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Influence of Light Intensity and Temperature on the Development of Early Life Stages of *Ascophyllum nodosum* (Phaeophyceae)

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Abstract: *Ascophyllum nodosum* is an ecologically and economically important species forming marine forests in temperate regions. In Europe, this brown seaweed reaches its southern distribution limit in the north of Portugal, where populations are under climatic pressure. Conservation and restoration actions are essential to preserve the important ecological roles of these populations, including biodiversity enhancement. In this study, we assessed the effect of temperature and light on the development of the early life stages of *A. nodosum*, from gamete germination and rhizoid development to germling growth, in order to support the establishment of nursery protocols for producing seedlings that can be used in reforestation actions. We found that for this population, temperature around 12 °C and low-light conditions (40 µmol m^{-2} s^{-1}) favor gamete germination, rhizoid development, and initial germling growth during the first month, after which higher light supply (>80 µmol m^{-2} s^{-1}) is needed to sustain further growth stages. The results obtained in this study are relevant for the establishment of nursery methods for *A. nodosum* and should be complemented by further studies to determine more precisely the light and nutrient requirements to optimize growth in the germlings' later growth stages.

Keywords: fucoids; marine forests; germination; reforestation; nursery

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1. Introduction

Canopy-forming seaweeds, mainly brown macroalgae like fucoids and kelps, play a crucial role in creating diverse and complex ecosystems in rocky shores. Acting as ecological engineers, they sustain a multitude of species by providing them with a habitat, shelter, and food, thereby increasing productivity and affecting nutrient cycling [1].

Several stressors, both natural and anthropogenic, can dramatically shape these macroalgal assemblages by affecting their distribution, abundance, and diversity [2]. Even if macroalgal communities may adapt to natural disturbance, their resistance and resilience to sporadic events, such as those caused by anthropogenic pressures, is largely dependent on the traits of individual species, such as recruitment and settlement rates [3,4]. In species with low dispersal ability and a low recruitment rate, like most fucoids, the natural recovery of canopy stands after loss is infrequent, if not impossible, in many populations [5,6]. During the last decades, climate change, pollution, resource depletion, the introduction of non-native species, and habitat destruction have all contributed to the decline of canopy-forming species along the coasts of Europe, with reductions in abundance and extinctions in several locations [7]. Despite substantial conservation efforts, most of these degraded ecosystems have yet to recover, underscoring the necessity for proactive restoration measures [8].

As a result, there has been a growing interest in marine forest restoration, accentuated in recent years by the EU Biodiversity Strategy 2030, which envisions reversing the decline of these habitats through the active reintroduction of individuals to create self-sustainable populations [9]. Active restoration has traditionally involved several techniques, including transplantation, seeding, and the introduction of artificial habitats [10,11]. Out-planting, which consists of producing juveniles from fertile material in nurseries for deployment in the field, is one of the techniques recommended for species with low dispersal capacities, such as fucoids, or threatened species, to avoid the depletion of natural donor populations [12].

Ascophyllum nodosum (Linnaeus) Le Jolis 1863, one of the main canopy-forming species in the North Atlantic Ocean, is a brown seaweed belonging to the family Fucaceae, closely related to the *Fucus* genus. In Europe, this species is found between the Arctic circle and Portugal, its southern distribution limit, in sheltered intertidal rocky shores [13,14]. Reproduction usually occurs during winter, although variations might arise due to fluctuations in atmospheric and seawater temperature [14]. *Ascophyllum nodosum* is a long-lived species with limited dispersal ability and a low recruitment rate, with its recruits having extremely slow growth rates, ranging 1–2 mm in the first year and reaching 1.5 cm after 2 years, when compared to those of other fucoids such as *Cystoseira* spp, which can grow up to 1 cm per month [5,14–17]. However, these species have a high potential to produce gametes and zygotes under optimal cultivation conditions [5].

In northern Portugal, *A. nodosum* occurs in a single marginal population over a rocky intertidal area that is approximately three kilometers long and tens of kilometers from the nearest northern populations [13,18]. Marginal populations are often reduced in size, and the already sub-optimal local conditions can drastically diminish their ability to deal with further disturbances [19,20]. These populations may also exhibit unique ecological adaptations with significant conservation value, especially under a changing climate scenario [19,21,22].

Given its life history traits and the high sensitivity to stressors, out-planting seems to be a viable option for the sustainable restoration of *A. nodosum*. This method has already been performed with *Cystoseira* spp. and other large fucoids [23–25], but, as far as we know, it has never been tested on *A. nodosum*.

The objective of this study was to contribute to the development of nursery protocols for producing seedlings of *A. nodosum* for out-planting, by assessing the influence of temperature and light intensity on the settlement and growth of *A. nodosum*'s early life stages.

2. Materials and Methods

In January 2023, fertile receptacles of *A. nodosum* were collected from the southernmost population in Europe, located in northern Portugal (Praia Norte, Viana do Castelo: 41.6970° N, 8.8510° W; Figure 1). Sampling was performed randomly across an approximate area of 100 m^2 within the population. Five to ten receptacles were collected from each individual.

Figure 1. Location of the *Ascophyllum nodosum* population at its southern limit of distribution in the north of Portugal.

At the laboratory, receptacles were separated by sex, after microscopic identification of female (oogonia) or male (spermagonia) gametangia, and stored under dark, humid, and cold (5 °C) conditions for 24 h. Receptacles from 16 individuals (7 female and 9 male) were used in the experiment.

The following day, receptacles were left to air-dry for two hours at room temperature [26]. Next, male and female receptacles were placed together on a 0.5 cm diameter mesh within a beaker filled with sterile seawater to induce gamete release. After one hour, the receptacles were removed, and the resulting gamete suspension was distributed into six-well plates at 10 mL per well. To examine the effects of varying cultivation conditions, a factorial laboratory experiment was conducted, combining different temperature and light levels. Three temperatures covering the range of the natural local temperature regime (12, 15, and 18 °C) and two light intensity levels (low: 40 μmol m^{-2} s^{-1} and high: 80 μmol m^{-2} s^{-1}) were selected. Six combinations of the cultivation conditions, consisting of two crossed levels of each cultivation condition (12H, 12L, 15H, 15L, 18H, 18L), were tested in a two-way crossed design. For each combination of factors, 6 replicates were used.

To ensure nutrient provision, Provasoli's Enriched Seawater [27] was added weekly. Germanium dioxide (GeO_2) was also added to the nutrient medium to prevent diatom growth.

The initial egg density and germination percentage were assessed after the first week by counting the number of eggs released and their development into embryos, respectively, in each well. Initial growth was evaluated by measuring the length of rhizoids three weeks after release. After one month in culture, the rhizoids had significantly elongated, making accurate measurement difficult. For this reason, embryo growth was assessed by measuring the occupied area each month. The cultivation experiment lasted three months (until April 2023).

Measurements were always performed by processing photographic data using the software ImageJ (version 1.54g). For each replicate, 6 fields of vision were randomly selected in photos obtained by a Nikon Eclipse TE300 (Nikon Instruments Inc., Melville, NY, USA) inverted microscope using a Nikon Digital Sight DS-U3 image acquisition system.

Statistical Analyses

Prior to statistical analysis, assumptions of data normality were assessed using the Levene's test and data were log-transformed when necessary [28].

To evaluate the effect of the different cultivation conditions on germination percentage, a two-way ANOVA was performed with temperature (3 levels, fixed: 12, 15, and 18 °C) and light intensity (2 levels, fixed: low light, high light) as factors. Unfortunately, due to a malfunction of the climatic chamber set at 15 °C, analysis of growth data was carried out only for temperatures 12 and 18 °C. The same statistical design was performed to assess the effect of temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on the length of rhizoids. To avoid problems of independence, we ran separated analyses of embryos growth (areas) for each experiment month (February, March, and April). All measurements were expressed as average ± standard deviation. All the analyses were carried out using GraphPad Prism version10.0.0. Tukey's tests were used for post hoc comparisons of significant means.

3. Results

3.1. Egg Release and Development Stages

Gamete release occurred within 1 h after rehydration. The concentration of released eggs was 10.95 per mL and the egg diameter ranged from 65.2 to 76.1 µm, averaging 71.2 ± 4.4 µm (Figure 2A). The first division of the fertilized eggs occurred in the first 24 h after release, starting germination (Figure 2B). Germination was followed by the development of rhizoids (Figure 2C), and as the germlings continued to grow, several rhizoids developed and elongated, and their body area increased (Figure 2D–F).

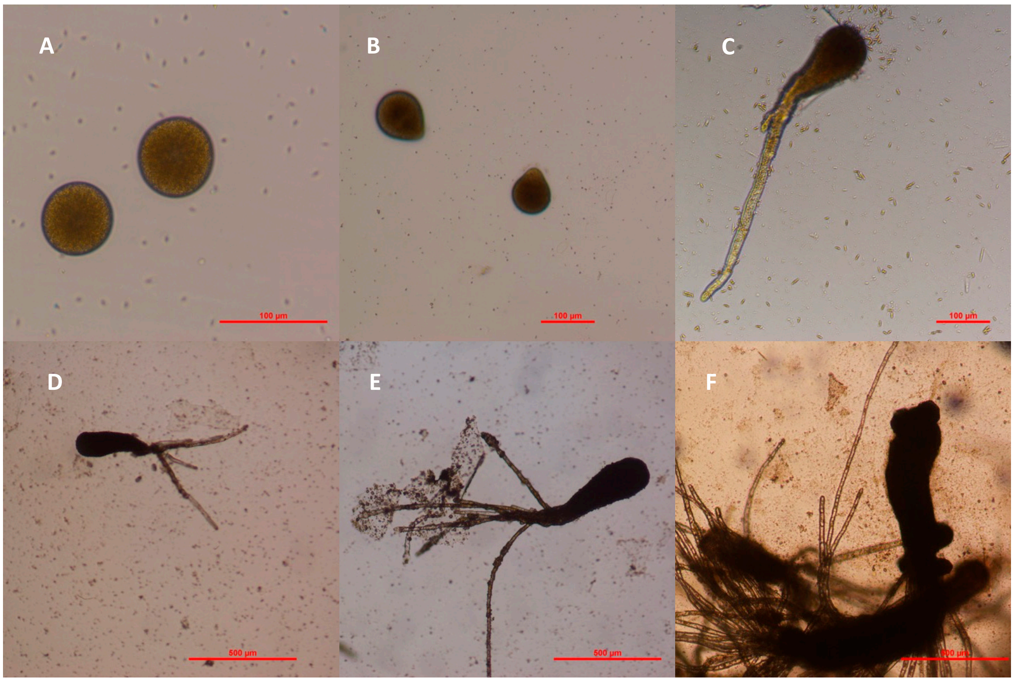

Figure 2. Initial development stages of *Ascophyllum nodosum*. (**A**) Two female gametes surrounded by male gametes released after one hour. (**B**) Germinating zygotes, after one week. (**C**) Embryo with rhizoid 2 weeks after fertilization. (**D**) Embryo at the end of the first month. (**E**) Embryo at the end of the second month. (**F**) Embryos at the end of the experiment.

3.2. Germination

A high percentage of germination was observed in all treatments (Figure 3). A significant interaction was obtained between the factors light intensity and temperature on the percentage of germinated eggs ($p = 0.016$) (Table 1). Tukey's multiple comparisons showed a significant difference only between the eggs at 15 °C and 18 °C at a lower light intensity (15 L vs. 18 L: 98.8 ± 0.6% vs. 93.4 ± 4.2%; $p = 0.0069$; Table S1).

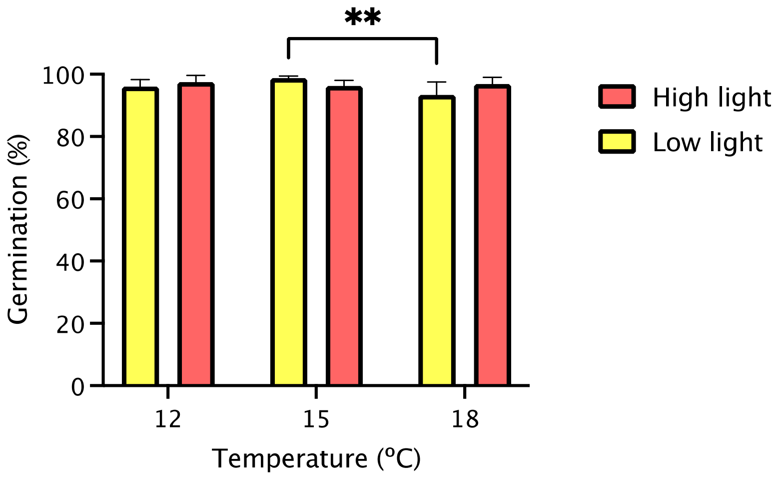

Figure 3. Percentage of germinated eggs (mean ± SD), at the three different temperatures and two light intensities. The asterisks indicate significant differences from Tukey's pairwise comparisons (**—$p \leq 0.01$).

Table 1. Results for the two-way ANOVA testing the effect of the factors temperature (3 levels, fixed: 12, 15, and 18 °C) and light intensity (2 levels, fixed: low light, high light) on egg germination.

Source	*df*	*SS*	*MS*	*F*	*p*
Light_intensity = L	1	5.74	5.74	0.98	0.331
Temperature = T	2	35.34	17.67	3.01	0.064
L × T	2	56.18	28.09	4.79	**0.016**
Error	30	176.10	5.87		
Corrected Total	35	273.35			

3.3. Growth

3.3.1. Rhizoid Length

The two-way ANOVA highlighted a significant effect of the interaction between light intensity and temperature on the length of rhizoids ($p < 0.001$, Table 2). Tukey's multiple comparisons (Table S2) showed that at 12 °C the length of rhizoids was significantly higher at low light compared to at high light (473.1 ± 17.0 µm vs. 223.4 ± 40.4 µm, $p < 0.0001$), while at 18 °C the differences between light levels were not significant (Figure 4). Furthermore, the length was significantly higher at 12 °C compared to at 18 °C for both light intensities (high light: 223.4 ± 40.4 µm vs. 83.9 ± 26.2 µm, respectively, $p = 0.0012$; low light: 473.1 ± 17.0 µm vs. 119.0 ± 25.7 µm, respectively, $p < 0.0001$).

Table 2. Results for the two-way ANOVA testing the effect of the factors temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on rhizoid length.

	df	*SS*	*MS*	*F*	*p*
Light_intensity = L	1	81,065.48	81,065.48	99.22	**<0.001**
Temperature = T	1	243,566.93	243,566.93	298.12	**<0.001**
L × T	1	46,016.69	46,016.69	56.32	**<0.001**
Error	12	9804.14	817.01		
Corrected Total	15	380,453.23			

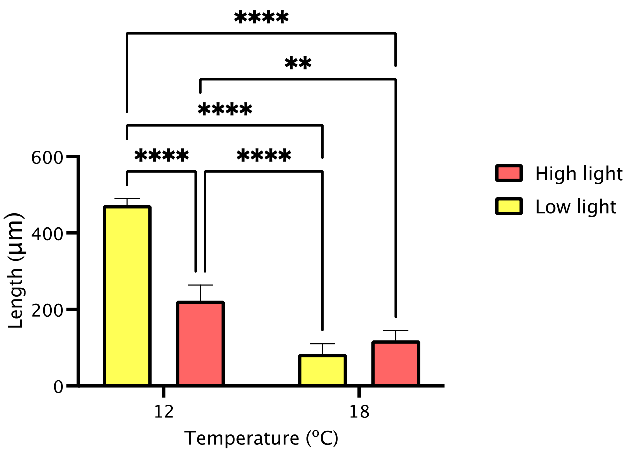

Figure 4. Length of rhizoids (mean ± SD) at two different temperatures and light intensities. The asterisks above bars indicate significant differences from Tukey's pairwise comparisons (**—$p \leq 0.01$; ****—$p \leq 0.0001$).

3.3.2. Embryo Area

An increase in embryo size was observed from February to March in all treatments, remaining constant afterwards (Figure 5A). Differences in area between treatments were assessed for each month, February, March, and April.

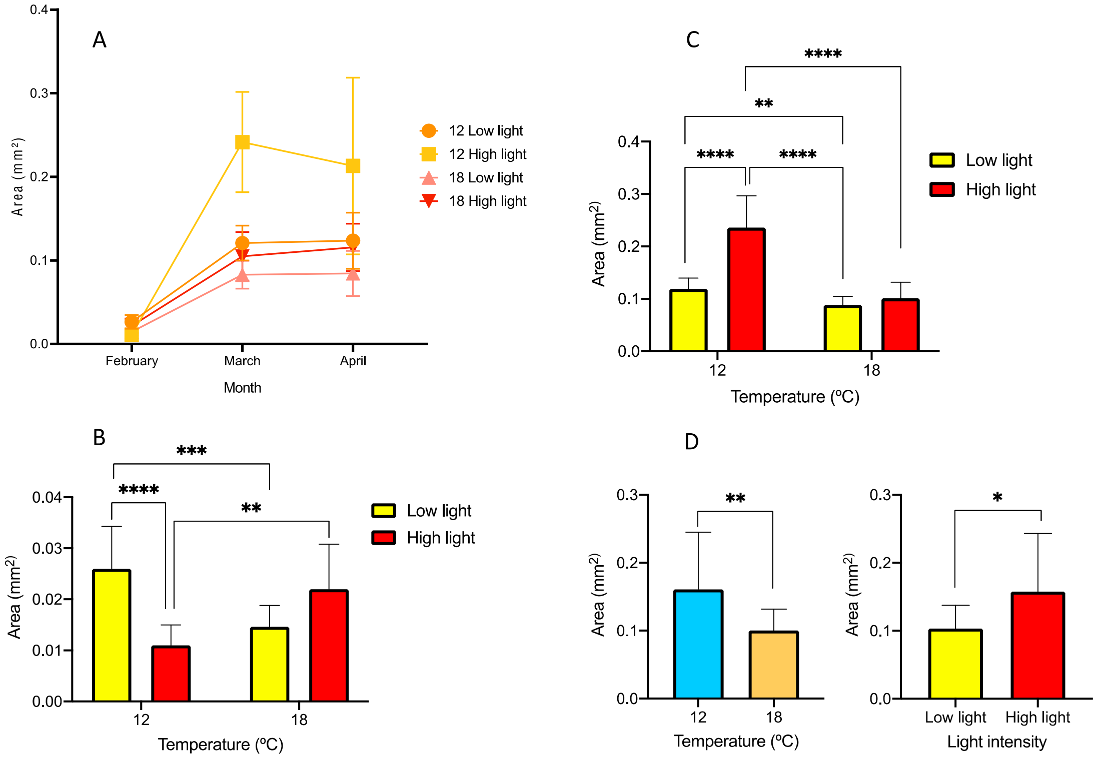

Figure 5. Development of embryos during 3 months at two different temperatures and light intensities. (**A**)—embryo area (mean ± SD) evolution with time, (**B**–**D**)—embryo area (mean ± SD) at the first (February 2023), second (March 2023), and last experiment month (April 2023) in the different conditions tested. The asterisks above bars indicate significant differences from Tukey's pairwise comparisons (*—$p \leq 0.05$; **—$p \leq 0.01$; ***—$p \leq 0.001$; ****—$p \leq 0.0001$).

In February, ANOVA highlighted a significant interaction effect between the factors of light intensity and temperature ($p < 0.001$, Table 3) concerning their influence on the embryo area. Tukey's multiple comparisons (Table S3) showed that the area of embryos kept at 12 °C was significantly higher in low light when compared to in high light (0.026 ± 0.008 vs. 0.011 ± 0.004 mm^2, $p < 0.001$, Figure 5B), while at 18 °C the differences between light intensities were not significant. Under low light, the embryos were significantly larger at 12 °C when compared to at 18 °C (0.026 ± 0.008 vs. 0.022 ± 0.009 mm^2, $p = 0.0009$, Figure 5B). The opposite was observed under high light, with larger embryos at 18 °C than at 12 °C (0.022 ± 0.009 vs. 0.011 ± 0.004 mm^2, $p = 0.0027$, Figure 5B).

Table 3. Results for the two-way ANOVA testing the effect of the factors temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on the area of embryos in February.

Source	*df*	*SS*	*MS*	*F*	*p*
Temperature = T	1	2,969,277.61	2,969,277.61	0.07	0.798
Light_intensity = L	1	185,769,146.15	185,769,146.15	4.16	**0.048**
T × L	1	1,412,922,613.31	1,412,922,613.31	31.61	**<0.001**
Error	40	1,788,204,485.00	44,705,112.13		
Corrected Total	43	3,389,865,522.07			

At the second sampling time (March 2023), the ANOVA also indicated a significant effect of the interaction between the factors light intensity and temperature on the embryo area ($p < 0.001$, Table 4; Figure 5C). Tukey's multiple comparisons (Table S4) showed that the area at 12 °C under high light was significantly higher ($p < 0.0001$) than in all other conditions tested (0.236 ± 0.068 vs. 0.119 ± 0.021 mm^2—12 L; 0.081 ± 0.017 mm^2—18 L; and 0.101 ± 0.031 mm^2—18H). Under low light, the area was higher at 12 °C than at 18 °C (0.119 ± 0.021 mm^2 and 0.081 ± 0.017 mm^2, respectively; $p = 0.0025$).

Table 4. Results for the two-way ANOVA testing the effect of the factors temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on the area of embryos in March.

Source	*df*	*SS*	*MS*	*F*	*p*
Temperature = T	1	89,625,712,974.28	89,625,712,974.28	67.94	**<0.001**
Light_intensity = L	1	55,959,135,885.38	55,959,135,885.38	42.42	**<0.001**
T × L	1	28,291,829,689.33	28,291,829,689.33	21.45	**<0.001**
Error	44	58,043,693,184.93	1,319,174,845.11		
Corrected Total	47	231,920,371,733.92			

In the last experiment month (April 2023), both light intensity and temperature significantly influenced embryo area, with no significant interaction between the two factors ($p = 0.01$ and $p < 0.001$, respectively, Table 5; Figure 5D). Under high light, the embryos reached a higher area than under low light (0.158 ± 0.085 vs. 0.103 ± 0.034 mm^2); moreover, the area was higher at 12 °C than at 18 °C (0.161 ± 0.084 and 0.100 ± 0.032 mm^2, respectively).

Table 5. Results for the two-way ANOVA testing the effect of the factors temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on the area of embryos in April.

Source	*df*	*SS*	*MS*	*F*	*p*
Temperature = T	1	47,649,748,923.31	47,649,748,923.31	14.50	**<0.001**
Light_intensity = L	1	38,357,634,749.89	38,357,634,749.89	11.67	**0.001**
T × L	1	6,269,528,224.31	6,269,528,224.31	1.91	0.174
Error	48	157,719,876,895.98	3,285,830,768.67		
Corrected Total	51	249,996,788,793.49			

4. Discussion

There are global concerns growing over the loss of key habitat-forming organisms, such as large brown macroalgae of the order Fucales, and the resulting cascade of effects on the services these organisms provide. To tackle this problem there is an urgent need to develop effective best practices and restoration strategies. Reforestation efforts that depend on nursery-grown seedlings for out-planting require the development of protocols to enhance efficiency and reduce laboratory cultivation time by controlling key factors influencing seedling development and growth. Two of the main environmental drivers influencing seaweed reproduction and growth are temperature and light [29]. Different species may respond differently to these variables and also, for a given species, their influence may lead to differences in the life history traits of different populations, especially those at their distribution limits [19,30]. In fact, the seasonality that most populations display in the field is caused by the influence of these factors on reproduction, initial life stages development, and growth.

In order to develop methods for the establishment of nurseries where seedlings are produced for reforestation purposes, it is fundamental to understand the influence of light and temperature on the initial life stages in order to correctly manipulate those variables according to each of those stages. Relatively few studies have examined the effects of temperature and light on the early life stages of *A. nodosum*. Bacon and Vadas [31], in their study on the dynamics of gamete release in a Maine, USA, population, found that gamete release was closely correlated with seawater temperature at high tide. Release began at 6 °C and ceased at 15 °C, exhibiting both yearly and spatial variability in line with local water temperature patterns. In another study, Sheader and Moss [32] examined gamete germination and found that it occurred at temperatures between 4 °C and 20 °C, though with variable success rates. The highest germination rates consistently occurred at 10 °C across a wide range of light intensities, including in total darkness. Our results showed a high percentage of germination across all the temperatures and light intensities tested, indicating that germination occurs without major limitations between 12 and 18 °C. This population, located at the southern limit of the geographic distribution of the species, was found to have a higher investment in reproduction at the expense of individual growth and defense against herbivores, translating into higher fertility [19], which can account for the observed high germination efficiency within the conditions tested. Additionally, the highest germination rate was observed at 15 °C, a temperature typical of the northern coast of Portugal. This suggests that the local population may be adapted to higher temperatures compared to populations further north, which may be a valuable advantage against the impacts of climate change, considering the foreseen global temperature increase.

After germination, an essential step for germling survival is their settlement, facilitated by the development of rhizoids that anchor them to the substrate [33]. Our results suggest that this development stage is favored by low light, consistent with the findings of Sheader and Moss [32]. Preference for low light is supported by field observations, since germlings usually settle under a canopy, which provides shade to protect this fragile development stage against photoinhibition, heat, and dissection stress [34]. Coverage with other macroalgae was also found to be important in the earlier stages of reproduction and settlement for other fucoid species [35–38]. Besides light, a canopy also reduces temperature rises during emersion periods, which can be lethal to juvenile individuals [39,40]. Considering temperature, lower temperature (12 °C) led to higher rhizoid length, contrary to the findings by Sheader & Moss [32], who found that rhizoid length increased with increasing temperature, from 0 to 20 °C.

Once germlings settle, their thallus starts increasing in size, and our results suggest that the effect of light is different between the initial and later thallus growth stages. After one month in culture, larger germlings were obtained at lower temperature (12 °C) and reduced light intensity, as during the rhizoid development stage, indicating that during the first month, growth under a canopy is advantageous. During the second month, however, germlings reached higher sizes under high-light conditions, which may be explained by an increase in light requirements as thalli grow to fulfill the juvenile and adult needs for photosynthesis. In *A. nodosum*, photosynthesis is light saturated at 200 µmol m^{-2} s^{-1} [41], higher than the high-light conditions defined for our study. These observations are also in agreement with data obtained by Sheader & Moss [32] who also found that high light increased thallus growth in a later stage.

Although the slow growth rate in this species is acknowledged [14], the significant growth between February and March followed by the very low growth obtained during the third month may indicate that the cultivation conditions did not meet the requirements of the species at this later stage. Limitations to further growth may have occurred besides light, namely insufficient space and/or nutrients. Our results suggest that, from the third month forward, light should be further increased and nutrient supply should be adjusted to optimize growth.

In conclusion, our study found that for this population of *A. nodosum*, gamete germination, rhizoid development, and initial growth (first month) respond well to 12 °C temperature and low light, although with interaction between these factors. In the second month, light intensity should be increased to 80 µmol m^{-2} s^{-1}, and afterwards both the light and nutrient supply needed to fulfill the growing germlings' requirements should be adjusted. Further studies are thus needed to determine more precisely what the light and nutrient requirements are to optimize growth in the third month until they reach approximately 2.5 mm, which was considered, for other fucoid species, an adequate size for out-planting [12].

For population conservation purposes, additional studies are needed to understand how the interacting factors light and temperature can affect this population and better assess the role of canopy cover over the later stages of recruitment. Since recruitment and growth in this population may decrease with the predicted temperature increase in a scenario of climate change, studying the effect of higher temperature ranges on germlings and juvenile development is essential to understand how temperature shifts will further affect this population, considering their marginal location. Also, assessing the effect of higher temperature on growth and survival may also be useful for temperature-resistant strain identification and selection. These strains can then be used in reforestation actions by applying nursery seedlings production and out-planting methods.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/phycology5010004/s1, Table S1: Tukey's multiple comparisons following the two-way ANOVA testing the effect of the factors temperature (3 levels, fixed: 12, 15, and 18 °C) and light intensity (2 levels, fixed: low light, high light) on egg germination; Table S2: Tukey's multiple comparisons following the two-way ANOVA testing the effect of the factors temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on rhizoid length; Table S3: Tukey's multiple comparisons following the two-way ANOVA testing the effect of the factors temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on the area of embryos in February; Table S4: Tukey's multiple comparisons following the two-way ANOVA testing the effect of the factors temperature (2 levels, fixed: 12 and 18 °C) and light intensity (2 levels, fixed: low light, high light) on the area of embryos in March.

Author Contributions: Conceptualization, I.C. and D.B.; methodology, I.C., D.B. and S.C.; formal analysis, A.C., S.C., D.B. and I.C.; investigation, A.C. and S.C.; resources, F.A., I.S.-P. and I.C.; writing—original draft preparation, A.C. and S.C.; writing—review and editing, I.C., D.B., S.C. and F.A.; visualization, A.C., S.C. and I.C.; supervision, I.C. and D.B.; project administration, F.A., I.S.-P.

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Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Raw data will be made available by the authors on request.

Conflicts of Interest: The authors declare no conflicts of interest.

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