

## Interactions between temperature, light and chemical promoters trigger seed germination of the rare Azorean lettuce, *Lactuca watsoniana* (Asteraceae)

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### Abstract

*Lactuca watsoniana* is an Azorean endemic plant, listed as a priority species for conservation. Although there is a considerable amount of data regarding germination in the genus *Lactuca*, little is known about seed germination in *L. watsoniana*, which is an important gap for defining conservation strategies. Seed viability (tetrazolium test) declined from 100 to 30% after four months storage at room temperature over silica gel. We tested the effects of 1) incubation temperature (three alternating temperature regimes); 2) gibberellic acid concentration (four levels); 3) addition of ethephon; and 4) type of light (three modalities) on seed germination (fresh seeds). There was a significant effect of incubation temperature, gibberellic acid concentration and type of light on percentage germination. There were also significant interaction effects of gibberellic acid with all the other studied factors, and the type of light with temperature regime. The optimal treatment was 0.1 mg l<sup>-1</sup> gibberellic acid, ethephon and a red filter at 15/10°C, resulting in 95% germination. Our study allowed the production of hundreds of seedlings that were reintroduced into the original population.

### Introduction

The genus *Lactuca* L. (Asteraceae, Cichorieae, subtribe Lactucinae) in its currently accepted circumscription comprises about 230 species, distributed in Europe, Africa, Asia and North America (Kilian *et al.*, 2009). One of them, *Lactuca sativa* L., lettuce, is a crop species of considerable economic importance (Wang *et al.*, 2013). While there is a lot of literature on lettuce (Globerson, 1981; Baskin and Baskin, 1998; Górski *et al.*, 2013), its wild relatives are not well studied, even though they are of potential value as sources for desirable traits in lettuce breeding (Sharrock *et al.*, 2014).

The Azorean lettuce, *Lactuca watsoniana* Trelease, is a perennial herb, endemic to the Azores archipelago in the Northern Atlantic. The species is restricted to only five of the nine islands of the archipelago (São Miguel, Terceira, São Jorge, Pico and Faial) and estimates of its total population size are 1,000 to 2,000 individuals (Silva *et al.*, 2009, 2010). It is therefore considered a priority species for conservation and was listed

as endangered on the IUCN Red List 2013. Furthermore, it was included in Annex II of the EC Habitats Directive and is protected under the Berne Convention. The species is threatened by habitat loss and degradation resulting from changes in land use, namely expansion of pasture, invasion by exotic species, consumption by introduced herbivores and disturbance of sensitive areas by tourists and locals. The remaining geographically isolated populations have a small number of individuals and are likely to suffer from inbreeding and loss of genetic diversity (Lowe *et al.*, 2005; Silva *et al.*, 2009). However, little is known about the current status of the populations, their reproductive output and genetic variability.

*Lactuca watsoniana* is currently restricted to the steep slopes of craters and ravines in temperate juniper rain forest between 600-800 m a.s.l. (Schaefer, 2005; Silva *et al.*, 2009). Flowering occurs from the end of August to early-September with pure white flowers developing in large corymbose panicles and 8-15 florets per capitulum. The achenes are 4-6 mm in length, ovate, 5- to 8-ribbed, with a beak of about 2 mm length and a hairy pappus. They are yellowish first but turn black when fully ripe in September (Schaefer, 2005).

The effect of light and plant growth regulators on germination of *L. sativa* seeds has been studied in great detail for many years (Globerson, 1981). In fact, *L. sativa* and its cultivars have been used as models for studying the presence and behaviour of the phytochrome system in seeds. Germination of *L. sativa* seeds is very sensitive to the presence of light as well as to the quality of light (Baskin and Baskin, 2014). Both, lettuce (*L. sativa*) and prickly lettuce (*L. serriola*) show a strong inhibition by far-red light irradiation (Górski *et al.*, 2013). Lee (1989) tested the effect of growth regulators to overcome this light induced germination inhibition, and Globerson (1981) studied germination of lettuce seeds under red light.

*Lactuca sativa* seeds also show thermoinhibition at temperatures above 28-30°C (Berrie, 1966; Sung *et al.*, 1998; Gonai *et al.*, 2004; Bufalo *et al.*, 2012), while *L. serriola* L. seeds were found to enter skotodormancy (secondary dormancy) during prolonged incubation of imbibed seeds in the dark at 25°C (Small and Gutterman, 1992). Thermoinhibition plays an ecologically important role in the detection of the appropriate seasonal timing for germination of soil-buried seeds in winter annual plants (Baskin and Baskin, 2014), and it often causes delayed or poor germination of crops, flowers and vegetables that have relatively low optimal temperatures for seed germination (Reynolds and Thompson, 1971; Abeles, 1986; Gallardo *et al.*, 1991). Both, thermoinhibition and skotodormancy in *L. sativa* can be broken by a combination of red light and addition of hormones (Speer and Vidaver, 1974; Bewley, 1980; Kristie *et al.*, 1981; Nascimento, 2000).

Gibberellins have been found to stimulate germination of *L. sativa* achenes in the dark (Kahn *et al.*, 1957), but considerable variation was reported in the activity of different gibberellins (Reynolds and Thompson, 1973). Hall and Galsky (1973) reported that combinations of low concentrations of gibberellic acid (GA<sub>3</sub>) and cyclic-AMP significantly increased germination over the levels obtained with either compound alone.

Ethephon has been shown to promote seed germination (Abeles, 1986), and in some cases, ethylene promoted germination of lettuce in the dark only in the presence of gibberellin (Dunlap and Morgan, 1977). Burdett and Vidaver (1971) found that both ethylene and gibberellins were necessary to stimulate germination of *L. sativa* at high temperature.

In previous seed germination studies devoted to *L. watsoniana* (Maciel, 1995, 1996), relatively low germination (20-30%) was obtained, possibly due to the occurrence of seed tegument inhibition. However, in these studies, a low number of seeds was used and the experimental design did not allow for an understanding of the complex interactions that might affect *Lactuca* germination.

Here, we test the effects of different concentrations of GA<sub>3</sub>, different alternating temperature regimes, the addition of ethephon and various light wavelengths on the germination of *L. watsoniana*. Our aim is to improve understanding of seed germination mechanisms in *L. watsoniana* and to establish an efficient propagation protocol, which could be used in ex situ conservation programmes.

## Materials and methods

### *Seed sampling*

Achenes (hereafter seeds) were harvested at the end of September 2012 from 19 individuals in a single population located on Gruta dos Montanheiros in the central part of Pico Island, Azores. The source population consisted of approximately 45 individuals of which 25 had flowered. Seeds were manually cleaned and inspected for size and colour. All underdeveloped and collapsed seeds were rejected, resulting in a total of 2,840 fresh, mature seeds that could be used for the tests.

### *Embryo characteristics and seed viability*

To evaluate the developmental stage of the embryo, total achene length was measured on a sample of 30 seeds, using a digital calliper ( $\pm 0.02$  mm) and a stereomicroscope. Seed viability was determined by the tetrazolium test according to AOSA (2000). The tetrazolium test was conducted using a sample of 10 seeds, immediately after field collection and at 30-day intervals for a period of four months. Seeds were stored at room temperature (approximately 20°C), in a closed container with silica gel. The type of seed embryo morphology was determined under a stereomicroscope using tetrazolium-stained seeds.

### *General conditions for seed germination and seedling development*

Germination assays were performed in sealed (with Parafilm®) 120 mm-diameter Petri dishes inside growth chambers with automatic temperature control ( $\pm 1^\circ\text{C}$ ) and a light period of 12 hours per day provided by six fluorescent lamps with a photosynthetic photon flux density (PPFD) of 19-22  $\mu\text{mol m}^{-2} \text{second}^{-1}$ . Seeds were imbibed with the respective GA<sub>3</sub> solution for 12 hours in a 50 ml beaker and then placed on 90 mm Filtres Laurent-Prat Dumas® filter paper moistened with distilled water or with 1.0 mg l<sup>-1</sup> ethephon (absence and presence of ethephon, respectively). Seedlings were kept inside the growth chambers under the same conditions as the germination test, until cotyledon emergence and then transferred into Jiffy® trays, in a large growth chamber with two fluorescent lamps with maximum luminous flux of 3350 lm and a light period of eight hours.

### *Experimental design*

A fully factorial design with four factors was tested: three light regimes (white, red and green); three alternating temperature regimes (20/15°C; 15/10°C and 10/5°C), four concentrations of GA<sub>3</sub> (0, 0.1, 1 and 10 mg l<sup>-1</sup>), and presence/absence of ethephon, giving a total of 72 treatments, with three replicates per treatment and 13 seeds per replicate.

Modification of the incubation light wavelength was achieved by completely covering Petri dishes with green or red cellophane sheets. The spectral emission of filtered light was measured to ensure that seeds were subjected to the correct wavelengths (green filter 420 nm, red filter 536 nm).

Seed germination was recorded daily for 80 days by examining radicle emergence, under the same light conditions as the germination test, without exposure to white light. Time between radicle and cotyledon emergence was monitored in all treatments. Development of 60 seedlings grown in Jiffy® trays was monitored for two months, by measuring the length between the insertion point of the cotyledonal leaves and the seedling apical meristem.

### *Statistical analysis*

Germination percentages were calculated after 14 weeks of incubation. Percentages were arc-sine-transformed following Zar (1999) and normality assessed with the Kolmogorov-Smirnov test. Since transformed data followed the usual parametric tests assumptions (i.e. normality, homoscedasticity), multifactor ANOVA was performed to test for main effects and interactions for GA<sub>3</sub> concentration, ethylene, light wavelength and incubation temperature). A contrast analysis was used as a multiple comparison procedure. Statistical analyses were performed using SPSS 18.0 and Microsoft Office Excel 2003.

For treatments with significantly higher germination than the control (10/15°C, 0 mg l<sup>-1</sup> GA<sub>3</sub>, absence of ethephon, white light), we fitted cumulative germination curves using the Gompertz sigmoid function (Laterra and Bazzalo, 1999; Moura and Silva, 2010) and used the estimated model to obtain T<sub>50</sub> values (i.e. the time until 50% of sown seeds have germinated).

## **Results**

### *Seed characteristics and viability*

Mean seed length was 5.30 ± 0.36 mm (5.98-4.50 mm; n = 30), mean seed width was 1.87 ± 0.17 mm (2.14-1.41 mm; n = 30) and mean pappus length was 4.32 ± 0.71 mm (5.99-2.83 mm; n = 30). *Lactuca watsoniana* seeds showed an axile, foliate, investing-like type of embryo (Vozzo, 2002): the embryo is erect and relatively large, occupying all the lumen, central rather than peripheral, the endosperm is missing or very reduced. The cotyledons are expanded, thick and encase small hypocotyls, however, they do not overlap, as in typical investing embryos. Fresh seeds were 100% viable, but viability decreased progressively with storage time. After four months storage at 20°C, about 70% of the seeds had lost viability.

*Seed germination*

A multifactorial ANOVA applied to the complete set of results showed a significant effect on germination percentage (figure 1) of temperature regime ( $F = 8.68$ ;  $P < 0.001$ ), GA<sub>3</sub> concentration ( $F = 28.11$ ;  $P < 0.001$ ) and type of light ( $F = 37.83$ ;  $P < 0.001$ ), and that there was a significant interaction between all four factors ( $F = 3.05$ ;  $P = 0.001$ ). In addition: i) ethephon interacted significantly with GA<sub>3</sub> concentration ( $F = 54.28$ ;  $P < 0.001$ ) but only had a promoting effect on germination in treatments without GA<sub>3</sub> (figure 2A); ii) incubation temperatures interacted significantly with GA<sub>3</sub> ( $F = 2.98$ ;  $P = 0.01$ ) and the higher temperature regimes were more effective when 0.1 mg l<sup>-1</sup> GA<sub>3</sub> were used (figure 2B); iii) green light had a negative effect on germination percentage, which was only overcome by the addition of 10 mg l<sup>-1</sup> GA<sub>3</sub> ( $F = 6.74$ ;  $P < 0.001$ ), and red

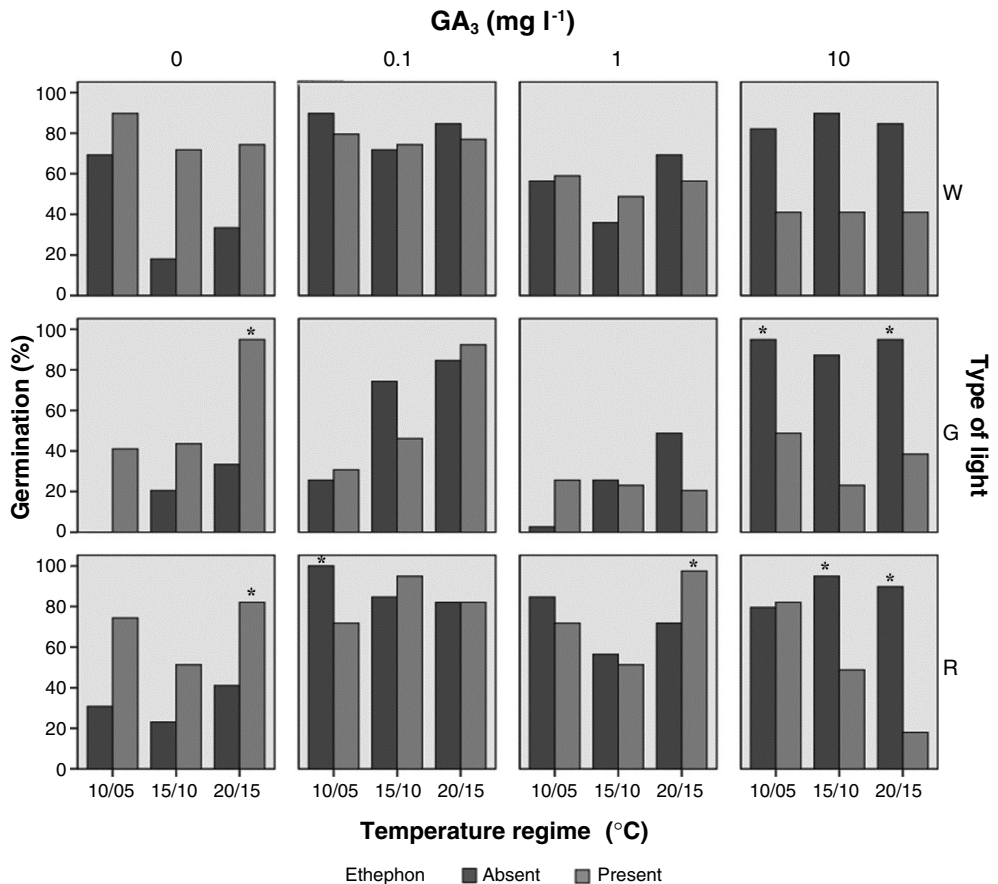


Figure 1. Germination of *Lactuca watsoniana* seeds at three different temperature regimes; four concentrations of GA<sub>3</sub>; white (W), green (G) or red (R) light; and presence or absence of ethephon. \* Treatments that showed a significantly higher germination percentage than the control (10/15°C, 0 mg l<sup>-1</sup> GA<sub>3</sub>, absence of ethephon, white light), as indicated by a contrast analysis applied after ANOVA.

light generally resulted in higher germination than white light, except in the absence of GA<sub>3</sub> (figure 2C); and iv) the type of light interaction with the temperature regimes ( $F = 9.29$ ;  $P < 0.001$ ) resulted in similar germination percentages at 20/15°C, under the three light regimes, while a negative effect on germination was observed at 10/5°C, when green light was used (figure 2D).

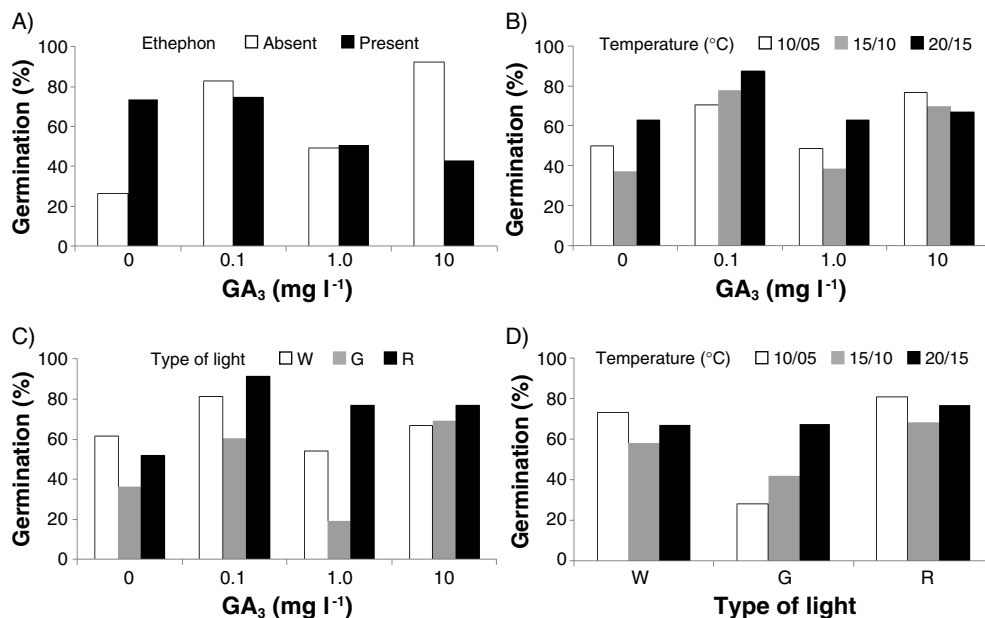


Figure 2. Interaction plots resulting from a multifactorial ANOVA of arc-sine-transformed germination percentage of *Lactuca watsoniana* seeds, taking into account the effects of ethephon, incubation temperature, GA<sub>3</sub> concentration and type of light. A) Ethephon vs. GA<sub>3</sub> (mg l<sup>-1</sup>); B) temperature regime vs. GA<sub>3</sub> (mg l<sup>-1</sup>); C) type of light vs. GA<sub>3</sub> (mg l<sup>-1</sup>); D) temperature regime vs. type of light.

To determine the best treatments, a contrast analysis comparing the control treatment (10/15°C, 0 mg l<sup>-1</sup> GA<sub>3</sub>, absence of ethephon, white light) with the remaining treatments was conducted. Five out of the eight best treatments were under red light and three under green light (figure 1). Considering the estimated T<sub>50</sub> values calculated by fitting the Gompertz model to the cumulative germination data (figure 3), the best result with a T<sub>50</sub> of 11 days and 95% total observed germination was achieved using 0.1 mg l<sup>-1</sup> GA<sub>3</sub>, ethephon and the red filter at 15/10°C.

### Seedling development

The time between radicle emergence and appearance of the cotyledons varied with treatment. In general, it occurred between 3 and 8 days (mean value of  $5.6 \pm 1.4$  days). The fastest growth ( $3.9 \pm 0.8$  days) was observed at the 20/15°C incubation temperature under white light. However, due to the bad response of seedlings to high temperatures,

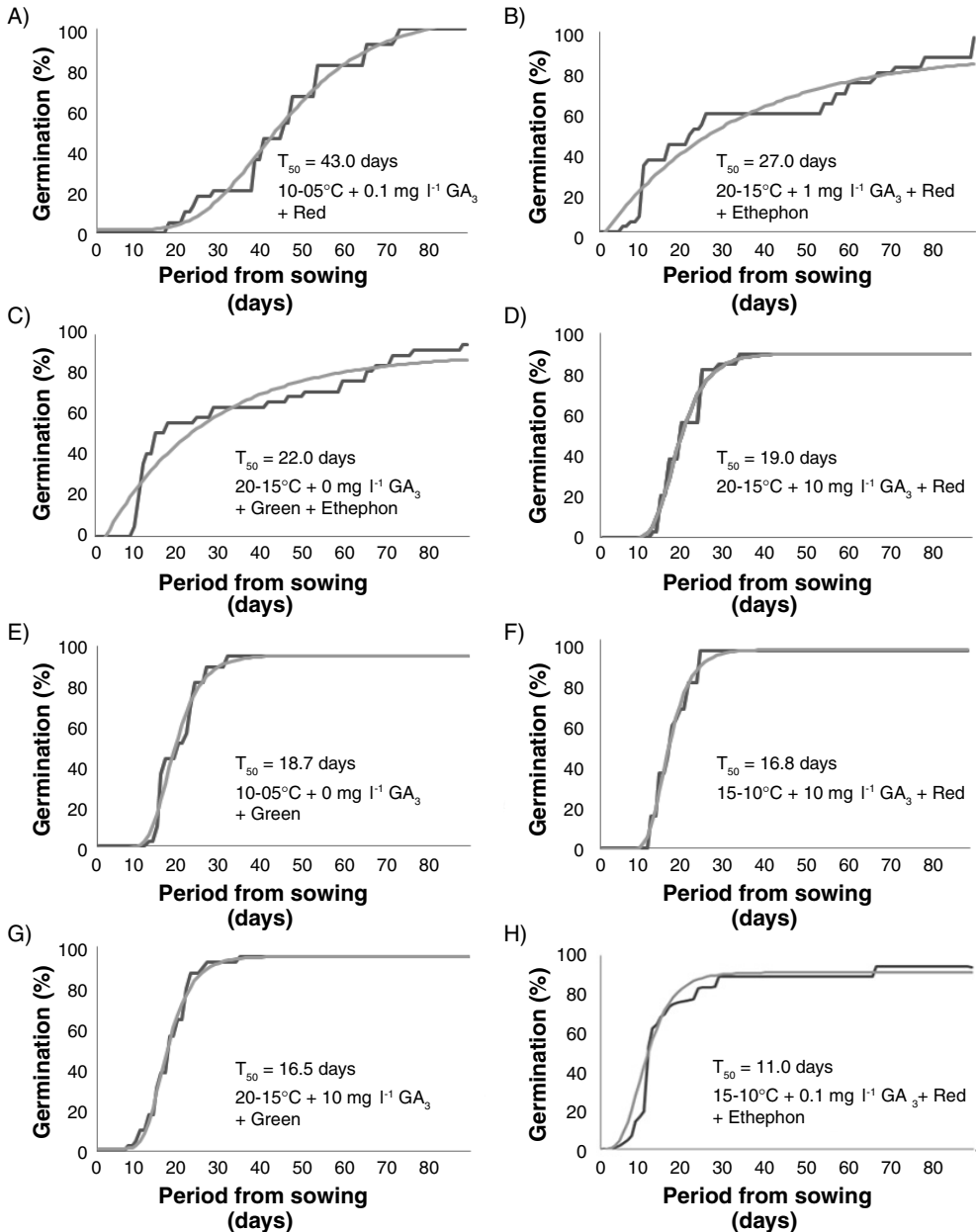


Figure 3. Observed (dark grey) and expected (light grey) accumulated germination curves for the eight treatments that showed a significantly higher germination percentage than the control (10/15°C, 0 mg l<sup>-1</sup> GA<sub>3</sub>, absence of ethephon, white light) as indicated by a contrast analysis applied after ANOVA. Expected curves and  $T_{50}$  values estimated by the Gompertz model.

immediate planting in soil or transfer to a lower temperature regime (10/05°C) until planting in soil is required after the cotyledons emerge. In the subsequent two months, the seedlings grew to a mean height of  $3.1 \pm 1.2$  mm, while the epicotyl diameter more than tripled in size (from 1 mm to approximately 3.5 mm).

Overall, we obtained a total of 1,686 seedlings out of the 2,840 seeds initially used in the assay. However, mortality between radicle emergence and the planting phase was relatively high (approximately 20%).

## Discussion

Freshly-mature seeds of *L. watsoniana* were non-dormant. The small size and mass of *L. watsoniana* seeds, with a low amount of endosperm and thin tegument might explain why this species does not show any dormancy. Similar observations have been made in two other small-seeded Azorean endemics from the Asteraceae, *Leontodon filii* (Hochst. ex Seub) Paiva and Ormonde and *L. rigens* (Dryand.) Paiva and Ormonde (Maciel, 1995, 1996; Pereira *et al.*, 2011). In contrast, most Azorean plant species with larger seeds and thicker tegument (mainly tree species) do show dormancy (Moura *et al.*, 2010; Martins *et al.*, 2012; Moreira *et al.*, 2012). Small seeds have several advantages over larger ones, such as being better suited to anemochorous and hydrochorous dispersal, and formation of a soil seed bank which can be important for recolonisation in instable habitats (Baskin and Baskin, 2014; Saatkamp *et al.*, 2009). The tetrazolium test showed a progressive loss of viability of *L. watsoniana* seeds (seed viability dropped from 100% in fresh seeds to 30% after four months of storage). This is in agreement with the results obtained by Maciel (1994, 1996) who found germination rates of 20-30% in 4-month-old seeds stored at room temperature. This loss of viability emphasises the importance of the use of fresh seeds in germination tests and might imply the absence of soil seed banks for *L. watsoniana* (Yu *et al.*, 2007; Wijayratne and Pyke, 2012). It also suggests that additional tests are required to find the best conditions for short- and long-term seed storage.

Besides the main effects of temperature regime, GA<sub>3</sub> and type of light, we also found a number of complex interactions between those factors. These are in agreement with studies in *L. sativa*, which also pointed out the importance of the interaction between growth regulators, temperature and light in the germination of different cultivars (Speer *et al.*, 1974; Dunlap and Morgan, 1977; Kristie *et al.*, 1981; Lee, 1989). For instance, according to Nascimento *et al.* (2004), ethylene may interact with light or gibberellins to promote germination at high temperature, and Speer *et al.* (1974) showed that ethylene and GA<sub>3</sub> are highly active when used in combination with red light. A possible explanation is that ethylene enhances the penetration of the other substances such as GA<sub>3</sub>. The action of gibberellins in lettuce seed germination might be through promotion of ethylene synthesis, and ethylene might then stimulate germination by other mechanisms (Stewart and Freebairn, 1969). However, in our study, ethephon showed a positive effect only when no GA<sub>3</sub> was added and interacted negatively with the highest concentration of GA<sub>3</sub>.

Five of the eight best treatments found in our study used red light. This positive effect of red light on the germination of *L. watsoniana* agrees with earlier studies of Speer *et*



*al.* (1974) where a combined treatment of red light and GA<sub>3</sub> promoted germination of *L. sativa* seeds. There was a generally negative effect of the green wavelength on germination percentage, with three exceptions: when combined with increased temperature (20/15°C); when combined with the highest GA<sub>3</sub> concentration; with the addition of ethephon. Previous reports had shown that the addition of ethephon could overcome the occurrence of the inhibitory influence of blue to green light on seed germination of *L. sativa* (Lee, 1989). The importance of the interactions is obvious from the germination progress curves: the T<sub>50</sub> was quite variable, ranging from 11 to 43 days but the fastest rates corresponded to 15/10°C, 0.1 mg l<sup>-1</sup> GA<sub>3</sub>, with the addition of ethephon and red light. Gibberellic acid at the concentration of 10 mg l<sup>-1</sup>, apparently does not interact positively with any of the other factors, but resulted in relatively low T<sub>50</sub> (approximately 16 days).

Cotyledon development in *L. watsoniana* required specific temperature conditions since high temperatures induced high mortality in this critical phase. This is similar to observations made in other endemic plants such as *Picconia azorica* (Tutin) Knobl. (Oleaceae) where low temperatures also promoted seedling growth (Martins *et al.*, 2012).

We also observed that increase in diameter and woodiness of seedling epicotyls was faster than seedling growth in height. Preliminary vegetative propagation data (E.F. Dias, unpubl.) show that within two years the plants only grow to a height of about 120 mm and a diameter of 27 mm. On the other hand, the vegetative part of the largest mature plants is about 400 mm high, and the synflorescence can reach up to 1.5 m. Thus, *L. watsoniana* grows slowly and therefore large plants in nature probably have a considerable age. Natural *L. watsoniana* populations are confined to open spaces like steep slopes of craters, ravines and clearings in temperate juniper rain forest, with soil temperatures that oscillates between 10/15°C during seed maturation (E.F. Dias, unpubl.), which fits well to our experimental results. Based on our results new seedlings in nature should appear about three weeks after seed release, if all the ecological conditions are ideal.

## Conclusions

Similarly to other *Lactuca* spp., *L. watsoniana* seeds respond to a complex array of interactions, where red light, gibberellic acid, ethephon and temperature seem to play the most important role. In order to obtain high germination success in *L. watsoniana*, three rules should be followed: a) collect only well-developed black seeds; b) use fresh seeds stored at room temperature for a maximum of one month; c) germinate seeds with 0.1 mg l<sup>-1</sup> GA<sub>3</sub>, ethephon, a red light filter and a temperature regime of 15/10°C.

We believe that our germination protocol will be useful for the restoration of the most depauperate and endangered Azorean lettuce populations, where number of individuals is very low. The newly produced plants from our study have already been reintroduced to the source population in 2013. However, simply producing additional plant individuals does not help against habitat loss and predation through introduced herbivores like rabbits and goats. These most important threats of *L. watsoniana* can only be addressed in comprehensive species management plans, which are urgently needed not only for *L. watsoniana* but also for many other Azorean endemics.

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