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Research Article

Potential Propagation by Seed and Cuttings of the Azorean Native *Calluna vulgaris* (L.) Hull

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This work investigates the potential propagation by seed and cuttings of the Azorean native *Calluna vulgaris* (L.) Hull. for landscape conservation. With that purpose we have performed several germination and cuttings trials, using plant material from wild populations of this species. In the germination trials, we tested the effects of photoperiod length (8 and 16 h), temperature (10, 15, 20, and 20–10°C), seed age (6, 108, and 270 days), temperature of seed storage (4°C and room temperature), and seed surface sterilization on the germination characteristics. In the cuttings trials, we tested the effects of stem cutting type, cultural conditions, cuttings' harvest month, and rooting substrates on the rooting percentages. The best percentages of germination, 93 and 90%, were obtained with fresh seeds and surface sterilized and sown under an 8 h photoperiod and with temperatures of 10°C or 15°C, respectively; germination after seed storage during 270 days is significantly superior (71%) when seeds are stored at 4°C. The best percentages of rooting were achieved for straight (96%) or heel cuttings (90%) harvested in March, planted on soil from natural stands of *C. vulgaris* and *Erica azorica* Hochst., outdoors in half shade, and partially covered with transparent polyethylene film.

1. Introduction

Calluna vulgaris (heather) occurs throughout a broad geographical and climatic range; it is a native species of the Azores archipelago [1], Europe, Asia Minor [2], and Northwest of Africa [3], and it was introduced on Madeira Island [4], Canada, United States, Falklands and Crozet islands, Australia, and New Zealand [5]. *C. vulgaris* is a keystone species not only in Europe's ecosystems [6, 7] but also at the Atlantic islands of Azores [8]. This species is present on all the islands of the archipelago and can be found up to 2300 m altitude at Pico Island [8, 9]. Since the beginning of settlement in the fifteenth century, the natural vegetation of the Azores has been undergoing a gradual change in terms of its composition and structure, as a result of human activities and the corresponding introduction of numerous plant and animal species. Some of these species have escaped from culture and become naturalized or invasive [10]. Presently only 18.3% of the vascular plants that occur in the Azores are considered to be native [11]. Nevertheless, in the last years, several efforts have been directed to the protection and restoration of the Azorean landscapes and habitats, as in the project "Sustainable Laurissilva"—LIFE07 NAT/P/000630 [12] or in the "Furnas Monitoring and Research Centre-CMIF", both at São Miguel Island [13].

Since the first LIFE financed project directed to the habitat restoration of the Azorean bullfinch, the Regional Forestry Services holds the production of the autochthonous trees species, excluding the production of small shrubs like *C. vulgaris* [14]. Nevertheless, this is a species of interest to the current project of LIFE Program "Lands of Priolo"—LIFE12 NAT/PT/000527 [15] and to the "CMIF." At the Azores the first attempts to germinate seeds in trays (Life project, Filipe Figueiredo, personal communication, 2013) or direct sowing in field (CMIF, Malgorzata, personal communication, 2012), failed to produce high quantities of plants or in field establishment of new plants. The absence of data regarding the germination characteristics and the success of propagation by cuttings of the Azorean heather leads us to study the potential propagation of *C. vulgaris* Azorean wild populations.

Today heathlands restoration combines different techniques including mechanical disturbance, low intensity grazing and fires, transference of soil blocks with plants and/or seeds, seeding using fruiting branches or capsules, and planting potted plants grown in nurseries [16–19]. In the United Kingdom restoration techniques involving the sowing of *C. vulgaris* seeds by hydroseeding or direct broadcast lead often to germination levels below 10% due to seed dormancy [20]. Because of its ornamental value, *C. vulgaris* has been propagated by seed, cuttings [21–24], and *in vitro* culture [25]. Germination of *C. vulgaris* seeds is affected by pH, light, and temperature but the germination characteristics and the presence of dormancy differ among the seed lots used [20]. Seed lots differ from each other according the climatic severity of the home site [26], the storage conditions of the seeds [20, 27], and their age [28, 29]. *C. vulgaris* seeds arepositively photoblastic [28, 30–32] and germination around a pH 3.8–4.0 [33] and 20°C is generally high [20, 26, 27, 30, 34]. Higher germination levels in fresh seeds compared with stored ones were reported by Pons [35] for this species,while Grimstad [27] found that seed conservation to 3°C for 16 months slightly improved germination. Seed dormancy of *C. vulgaris* is broken by gibberellic acid treatments (GA_3, GA_4, GA_7) alone [27, 30] or in combination with smoke solutions during the imbibition periods [20]. Cuttings have been the preferred technique to propagate the ornamental clones of *C. vulgaris* not only because of the maintenance of the phenotypic characteristic but also because small seeds (0.55–0.65 × 0.35–0.45 mm) [31] and seedlings are difficult to manipulate, and the time elapsed between a seedling and a flowering plant is longer [36]. *C. vulgaris* can be propagated in any season of the year, if vegetative straight or heel cuttings are properly disinfected, planted in suitable substrates, and placed in greenhouses with control of relative humidity, temperature, and fungal diseases [22–24, 37, 38]. Both *in vitro* produced shoots [25] and stem cuttings need no exogenous application of auxins to get rooted [36–38]. Garcia et al. [36] recommend a mixture of peat : perlite (1 : 1, v/v) as rooting substrate and cell trays with 35 cm^3 of capacity and 5 cm high, while Iglesias-Díaz and González-Abuín [38] use a mixture of peat : bark : sand (1 : 2.5 : 1.5, v/v) in cells with 3 × 3 × 5 cm.

Previous studies on *C. vulgaris* have provided data on differentiation into ecotypes depending on altitude [39–41], while methods of allozyme and chloroplast DNA analysis have made possible to reveal geographic variation and differentiation of populations [42–44]. Taking into account the specific climatic, soil, and biotic conditions of the Azorean islands, where the isolated populations of *C. vulgaris* have been growing for the last thousands years, we have put forward the hypothesis that some ecotypic differences for characteristics of adaptive significance may have arisen, including those related to the germination characteristics.

In order to identify these potential differences that are useful to the regional projects dealing with the propagation of the Azorean heather, we studied the effect of light and temperature regime on seed germination and the effect of storage period, storage temperature, and surface sterilization of seeds on seed germination. In addition we studied the effect of cutting's harvesting month, cutting type, substrate, and cultural conditions on rooting induction.

2. Methods

2.1. Germination Trials

2.1.1. Plant Material. Seeds were harvested at Tronqueira, a region belonging to the Natural Park of São Miguel Island, and under intervention of the "Lands of Priolo" LIFE project. Capsules of *C. vulgaris* were manually collected in September, over 100 different shrubs (nearly 20 m between each shrub). In the next day, plump seeds were selected under a stereomicroscope, placed inside paper bags, and stored in hermetic glass jars with silica gel at room temperature or at 4°C, until their use. Also, a seed lot harvested at December 2012 with two months of storage at room temperature used to sow in restoration areas was kindly provided by CMIF.

2.1.2. Seed Sterilization and Germination. Seed surface sterilization was only performed with a lot of 108 days stored seeds, since nonstored seeds presented a percentage of germination above 90 without sterilization. Before sowing, seeds were soaked in a 0.6% benomyl solution (Benlate) for 30 min, rinsed 3 times with sterile distilled water, soaked in a 10% commercial bleach solution with 0.01% Tween 20 (Sigma) for 20 min, and finally rinsed six times with sterile distilled water. All the seeds were sown in Petri dishes, but surface sterilized seeds were sown on sterilized material. Lots of 100 seeds were placed on filter paper (Whatman number 1) moistened with distilled water, in 12 cm diameter Petri dishes sealed with laboratory film (parafilm "M"). The germination trials were carried out in germination chambers with automatic control of light (light intensity ~56 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and temperature (±1°C). Two photoperiods (8 or 16 hours of light in each 24 hours) and the following temperature regimens were tested: continuous temperatures of 10°C, 15°C, and 20°C and alternating temperatures of 20°C (during the 8 h of light) and 10°C (during the 16 h of darkness).

2.1.3. Data Analysis. The number of seeds showing radicle emergence was recorded every week. For each different temperature and light regime, four replicates of 100 seeds were set up [45]. The "mean time of germination" was calculated according to the formula: mean time of germination = $\sum(n_i * d_i)/N$ (where n is the number of germinated seeds on day i, $d$the number of days from the beginning of the test, and N is the total number of germinated seeds) [46]. For the variable "percent of germination", a χ^2 test was used to analyze the contingency tables and, when the replicate results were homogeneous, the total χ^2 was used. A Levene test was used to test the homogeneity of the variables "number of days to first radicle emergence" and "mean time of germination." When homogeneity was encountered, data were statistically compared using the student's t-test or the one-way analysis of variance (ANOVA) and, when the null hypothesis was rejected, the Tukey multiple comparison test was used. When homoscedasticity was not verifiable, data were statistically

Table 1: The effect of light regime on the germination characteristics of *Calluna vulgaris* seeds at 15°C. Storage period at room temperature before trial: 6 days. Mean values ± standard deviations. In each column, groups that showed significant differences ($P < 0.05$) are indicated with different letters.

Photoperiod (hours of light per day)	Number of seeds	Days to first radicle emergence	Mean time of germination (days)	Percent germination (at day 126)
8	4 × 100	15 ± 1.7^a	34 ± 3.0^a	90 ± 7.0^a
16	4 × 100	21 ± 3.8^a	41 ± 3.9^a	70 ± 7.9^b

Table 2: The effect of temperature regime on the germination characteristics of *Calluna vulgaris* seeds under a photoperiod of 8 h. Storage period at room temperature before trial: 6 days. Mean values ± standard deviations. In each column, groups that showed significant differences ($P < 0.05$) are indicated with different letters.

Temperature regime (°C)	Number of seeds	Days to first radicle emergence	Mean time of germination (days)	Percent germination (at day 126)
10	4 × 100	17 ± 2.0^a	44 ± 3.1^a	93 ± 5.8^a
15	4 × 100	15 ± 1.7^a	34 ± 3.0^b	90 ± 7.0^a
20	4 × 100	15 ± 1.5^a	33 ± 5.2^b	65 ± 6.8^b
20–10	4 × 100	17 ± 1.0^a	32 ± 2.8^b	74 ± 6.5^b

compared using the Mann-Whitney test or the Kruskal-Wallis test for nonparametric analysis of variance and, when the null hypothesis was rejected, a nonparametric Tukey-type test for multiple comparisons was used.

2.2. Cutting Trials

2.2.1. Plant Material. Stem cuttings were collected from approximately 200 adult shrubs of a wild population at Lombadas (São Miguel Island), in October 2011, February 2012, and March 2012. Cuttings were sprayed with tap water, placed inside polyethylene bags, and transported in approximately 40 minutes to the laboratory. The cuttings were then placed in tap water and the following types of cuttings with 4 to 7 cm long were prepared: straight, mallet, and heel cuttings from vegetative stems. In October straight cuttings from stems with remains of dry capsules were also done and identified as cuttings from reproductive stems. Foliage was removed from the basal 2-3 cm of each cutting and in the reproductive stems the remains of dry capsules were also removed.

2.2.2. Rooting Conditions. Four types of rooting substrates were tested: (a) native soil, (b) native soil: horticultural coarse perlite (http://www.granja.pt) (3 : 1), (c) blond peat (Tref Substrates BV): perlite (1 : 1), and (d) blond peat: perlite: vermiculite (Verlite) (2 : 1 : 1). Native soil was collected in areas with *C. vulgaris* and *Erica azorica* shrubs at Lombadas on landslides near the road. Native soil was selected as rooting substrate, since all the produced plants are meant to return to their native habitat; this substrate is available for the project teams at no cost and also possesses the symbionts that can colonize *C. vulgaris* roots. In order to improve drainage of the native soil substrate, horticultural coarse perlite (http://www.granja.pt) was added to that substrate. The mixture recommended by Garcia et al. [36] was also tested, alone or with vermiculite to improve water retention. The substrates were placed in plastic trays with 40 cells, each one with 35 cm^3 and trays were partially covered with transparent polyethylene film. Two cultural conditions were tested: growth chamber (20°C, 16 h photoperiod, and 85% ± 3 environmental relative humidity) and outdoors in half shade from March to June (81.4% mean relative humidity, 16.5°C mean temperature). Rooting substrates were maintained moist by applying water manually.

2.2.3. Data Analysis. The study was carried out with four replicates in a randomized blocks design. Ten cuttings per replicate were used. The number of cuttings showing roots was recorded after 12 weeks in culture. For the variable "percent of rooting," a χ^2 test was used to analyze the contingency tables and, when the replicate results were homogeneous, the total χ^2 was used.

3. Results

3.1. Germination Trials

3.1.1. Effect of Light Regime on Seed Germination. The shortest photoperiod (8 h/day) significantly increased the germination percentages but did not significantly decreased the number of days to first radicle emergence or the mean time of germination (Table 1).

3.1.2. Effect of Temperature on Seed Germination. The significantly highest percentages of germination were obtained with the temperatures regimes of 10°C and 15°C, but the temperature regime of 10°C significantly delayed the mean time of germination. The number of days to first radicle emergence was not significantly different within the temperatures regimes tested (Table 2).

3.1.3. Effect of Storage Period on Seed Germination. Seeds with 6 days of storage presented significantly superiors percentages of germination and mean times of germination.

Table 3: The effect of storage period at room temperature on the germination characteristics of *Calluna vulgaris* seeds under a photoperiod of 8 h and a temperature regime of 10°C. Mean values ± standard deviations. In each column, groups that showed significant differences ($P < 0.05$) are indicated with different letters.

Storage period at room temperature before trial (days)	Number of seeds	Days to first radicle emergence	Mean time of germination (days)	Percent germination (at day 112)
6	4 × 100	17 ± 2.0[a]	44 ± 3.1[a]	93 ± 5.8[a]
108	4 × 100	7 ± 2.0[a]	30 ± 1.5[c]	52 ± 13.0[b]
270	4 × 100	12 ± 3.0[a]	37 ± 2.2[b]	41 ± 16.3[b]

Table 4: The effect of storage temperature on the germination characteristics of *Calluna vulgaris* seeds under a photoperiod of 8 h and a temperature regime of 10°C. Mean values ± standard deviations. In each column, groups that showed significant differences ($P < 0.05$) are indicated with different letters.

Storage period and temperature	Number of seeds	Days to first radicle emergence	Mean time of germination (days)	Percent germination (at day 112)
270 days at 4°C	4 × 100	11 ± 2.5[a]	34 ± 5.6[a]	71 ± 7.9[a]
270 days at room temperature	4 × 100	12 ± 3.0[a]	37 ± 2.2[a]	41 ± 16.3[b]

The number of days to first radicle emergence was not significantly different within the storage periods tested (Table 3). The germination test (15°C, 8 h photoperiod), using the 2-month stored seed lot provided by CMIF, resulted in a mean germination percentage of 5.1 ± 3.6 with 49.0 ± 8.1 days to first radicle emergence and a mean time of germination of 54.3 ± 8.8 days.

3.1.4. Effect of Storage Temperature on Seed Germination. Seeds stored at 4°C showed significantly superior percentages of germination regarding those stored at room temperature, but the number of days to first radicle emergence and the mean time of germination were not significantly different (Table 4).

3.1.5. Effect of Surface Sterilization on Seed Germination. At the end of this germination test (day 112), only part of the nongerminated seeds without surface sterilization presented contamination, while none of the surface sterilized seeds presented contamination. Seeds surface sterilized showed significantly superior percentages of germination regarding the control, while the velocity of germination was not affected (Table 5).

3.2. Cutting Trials

3.2.1. Effect of Stem Cutting Types on Rooting Response. The rooting response was influenced by the type of stem cutting. Significantly inferior percentages of rooting were found for mallet cuttings, either for cuttings harvested in October and planted in peat : perlite (1 : 1) or for cuttings harvested in March and planted in native soil : perlite (3 : 1) substrate, both under a temperature of 20°C and a 16 h photoperiod (Table 6).

3.2.2. Influence of Cultural Conditions on Rooting Response. For a total of 160 heel cuttings harvested in March and planted in native soil : perlite (3 : 1) substrate, the rooting percentages were not significantly affected by cultural conditions: 90.0% ± 13.5 at the growth chamber (20°C, 16 h photoperiod) and 87.5% ± 11.9 outdoors.

3.2.3. Influence of Harvest Month on Subsequent Rooting Response. For a total of 80 heel cuttings, planted outdoors in native soil : perlite (3 : 1) substrate, the rooting percentages were significantly influenced by the harvest month: 95.0% ± 10.0 for cuttings harvested in March and 70.0% ± 5.0 for cuttings harvested in October.

3.2.4. Effect of Substrates on the Rooting Response. For heel cuttings harvested in March and planted outdoors, the rooting response was influenced by the substrates used. The rooting percentages were significantly superiors for cuttings planted in native soil or native soil : perlite (3 : 1) substrates (Table 7).

4. Discussion

4.1. Germination Trials. Germinationof *C. vulgaris* seeds is light dependent; however, their exposure to continuous light [20, 30] or long photoperiods (16 h) from white fluorescent tubes reduces the percentages of germination. This behavior is similar to some other species of seeds, which germinate under short days despite their dormancy being broken by light [47]. Spindelböck et al. [26] studying the strategies of *C. vulgaris* seed germination in the Norwegian climates demonstrated that conditional cold avoidance explains the between-population variation in the germination characteristics. Unlike Juntilla [30] using continuous light, or Spindelböck et al. [26] using a 16 h photoperiod, the percentages of germination here obtained using a short photoperiod (8 h) increased with decreasing temperature within the temperature range of 20, 15, and 10°C. In fact, the preferred germination temperatures of the Azorean *C. vulgaris* seeds

Table 5: The effect of surface sterilization on the germination characteristics of *Calluna vulgaris* seeds under a photoperiod of 8 h and a temperature regime of 10°C. Storage duration: 108 days. Mean values ± standard deviations. In each column, groups that showed significant differences ($P < 0.05$) are indicated with different letters.

Surface sterilization	Number of seeds	Days to first radicle emergence	Mean time of germination (days)	Percent germination (at day 112)
Yes	4 × 100	7 ± 2[a]	30 ± 2.6[a]	78 ± 14.1[a]
No	4 × 100	7 ± 2[a]	30 ± 1.5[a]	52 ± 13.0[b]

Table 6: Rooting percentages obtained on different types of cuttings of *Calluna vulgaris* under a photoperiod of 16 h and a temperature regime of 20°C. Mean values ± standard deviations. In each column, groups that showed significant differences ($P < 0.05$) are indicated with different letters.

Type of stem cutting	*N*	October Peat : perlite (1 : 1)	*N*	March Native soil : perlite (3 : 1)
Heel	4 × 10	50.0 ± 14.1[a]	4 × 20	90.0 ± 13.5[a]
Reproductive	4 × 10	30.0 ± 7.1[ab]	—	—
Straight	4 × 10	25.0 ± 7.1[bc]	4 × 20	96.3 ± 4.8[a]
Mallet	4 × 10	10.0 ± 7.1[c]	4 × 20	70.0 ± 16.3[b]

Table 7: The effect of substrate type on the rooting percentages of *Calluna vulgaris* heel cuttings harvested in March and planted outdoors. Mean values ± standard deviations. Groups that showed significant differences ($P < 0.05$) are indicated with different letters.

Substrate	*N*	Percent rooting
Native soil	4 × 10	80.0 ± 10.0[a]
Native soil : perlite (3 : 1)	4 × 10	95.0 ± 10.0[a]
Peat : Perlite : vermiculite (2 : 1 : 1)	4 × 10	62.5 ± 9.6[b]
Peat : perlite (1 : 1)	4 × 10	45.0 ± 12.9[c]

(10 to 15°C) are present throughout the year in the vast majority of *C. vulgaris* distribution area [48–50]. Since the Azores islands benefit from a relatively stable temperate oceanic climate, the absence of frost and temperatures lower than 6°C during the winter in the distribution area of the Azorean *C. vulgaris* [49–51] is reflected in the absence of cold avoidance strategy that the seeds of Norwegian populations of this species possess. Although seed germination photoperiodic requirements depend on the temperature [52], from a functional viewpoint the preferences of the Azorean *C. vulgaris* seeds by a short photoperiod (8 h) and temperatures between 10 and 15°C prefigure the possibility of germination during autumn and winter and represent a characteristic of adaptative significance. Under a suitable photoperiod (8 h) and temperature (10 to 15°C) regimes most of the Azorean seeds of *C. vulgaris* will not exhibit dormancy, but in no more than 9 months of storage at room temperature (≈20°C) the germination capacity decreases more than 50%. Low temperature during seed storage is a current practice [45] to reduce seed metabolism and preserve their germination capacity. As previously referred by Grimstad [27] we found a positive effect in the storage of *C. vulgaris* seeds under a temperature of 4°C, resulting in 71% of germination. The long mean time of germination allows the development of fungi inside the Petri dishes when using stored seeds at room temperature, which ultimately has a negative effect on the final number of germinated seeds; in these circumstances the surface sterilization of the seeds and the use of sterile equipment revealed to be useful to perform the *in vitro* germination tests.

4.2. Cutting Trials. *C. vulgaris* has been propagated by cuttings and it is considered an "easy-to-root" species [21–24, 37]. In our study, it was possible to obtain rooted *C. vulgaris* plants using cuttings harvested in different months, different types of cuttings and substrates, and different cultural conditions. Nevertheless, superior percentages of rooting were obtained in native soil or native soil with improved permeability by the addition of perlite using straight cuttings and heel cuttings harvested in March. The cuttings mortality may have contributed to the absence of a surface sterilization treatment previous to plantation and the absence of any fungal disease control or mist cycles during the culture [38]. The best results obtained with native soil are best explained by the positive effect of beneficial soil microorganisms on plant disease suppression and plant growth promotion [53–55]. The superior rooting percentages obtained in spring are usually explained by the physiological adjustment associated with the seasonal biological rhythms that occur in temperate climates [39, 56]. Since wounding induces initial cell division from parenchymal cells, the physical (a bigger area ripped out) and histological characteristics of the wounded area in the heel cuttings in relation to the mallet cuttings may explain the superior percentages of rooting observed in cuttings harvested in less favorable months or cultivated on less suitable cultural conditions [57].

5. Conclusions

In this study we have found some ecotypic differences for characteristics of adaptive significance related to the germination characteristics. The Azorean seeds of *C. vulgaris* lack

a cold avoidance strategy since the germination preferences for lower temperatures and short days indicate the possibility of germination during autumn and winter seasons; therefore, the nondormant seeds can be sown immediately after harvest if temperatures of 10° until 15°C are provided. In restoration areas and under a perspective of annual seed harvesting and sowing, the seed storage at 4°C can be an easy procedure to store the seeds before they are sown and until the next season of seed production. At the Azores the very small seeds of the native *C. vulgaris* get buried with the frequent rains and are easily outcompeted with fast growing introduced herbaceous species on restoration areas; therefore, seeds should be sown in trays on commercial germination substrate and sheltered from rain. Also, the Azorean heather can be propagated at low cost by straight or heel cuttings harvested in March, planted on soil from natural stands of *C. vulgaris* and *Erica azorica* Hochst, and partially covered with transparent polyethylene film, outdoors in half shade.

Conflict of Interests

The authors declare that they have no conflict of interests.

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