endangered endemic plant of Sardinia and the phloroglucinol compounds identified in this species have been reported to inhibit Human Immunodeficiency Virus activity. Seed banks are a genetic repository that effectively preserve taxa of conservation interest and they hold knowledge about the biology and germination eco-physiology of the taxa they preserve. The main goals of this study were to investigate the germination requirements of *H. scruglii*, to evaluate the seed viability after eight years of long-term conservation and to suggest an efficient protocol for germination. Seeds stored at -25 °C in the seed bank were tested at temperatures from 5 to 30 °C and 25/10 °C. Base temperature ($T_b$) and thermal time ($\theta_{50}$) for germination were estimated. *H. scruglii* seeds germinated over a wide range of temperatures, responding positively to high temperatures and 25/10 °C. $T_b$ was estimated at 4.92 °C, and $\theta_{50}$ was estimated at 198.27 degree days. The high viability detected in seeds of *H. scruglii* allows us to suggest the use of seeds stored in a seed bank when fresh material for plant propagation is scarce. Our results provide new and useful baseline information for implementing conservation and multiplication strategies for this endangered medicinal plant.

**Keywords:** endangered species; Hypericaceae; medicinal plant; plant propagation; seed banking.
Introduction

A medicinal plant is any plant containing substances that can be used for the synthesis of useful drugs (World Health Organization; WHO 2001). It is well known that some species belonging to the genus *Hypericum* (Hypericaceae) have medicinal proprieties. For example, *H. perforatum* L. is widely used in Europe as a drug for the treatment of mild to moderate depression (Brolis et al. 1998; Fiebich et al. 2011). Accumulating scientific evidence, together with the various traditional uses of *Hypericum* species, suggests plants of this genus have potential to treat diseases other than depression (Stojanovic et al. 2013; Mandrone et al. 2017). More recently, an important discovery was made regarding *H. scruglii* Bacch., Brullo & Salmeri; Sanna et al. (2018) identified phloroglucinol compounds in this plant that are able to inhibit the activities of the Human Immunodeficiency Virus type 1 (HIV-1). The results obtained by Sanna et al. (2018) emphasize the importance of extending research into this plant, so the development of an effective protocol for the conservation and multiplication of *H. scruglii* is needed in order to reconcile the conservation interest of the species with research in the pharmaceutical field. In fact, in addition to its medicinal value, *H. scruglii* is an endemic plant species of Sardinia (Italy), that is categorized as Endangered in Global and Regional IUCN (International Union for Conservation of Nature) Red Lists (Bacchetta et al. 2010; Fois et al. 2014) and, absurdly, it is still not protected by local or international regulations. Given the growing importance of *H. scruglii*, the Sardinian Germplasm Bank (BG-SAR – University of Cagliari) ensures the conservation of this taxon, as well as other important plants, preserving them for the long-term (Porceddu et al. 2017c).

*Ex situ* conservation acts as a back-up for certain fields of plant diversity (Li and Pritchard 2009) and it is complementary to *in situ* conservation actions. The complementarity of *ex situ* and *in situ* conservation better safeguards endangered species (Fenu at al. 2020). However, the integration of the *ex situ* approach in an *in situ* program remains sporadically adopted (Fenu et al. 2020). Thus, the *ex situ* conservation strategies are often the only way for the preservation of some endangered
species (Maunder et al. 2004; Cochrane et al. 2007). Seed banking, as an integral part of the *ex situ* conservation, has a pivotal role in safeguarding plant species for long times and in developing standard viability monitoring protocols (Bewley and Black 1994). In addition to their role in *ex situ* conservation, seed banks are also a source of knowledge about the germination eco-physiology of the taxa they hold, by determining germination and multiplication protocols for many of these species (Bewley and Black 1994; Valderrábano et al. 2018).

To understand the reproductive mechanisms of *H. scruglii* and to suggest an efficient protocol for seed germination and multiplication, study of its germination ecophysiology under a wide range of temperature is fundamental. Temperature is one of the most important environmental conditions that control germination (Garcia-Huidobro et al. 1982; Probert 2000). The germination response in relation to accumulated temperature has been modelled using a thermal time (*θ*) approach (Covell et al. 1986; Pritchard and Manger 1990) and allows for the description of linear change in germination rate under different conditions and for the estimation of thresholds for the germination response (Porceddu et al. 2017b; Seal et al. 2018; Fernández-Pascual et al. 2019). As reported by Mattana et al. (2019) in their study on the critically endangered wild medicinal plant *Dioscorea strydomiana* Wilkin, limited data are available with regard to thermal thresholds for seed germination of wild native species.

In the present study, we investigated the seed germination ability of *H. scruglii*. Specifically, the aims of the work were (i) to identify the temperature requirements for germination of *H. scruglii* and to evaluate the viability of its seeds by determining the germination capacity after eight years of long-term conservation, (ii) to detect the base temperature (*T_b*) and the thermal requirements (*θ*) for seed germination and (iii) to suggest a germination protocol for this taxon. This knowledge is important because *H. scruglii* was recently recognized as a plant of high pharmaceutical value and in the future, there may be a need to propagate the species by seeds. This approach may be an example for other important medicinal plant species that require rapid conservation efforts to prevent and/or mitigate the extinction risk. The new data presented in this work would guarantee
greater protection of the species in nature, taking into account also that it is considered to be in danger of extinction.

**Material and Methods**

**Study species**

*Hypericum scruglii* is a vascular plant species distributed through central and south east Sardinia, in particular the Sarcidano, Barbagia of Seulo, Ogliistra and Quirra regions (Bacchetta et al. 2010). The species is generally associated with calcareous substrates such as limestone, conglomerate, travertine, sandstone and marl, where it grows exclusively on damp soil near springs or streams with freshwater (Bacchetta et al. 2010). It is a perennial herb 5–30 cm tall, herbaceous, prostrate to decumbent. Flowering occurs in late June or July and fruiting in August and September. The fruit consists of an ellipsoid capsule, tridentate with loculi longly apiculate, which contains numerous small brownish seeds, 0.7–0.9 mm long, with testa finely reticulate-scalariform (Bacchetta et al. 2010). The seeds of *H. scruglii* have been reported to exhibit orthodox storage behaviour (Royal Botanic Gardens, Kew 2020).

**Seed collection and treatment**

Collection of *H. scruglii* seeds was carried out in September 2011, in a locality of Laconi (Oristano province, Sardinia, Italy) named Santa Sofia. A sample of the collected seeds was transferred, as a "safety duplication", to the Millennium Seed Bank of the Royal Botanic Gardens, Kew, in accordance with the terms and conditions of the Memorandum of Collaboration between BG-SAR and Kew. Germination of 96% at 20 °C (seeds sown: 47) and of 100% at alternating temperatures 25/10 °C (seeds sown: 42), both with a light/dark regime of 8/16 h, were recorded in fresh seeds.
tested before long-term conservation in the seed bank (Royal Botanic Gardens, Kew 2020).

Following international standards for long term storage (Bacchetta et al. 2006, 2008), the seeds were gradually dried at 15 °C and 15% relative humidity (RH), in order to reach ca. 3%–5% internal seed moisture content, and then stored at -25 °C at BG-SAR (Porceddu et al. 2017c). Before the germination tests the stored seeds were removed from storage and brought to room temperature (ca. 20 °C and 40% RH) for 12 hours, in order to restore internal seed humidity.

Seed germination experiments

Four replicate samples of 25 seeds each were sown on the surface of 1% agar water, in 60 mm diameter plastic Petri dishes. They were incubated at a range of constant temperatures (5, 10, 15, 20, 25 and 30 °C) and one alternating temperature (25/10 °C), with 12 h of irradiance per day in germination chambers (Sanyo MLR-351) equipped with white fluorescent lamps (FL40SS.W/37 70–10 μmol m⁻² s⁻¹). In the alternating temperature regime, the 12 h light period coincided with the period with elevated temperature. All the germination tests were conducted in 2018. Germination of the seeds, defined as visible radicle emergence with length >1 mm, was scored three times a week.

Thermal time base temperature

Thermal time analysis was carried out for *H. scruglii* seeds germinating at constant temperatures from 5 to 30 °C. Estimates of time (*t₉₅* days) taken for cumulative germination to reach different percentiles (*g*) for successive increments of 10% germination were interpolated from the germination progress curves (Covell et al. 1986). Germination rate (1/*t₉₅*) was regressed, using a linear model, as a function of temperature according to the following equation:

\[
\frac{1}{t₉₅} \text{ (days}^{-1}) = \frac{(T₉₅ - T_b)}{θ},
\]
where \( T_b \) corresponds to the base temperature for germination and \( \theta \) to the thermal time (García-Huidobro et al. 1982). An average (± 1SD) of the x-intercept among percentiles was calculated for the sub-optimal temperature range (5–30 °C) to establish the \( T_b \) (Pritchard and Manger 1990).

Thermal time (\( \theta; \; ^\circ\text{Cd}, \; \text{degree days} \)) estimates were then calculated separately as the inverse of the sub-optimal regression equations (Covell et al. 1986).

**Data and statistical analysis**

The final germination percentages were calculated as the mean of the four replicates (± SD) based on the total number of filled seeds. Furthermore, the germination rate (\( T_{50} \)) was defined as the time (in days) required to reach 50% germination success.

Generalized Linear Models (GLMs) were used to evaluate the effects of incubation temperature on final seed germination percentage and on the \( T_{50} \). A logit link function and quasibinomial error structure was used to analyse seed germination percentages, whereas a log link function and Poisson error structure was used for analysing \( T_{50} \). In the subsequent ANOVA, \( F \) tests with an empirical scale parameter, instead of chi-squared, was used (Crawley 2007). Significant differences highlighted by the GLM were then analysed using *post-hoc* Tukey multiple comparison tests. All statistical analyses were carried out with R version 3.0.3 (R Core Team 2015).
Results

Seed germination

Seeds of H. scuruglii stored for eight years at -25 °C were able to germinate under a wide range of constant temperature regimes and at the alternating temperature tested (Fig. 1 A). High germination percentages (> 70%) were observed at all tested temperatures (Fig. 1 A). There was not statistically significant ($P > 0.05$) effect on final germination percentages (dependent variable) for temperature (Table 1). However, there was a statistically significant effect ($P < 0.001$) on the germination rate (calculated on the $T_{50}$ values) for temperature (Table 1). The post-hoc pairwise t-tests highlighted no statistical difference ($P > 0.05$) among 20, 25, 30 °C and 25/10 °C (here identifiable as “Group with letters a”) and between 10 and 15 °C (here identifiable as “Group with letters b”) (Fig. 1 B). However, the $T_{50}$ obtained at 5 °C was statistically different ($P < 0.05$) with respect to “Group with letters a” (i.e. 20, 25, 30 °C and 25/10 °C) and to “Group with letters b” (i.e. 10 and 15 °C) (Fig. 1 B). In general, seeds tested at temperatures above 5 °C needed ca. 30 days to reach $T_{50}$, while at 5 °C the value was reached in ca. 70 days. The higher temperatures (seeds of “Group with letters a”) accelerated germination, reducing the time to reach $T_{50}$ to ca. 10 days (Fig. 1 B). In addition to the germination results, we observed that the first stages of seedling development of H. scuruglii under controlled conditions (at 20 °C and in a sterilized soil substrate) did not manifest any evident problems (Fig. 2).

Base temperature and thermal time

Based on germination rate responses for each 10th percentile from 10 to 70% germination, it was possible to estimate the mean base temperature ($T_b$) for germination (Fig. 3 A). Average $T_b$ value for H. scuruglii was 4.92 ± 1.13 °C (Fig. 3 A). Figure 3 B shows the relationship between thermal time ($\theta_g$) and germination percentage. According to the linear regression equation obtained ($y =
0.29x - 8.92), the thermal time required for 50% germination ($\theta_{50}$) was estimated at 198.27 °Cd (Fig. 3 B).

**Discussion**

Our results show that the seeds of *H. scruglii* germinated under controlled conditions and they responded positively to both constant and alternating temperatures. Indeed, high germination percentages (> 70%) were recorded under all the conditions tested. As noted during the experiment, when considering the rate of germination, the species responds to treatments with high temperatures (from 20 to 30 °C) and alternating temperature (25/10 °C) reaching the $T_{50}$ values within 10 days.

There are relatively few complete studies on the germination biology and thermal requirements of *Hypericum* species. Pérez-García et al. (2006) found that germination temperatures had no significant effect on final germination percentages in *H. perforatum* and that the germination process ends within 30 days. Conversely, seeds of other *Hypericum* species need specific treatments (e.g. cold or warm stratification, gibberellins) and/or conditions (e.g. light, dark) in order to promote and ensure germination (Sánchez-Coronado et al. 2015; Carta et al. 2016; Rosbakh et al. 2020). Our study provides important new data for the germination requirements of *H. scruglii*, increasing knowledge regarding the germination biology of members of the genus *Hypericum*.

The results of our study demonstrate that the preservation methods and *ex situ* strategies implemented by the seed bank were effective forms of long-term storage (Porceddu and Bacchetta 2018). The outcome of this work may have positive implications for the preservation and conservation of *H. scruglii*, as it is evident that seeds stored at -25 °C for eight years have maintained their viability and a high germination capacity. This behaviour is in accordance with that detected in other *Hypericum* species; for example, seeds from species belonging to the same genus have been reported to maintain their viability for at least 7-13 years of long term storage (see Table 2).
The identification of a suitable range of incubation temperatures allowed us to estimate the minimum germination temperature and thermal requirements of *H. scruglii*. The base temperature for germination ($T_b$) of *H. scruglii* was estimated at 4.92 °C, and the thermal time to reach $\theta_{50}$ was estimated at 198.27 °Cd (degree days). To our knowledge, to date, no information is available in the literature on the base temperature and thermal time of *Hypericum* species, so this work is the first attempt at reporting thermal threshold data for seed germination in the genus. However, the $T_b$ and $\theta_{50}$ values estimated in this work for *H. scruglii* are consistent with the thermal thresholds reported for seed germination of Mediterranean species, in which base temperature ranges from -9 to 9 °C and thermal time from 22 to 357 °Cd (Picciau et al. 2019).

In light of the recently discovered medicinal potential of phloroglucinol compounds in *H. scruglii* to inhibit the activities of HIV-1 (Sanna et al. 2018), one of the main goals of this study was to suggest a germination protocol for this taxon, in the assumption that an efficient protocol for seed germination must be taken into account to ensure the availability of plants cultivated *ex situ* (Porceddu et al. 2017a). In general, propagation from seed is relatively inexpensive and usually effective, but often the particularities of germination requirements are unknown or only partially known (e.g. Cuena-Lombrana et al. 2016; Picciau et al. 2017). On the basis of our results, we suggest that the efficient protocol for seed germination of *H. scruglii* consists of seed incubation at 20-30 °C, or alternating temperature of 25/10 °C, under a photoperiod of 12 h light/12 h dark. A seed germination protocol facilitates propagation of the species at any time, guaranteeing the availability of the plants for multiple purposes, such as natural population reinforcement, phytopharmaceutical research, chemical and pharmacological studies. In addition, the propagation of plants in *ex situ* environments may bypass the natural phenological timing of the species, ensuring that the plant material is available when it is needed.

The high viability detected in seeds of *H. scruglii* stored for eight years under seed banking conditions, and the efficient protocol for seed germination for this species proposed in this work, allows us to suggest the use of stored seeds when fresh material for plant propagation is scarce or is...
not available (Godefroid et al. 2016). Especially in the case of endemic and threatened species, which may present problems related to the poor availability of material for plant propagation, seed banks can be important sources of plant materials (Godefroid et al. 2016; Cuena-Lombraña et al. 2020). The availability of plant materials in _ex situ_ germplasm banks could effectively contribute to the reduction of the plant-harvest pressure on wild populations, moving the attention of the collectors from plants to seeds. However, it is important to remember that germplasm collection, conservation and movement may be subject to international and national regulations, and this operation must be carried out by authorized and trained persons (Valderrábano et al. 2018).

**Conclusions**

This study demonstrates that seeds of _H. scruglii_ can achieve high germination percentages under laboratory conditions, even after at least eight years of long-term storage in a seed bank. The high viability detected in seeds of _H. scruglii_ allows us to suggest the use of seeds stored in seed bank when fresh material for plant propagation is scarce or is not available. These results provide new and useful baseline information for implementing conservation and multiplication strategies for this endangered medicinal plant.
References


**Tables**

**Table 1.** GLM results of seed germination (%) and germination rate ($T_{50}$) (dependent variables) for the Temperature (5, 10, 15, 20, 25, 30 °C and 25/10 °C) factor.

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<tr>
<th>Germination (%)</th>
<th>Df</th>
<th>Deviance</th>
<th>Resid.df</th>
<th>Resid.dev.</th>
<th>F</th>
<th>P</th>
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<td>Null</td>
<td>27</td>
<td>204.56</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Temperature</td>
<td>6</td>
<td>62.996</td>
<td>21</td>
<td>141.56</td>
<td>1.8853</td>
<td>0.1309</td>
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<th>Germination rate ($T_{50}$)</th>
<th>Df</th>
<th>Deviance</th>
<th>Resid.df</th>
<th>Resid.dev.</th>
<th>P</th>
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<td>Null</td>
<td>27</td>
<td>423.08</td>
<td></td>
<td></td>
<td>&lt; 2.2e-16***</td>
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<tr>
<td>Temperature</td>
<td>6</td>
<td>414.07</td>
<td>21</td>
<td>9.01</td>
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Table 2. Examples and related information for Hypericum species that maintain their viability for at least seven years of long-term storage.

<table>
<thead>
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<th>Species</th>
<th>Years of storage</th>
<th>Average germination change (%)</th>
<th>Source</th>
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<tr>
<td><em>H. reflexum</em> L.f.</td>
<td>13</td>
<td>100 to 97</td>
<td>Royal Botanic Gardens, Kew (2020)</td>
</tr>
<tr>
<td><em>H. androsaemum</em> L.</td>
<td>11</td>
<td>100 to 98</td>
<td>Royal Botanic Gardens, Kew (2020)</td>
</tr>
<tr>
<td><em>H. perforatum</em> L.</td>
<td>11</td>
<td>100 to 92</td>
<td>Royal Botanic Gardens, Kew (2020)</td>
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<tr>
<td><em>H. tomentosum</em> L.</td>
<td>8</td>
<td>-</td>
<td>Montezuma-De-Carvalho et al. (1987)</td>
</tr>
<tr>
<td><em>H. canadense</em> L.</td>
<td>7</td>
<td>82 to 80</td>
<td>Walsh et al. (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90 to 72</td>
<td></td>
</tr>
<tr>
<td><em>H. hirsutum</em> L.</td>
<td>7</td>
<td>81 to 72</td>
<td>Walsh et al. (2003)</td>
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Figure captions

**Fig. 1** A) Final germination percentages and (B) germination rate of *Hypericum scruglii* at each tested temperature (5, 10, 15, 20, 25, 30 °C and 25/10 °C). The points correspond to the mean of four replicates. Continuous lines show fitted Weibull functions, calculated using germination parameters. Dashed lines indicate the time to achieve the $T_{50}$s. Different letters in (B) indicate significant differences ($P < 0.05$) of $T_{50}$ by post hoc pairwise t-test comparisons.

**Fig. 2** Germinated seed (A), seedlings with cotyledons (B), and developed seedlings (C) of *Hypericum scruglii* propagated at the BG-SAR under controlled conditions.

**Fig. 3** A) Base temperatures ($T_b$), calculated for different germination percentiles of *Hypericum scruglii* seeds incubated at each sub-optimal constant temperatures (5, 10, 15, 20, 25 and 30 °C). Linear regressions of percentiles where $P > 0.05$ were not included; B) Thermal times ($\theta_g$, expressed in °Cd) calculated from germination time-courses from estimated $T_b$ of 4.92 °C. Thermal times to reach $\theta_{50}$ are shown in dashed line.
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85x108mm (300 x 300 DPI)
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