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ORIGINAL PAPER

An *ex-situ* approach for cultivating coralline algae in a restoration perspective

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Abstract

Mediterranean coralligenous reefs are hotspots of biodiversity, but they are declining due to multiple impacts, prompting a growing number of conservation and restoration efforts. Restoration through the outplanting of the habitat foundation species, crustose coralline algae (CCA, Rhodophyta, Corallinophycidae), is still largely unexplored, although they make a major contribution to building these valuable ecosystems. In this study, we tested the feasibility of *ex-situ* culture of *Lithophyllum stictiforme*, one of the most common CCA of the coralligenous reefs, with nutrient-enriched artificial seawater medium, contributing to the knowledge of early life stage development and calcification. Thalli fragments with conceptacles were collected at a depth of 28 m (Costa Paradiso, northern Sardinia). We tested two light intensities, (L^- 40 µmol photons $m^{-2}s^-1$, L^+160 µmol photons $m^{-2}s^{-1}$) and two temperatures, (T^- 14 °C, T^+ 20 °C), the lower settings were chosen to simulate conditions at the sampling site, while the higher values were explored as potential growth promoters. Spore settlement, density and growth were estimated for two-weeks. Calcification was analyzed at the end of the culture period using Scanning Electron Microscopy in combination with Energy Dispersive Spectroscopy (SEM-EDS). The L^- treatments had a positive effect on germination success and density, whereby the growth of the germination disc was enhanced in the L^-T^+ treatment. SEM-EDS showed that the marginal area of the germination discs had a higher Ca content than the core area, and that the L^- treatments promoted a higher cell wall Ca percentage.

Keywords Lithophyllum stictiforme · Habitat restoration · Biogenic reefs · Calcification · Mediterranean

Introduction

Coralligenous reefs are among the most important subtidal habitats in the Mediterranean for important ecosystem services they provide, acting as hot spots of marine biodiversity (Ådal et al. 2006; Ballesteros 2006; Piazzi and Ceccherelli 2020), providing habitat for economically valuable species (Canals and Ballesteros 1997; Ballesteros 2006), and producing carbonates (de Ville d'Avray et al. 2019; Cebrián et al. 2000; El Haïkali et al. 2004; Canals and Ballesteros 2007, Bracchi and Basso 2012; van der Heijden and Kamenos 2015). Their importance as marine calcifiers lies in their ability to precipitate calcium carbonate in their cell walls, predominantly as high-Mg calcite and, in the case of tropical species, aragonite (Nash and Adey 2017). Recently, crustose coralline algae (CCA) have garnered increased attention in the context of global environmental changes, mainly due to the susceptibility of their high-Mg calcite skeletons (McCoy and Kamenos 2015) and their capacity to adjust the geochemistry of their thallus to environmental

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conditions (Moberly 1968; Ulrich et al. 2021). Studies have repeatedly shown that Mg/Ca ratios in CCA can serve as indicators of seawater temperature (Kamenos et al. 2008; Diaz-Pulido et al. 2014; Ragazzola et al. 2020; Hetzinger et al. 2023) since the incorporation of Mg into calcite is an endothermic process, leading to an increase in the Mg/Ca ratio with rising temperatures (Mucci and Morse 1985; Rosenthal et al. 1997).

They are characterized by biogenic assemblages, from 20 to about 150 m depth, mainly formed by the accumulation of CCA, mostly *Lithophyllum*, *Lithothamnium* and *Mesophyllum*, which constitute a secondary substrate hosting highly diverse and stratified assemblages including erect algae, sponges, bryozoans, gorgonians, and other alcyonaceans (Ballesteros 2006). Spatial patterns of colonization of *L. stictiforme* thalli by epibionts have recently been detected and differences in diversity between assemblages recruited on the CCA and on rock have provided evidence of the importance of CCA in promoting the biodiversity of coralligenous reefs (Piazzi et al. 2022).

These reefs are currently facing significant threats that are leading to their decline (Ballesteros 2006; Bevilacqua et al. 2018). Overfishing, pollution, invasive species and climate change are disrupting the demography, phenology and biogeography of numerous species, jeopardizing the integrity of the entire ecosystem (Ballesteros 2006; Bevilacqua et al. 2021; Cebrian et al. 2021, Garrabou et al. 2022). Regression of coralligenous reefs is due to both impacts on the animal (e.g. gorgonians, bryozoans and sponges (Garrabou et al. 2009) and CCA (Piazzi et al. 2011; Martin et al. 2013b; Pinna et al. 2022). Given these challenges, both passive restoration (i.e. removal or mitigation of stressors) and active restoration strategies (i.e. transplantation of organisms), have been proposed as viable approaches to expedite the recovery of coralligenous reefs with the aim of promoting the recovery of the community structure, and thus restoring its functioning. The active restoration consists in transplanting sessile macroinvertebrates, mostly gorgonians, onto disturbed bioconstructions (Cerrano et al. 2018; Casoli et al. 2022), following Linares et al. (2008) techniques. As coralligenous restoration is still in its infancy, the effectiveness of transplants is quite uncertain due to the lack of long-term data and the scant number of case studies that have resulted in a wide range of survival outcomes (Linares et al. 2008; Fava et al. 2010; Montseny et al. 2019; Casoli et al. 2022). In this frame, the possibility of promoting the development of CCA by producing recruits from fertile material in hatcheries for release into the sea remains to be explored. In fact, several studies indicate that these calcifying macroalgae not only serve as a selective primary substrate but are also actively involved in the recruitment of a large number of invertebrate species by inducing larval settlement and metamorphosis (Lau et al. 2005; O'Leary et al. 2017; Gómez-Lemos et al. 2018; Seabra et al. 2019; Siboni et al. 2020; Jorissen et al. 2021). In the Mediterranean, *Lithophyllum stictiforme* (Areschoug) Hauck, one of the principal bioconstructors of coralligenous reefs (Garrabou and Ballesteros 2000; Ballesteros 2006; Piazzi et al. 2022), has been shown to positively influence the density and dynamics of settlement of some gorgonians (Zelli et al. 2020). The outplanting of *L. stictiforme* recruits could therefore provide natural substrates for the whole assemblage to develop.

One of the bottlenecks in planning large-scale restoration efforts is the need to outplant a large number of seedlings. Therefore, planning an efficient, effortless and cost-effective seedling production system that fits the breeding requirements of a specific species can be particularly challenging. However, the high potential of *Lithophyllum stictiforme* to generate gametes and zygotes under optimal conditions has been highlighted (Rodriguez-Prieto 2016), supporting the hypothesis that the cultivation of seedlings from fertile conceptacles of this species could be a reliable option for the restoration of coralligenous reefs without depleting natural populations. This technique has also been explored for canopy forming species restoration such as algae of the genus *Cystoseira sensu lato* (Falace et al. 2006, 2018; Verdura et al. 2018) and large fucoids (e.g. Hwang et al. 2007; Pang et al. 2009; Yu et al. 2012, Yoon et al. 2014).

With the perspective of cultivating CCA seedlings, the development of an effective protocol is essential. As temperature and light play a key role in the growth of macroalgae (Hurd et al. 2014), with responses being species-specific, they should be adequately addressed in *ex-situ* culture. As well, optimal growth conditions likely vary depending on the ontogenetic stage, and early-life stages may have different requirements than adults (Yoshioka et al. 2020). Several studies have been conducted on the optimal temperature and light requirements for both adult CCA growth (e.g. Adey 1970; Leukart1994, Rodríguez-Prieto 2016; Martin et al. 2024) and early-life stages (sporelings) (e.g. Jones and Woelkerling 1983; Ichiki et al. 2000; Kyoung et al. 2002; Song et al. 2013), Ordoñez et al. 2017; Yoshioka et al. 2020). However, to our knowledge, there is no information on the development of germination discs of *L. stictiforme*. Coralline algal spores in the early stages of growth follow a characteristic pattern of cell division forming a germination disc that is symmetrical and clearly visible even after few days of development (Chamberlain 1993). After being released, the spores settle on the substrate and anchor themselves to it thanks to the secretion of adhesive polysaccharides. Once adhered, the spores begin to germinate, producing flat and compact cells that are arranged radially, thus forming the germination disc, a stable and circular structure. This disc expands thanks to cell division and, in CCA, begins to

deposit calcium carbonate, which gives rigidity and resistance to the structure. From this base, the thallus of the alga will subsequently develop.

In this study, we therefore tested the feasibility of *ex-situ* culture of *L. stictiforme* using a crossed experimental design to investigate the effects of light and temperature conditions on development and calcification at the early life stage and to determine the optimal laboratory combinations.

Materials and methods

Sampling site

Thalli fragments of *L. stictiforme* ($N=40$), measuring approximately 15 × 15 cm and bearing developed conceptacles, were harvested by scuba divers from a depth of 28 m at Costa Paradiso (Northern Sardinia, Tyrrhenian Sea, N 41.06546°, E 008.95039°) in March 2023. The algae were stored in seawater in the dark for 24 h before being transported to the facilities of the University of Trieste (Italy) within 10 h in dark and cold conditions.

Experimental setup

The culture was established in environmentally controlled rooms and the photoperiod was set to 10:14 h (L: D), which corresponds to the conditions during sampling. We tested two light intensities and two temperatures in a crossed experimental setup. Light was provided by LED lamps (AM366 Sicce USA Inc., Knoxville, USA) at 40 µmol photons m^{-2} s^{-1} (L^-) and 160 µmol photons m^{-2} s^{-1} (L^+), while the temperature was set to 14 °C ± 0.5 (T^-) and 20 °C ± 0.5 (T^+). These two temperature values reflected variation in the Mediterranean site where the adult thalli were collected, due to the presence of a thermocline (a transition layer between the warmer water at the surface and the cooler deep water below). The air temperature was automatically set by the room controller and the water temperature was measured twice daily in each tank. The lower light and temperature values were chosen to reflect the environmental conditions at the sampling site, while the higher values were based on measurements at shallower depths within the sampling site where *L. stictiforme* can be found (Pinna et al. 2022). Each treatment combination was replicated in three tanks of 5 L each containing 12 glass slides (75 × 25 mm), that were arranged randomly within the laboratory under one lamp per light treatment in fixed position: to avoid any bias due to tank location, within each light treatment, tanks were rotated every two days (Fig. 1).

Thalli fragments were examined under the stereomicroscope to identify the sporophytes, and the presence of tetraspores was verified by removing the roof of randomly selected conceptacles for examination. The sporophyte fragments were then cleared from epiphytes with tweezers and rinsed with artificial seawater (Tropic Marin® Pro- Reef Sea salt). The thalli were then arranged in tanks suspended on rigid PVC grids (mesh size 25 mm) with the surface of the thalli facing the glass slides at the bottom of tanks. The tanks were filled with artificial seawater (37 PSU) supplemented with 10% Von Stosch's solution (Von Stosch 1963; Guiry and Cunningham 1984). Seawater pH, calcium (Ca), magnesium (Mg) and alkalinity (KH) were measured every three days. The pH measurements were performed using a pH meter (HI-5521-02, Hanna Instruments) calibrated on the total scale with Tris/HCl buffers (according to Dickson et al. 2007). [Ca], [Mg] and KH were determined using a high-precision titration test (Foundation™ Pro Test Kit). To ensure consistent chemical conditions in the culture medium, it was renewed every three days, with both tanks and slides repositioned to ensure randomized culture conditions.

Each slide was seeded with a *L. stictiforme* fragment of approx. 3 × 4 cm and then removed after 24 h. To prevent possible anoxia, the slides were lifted from the bottom during cultivation using a grid, while continuous oxygenation in the tanks was ensured by air pumps and bubblers. Seedlings were cultured for 14 days and data acquired at three time points post germination: two days (T1), seven days (T2) and fourteen days (T3). At each sampling time, three slides per tank for each treatment were collected and fixed in 4% formalin for the subsequent analyses. To guarantee independence of data, the slides sampled were never reused.

Collected data

The following data were collected during culture:

- *Germination success* (=*N. germination discs with at least one cell division/total number of spores x 100*) was assessed at T1 on 20 subareas, corresponding to 20 photos per slide (i.e., full field of view of the microscope at 4x magnification, area per field of view: 7.033 mm^2, total area per slide: 140.66 mm^2). Photographic sampling was done with an Axiocam 208 color camera mounted on a Zeiss Primostar 3 microscope.
- *Germination disc density* (i.e., *mean N. germination discs per slide*) was counted at T2 and T3 under an inverted microscope.
- *Germination disc growth*: at T3 the area of 20 randomly selected germination discs per slide was measured by ImageJ software (Schneider et al., 2012).
- *Germination discs growth stages*: seven growth stages were identified: I- undivided spore, II- up to four-cells, III-up to eight cells, IV- up to sixteen-cells, V- disc with

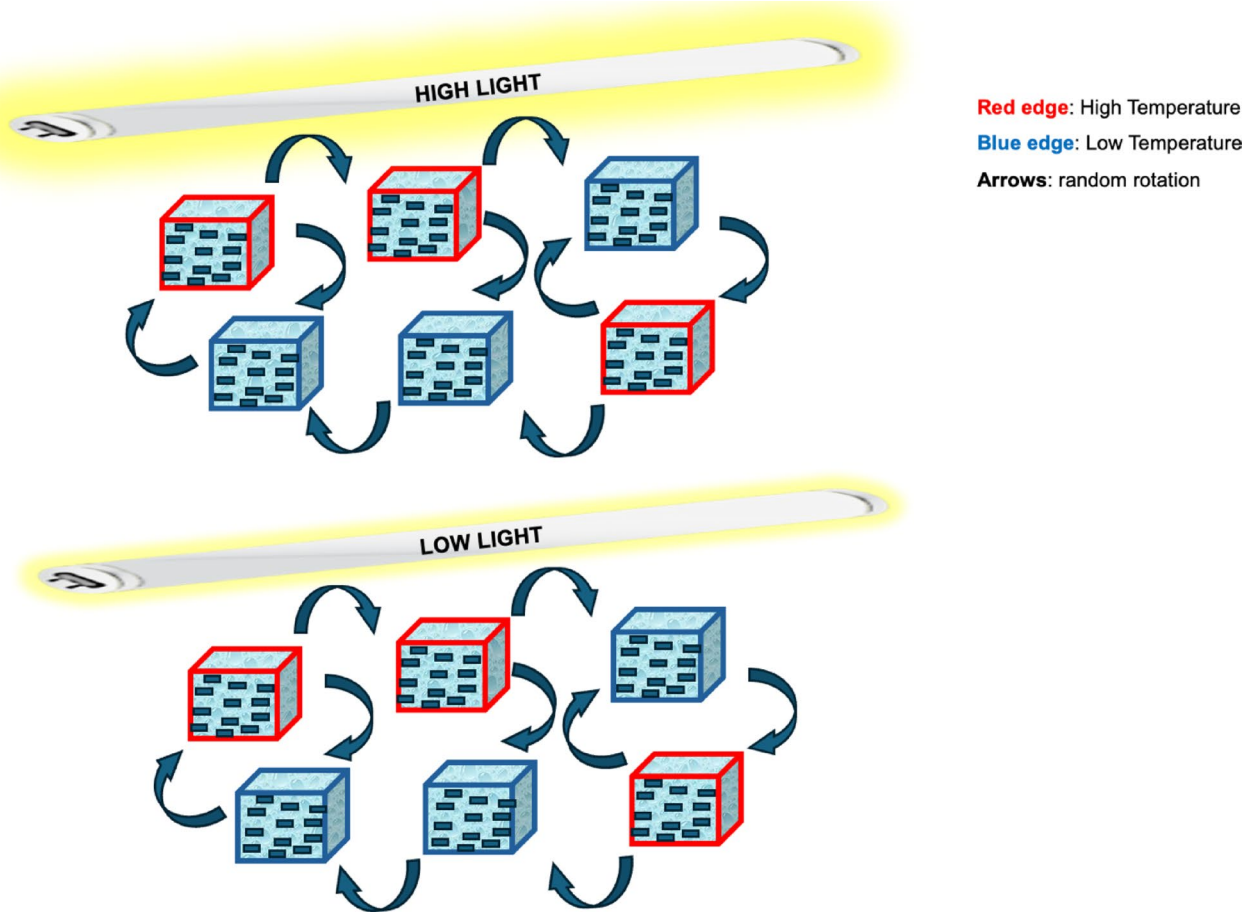

Fig. 1 Tank arrangement: in the mesocosm three tanks, each containing the slides, were randomly assigned to each combination of treatments. The arrows indicate that the tanks have switched position every two days

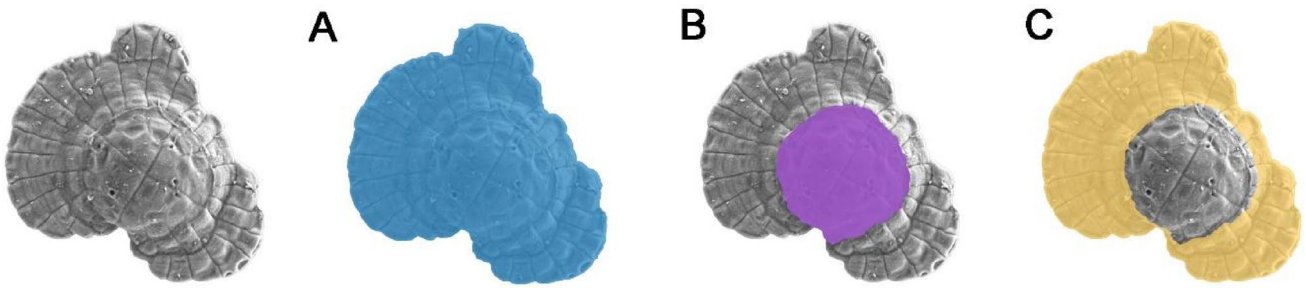

Fig. 2 SEM-EDS maps acquired at T3 on *L. stictiforme* germination discs over (**A**) the total germination disc area, (**B**) the core area and (**C**) the marginal area

meristematic peripheral cells, VI- disc with two rows of peripheral cells and VII- disc with three or more rows of peripheral cells. Development was assessed by counting live (red-colored) germination discs at different stages on 20 subareas, corresponding to 20 photos per slide (i.e., full field of view of the microscope at 4x magnification, area per field of view: 7.033 mm^2, total area per slide: 140.66 mm^2) at T1, T2 and T3.

To investigate the elemental composition and the distribution of calcification in the germination discs, Scanning Electron Microscopy in combination with Energy Dispersive Spectroscopy (SEM-EDS) analyses were performed at T3. To obtain the Ca and Mg atomic percentage, and the Mg/Ca atomic ratios, elemental maps were acquired on 3 randomly chosen germination disc per slide. The measurements were performed over the entire area of the germination disc, the central area and the marginal area (Fig. 2). For SEM-EDS

Table 1 Germination success. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on germination disc abundance at T1. In bold the Pseudo-F corresponding to a significant result. Variance homogeneity was assessed by PERMDISP test (Table 8)

Source of variation	df	MS	Pseudo-F	P(perm)	Perms
Temperature	1	0.0111	5.599	**0.038**	980
Light	1	0.0112	5.661	0.054	977
TeXLi	1	0.00009	0.047	0.832	983
Residual	8				

analysis, the slides were mounted on aluminum stubs coated with double sided carbon tape. Samples were carbon-coated using the pulsed rod evaporation method with the Q150T Es plus sputter coater (Quorum Technologies). Samples were analyzed with Gemini300 SEM (Zeiss) in secondary electron mode, at an acceleration voltage of 10 kV and a working distance of 8.5 mm. EDS analysis was performed at a working distance of 8.5 mm and an acceleration voltage of 10 kV, using an XFlash 610 M probe (Bruker), obtaining maps (with a scan time of 5 min) and semi-quantitative analysis of the regions of the germination discs (with a scan time of 1.5 min for each region).

Statistical analyses

Permutational analysis of variance (PERMANOVA) was performed to test the effects of temperature and light on germination success at T1, germination disc density (at T2 and T3), growth at T3 and development at each sampling time. For each variable tested, the design for the analysis consisted of two factors: Temperature (Te) and Light (Li), fixed and crossed, with three tanks for each combination of factors: tanks ($n=3$) were considered replicates for the germination success and germination disc density, while slides ($n=9$) were considered replicates for the growth and development at each sampling time. Significant effects were examined by performing a post hoc pairwise test. PERMANOVA and Pair-Wise tests were performed with PRIMER v7 software. Statistical analysis of the calcification data was performed using PRIMER Software. A PERMANOVA was also performed to test the effects of temperature, light and disc portion (core or peripheral) on germination disc Ca% and Mg/Ca content. The design consisted of three factors, fixed and crossed: Temperature (Te), Light (Li) and Disc Portion (DP) with two levels each. For all response variables analyzed, the homogeneity of variances was assessed using the PERMDISP test based on Euclidean distance with PRIMER Software (Anderson et al., 2006).

Results

Germination success of *Lithophyllum stictiforme* at T1 only depended on temperature ($p=0.038$, Table 1) with the higher percentage of germination discs with at least one cell division at T^+ (L^-T^+ 68.88±0.86, L^+T^+ 65.16 ±2.25) Conversely, light conditions did not affect the germination success (Fig. 3).

However, a significant effect of light was found on germination disc density with the low light condition promoting disc abundance, regardless the temperature (T2: L^-T^- 596.33 ± 433.36 and L^-T^+ 181.55 ± 13.91, T3: L^-T^- 290.44 ± 126.85 and L^-T^+ 92.88 ± 38.50) (Table 2; Fig. 4).

Germination of the spores followed the segmentation mode of the *Amphiroa* type, which was also described for *Lithophyllum* (Chihara, 1974) (Fig. 5). While no difference in segmentation pattern was observed among treatments, there was variability with respect to timing: multivariate PERMANOVA showed significant effects of temperature and light on seedling development through time (Table 3).

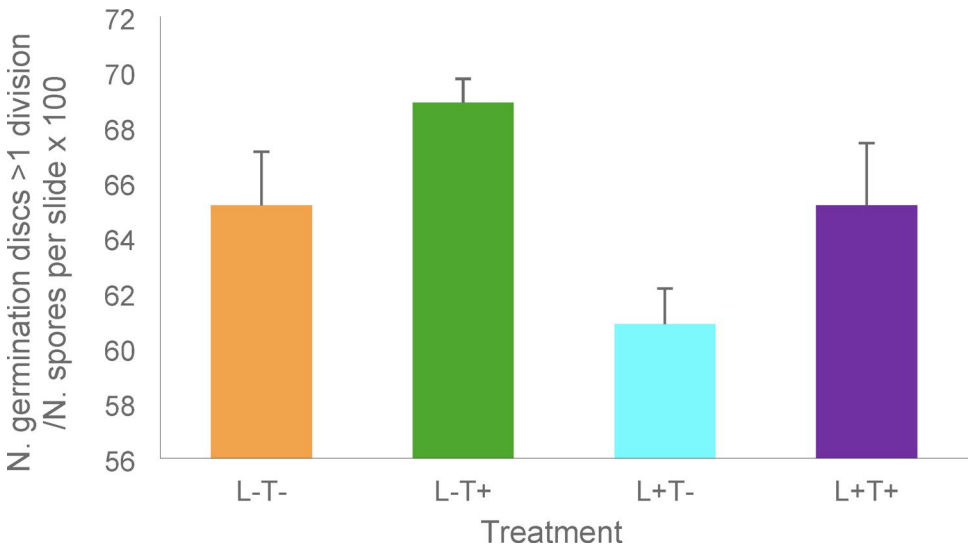

Fig. 3 Germination success. Percentage (mean + SE $n=3$) of germination discs with at least one cell division out of the number of spores per slide (over 140.66mm^2) for each treatment at T1: L^-T^-: low light-low temperature, L^-T^+: low light-high temperature, L^+T^-: high light- low temperature, L^+T^+: high light-high temperature

Table 2 Gemination disc density. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on disc survival at T2 and T3. In bold the Pseudo-F corresponding to a significant result. Variance homogeneity was assessed by PERMDISP test (Table 8)

Source of variation	df	Pseudo-F T2	Pseudo-F T3
Temperature = Te	1	3.458	1.527
Light = Li	1	**5.116**	**18.391**
TeXLi	1	0.359	0.761
Residual	8		

Table 3 Germination disc development. Results of multivariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on disc development. Pair-Wise tests on the interaction Temperature X Light. In bold the Pseudo-F corresponding to a significant result. Variance homogeneity was assessed by PERMDISP test (Table 8)

Source of variation	df	Pseudo-F T1	Pseudo-F T2	Pseudo-F T3
Temperature = Te	1	**9.190**	**9.929**	**14.863**
Light = Li	1	**16.308**	**24.854**	**13.393**
TeXLi	1	**5.635**	**9.807**	**3.745**
Residual	32			
Pair-Wise tests				
Temperature X Light				
	T1	**T2**	**T3**	
	T-L+=T-L-	T-L+≠T-L-	T-L+≠T-L-	
	T+L+≠T+L-	T+L+≠T+L-	T+L+≠T+L-	
	L-T+≠L-T-	L-T+≠L-T-	L-T+≠L-T-	
	L+T+=L+T-	L+T+=T-	L+T+≠L+T-	

Pairwise tests confirmed that a difference in development by the different treatments was detectable from the beginning of the experiment (T1). The first and second cell divisions occurred crosswise, resulting in a germination disc that was mostly four-celled (Fig. 5, stage II) two days after spore release (T1), although eight-celled (Fig. 5, stage III) and sixteen-celled stages (Fig. 5, stage IV) were also reached, especially in the $L^{-}T^{+}$ treatment. Univariate PERMANOVA showed significant differences between treatments for stages II and IV (Table 4A). In L^{-} treatment the development of stage IV was promoted at high temperature.

The differences between treatments increased over time (Fig. 6) as highlighted by pairwise tests at T2 and T3 at all treatment conditions (Table 3), with significant differences observed at stages IV, V and VI at T2 (Table 4B). The germination disc developed one row of meristematic peripheral cells (stage V) in all treatments except at $L^{+}T^{-}$. Under L^{-}, the stage VI was induced by high temperature and under T^{-} it was promoted by low light.

At T3, significant differences in the proportion of stages V, VI and VII were found (Table 4 C). In $L^{-}T^{+}$, stage VII was predominant (73.6% ± 8.3), while in $L^{+}T^{+}$ the percentage was lower (23.8% ± 6.3) and the germination discs remained mainly at stage IV. In $L^{-}T^{-}$, stage VII accounted for 13.8% ± 8 of the germination discs and in $L^{+}T^{-}$ this stage was not reached at T3. A significant effect of light was

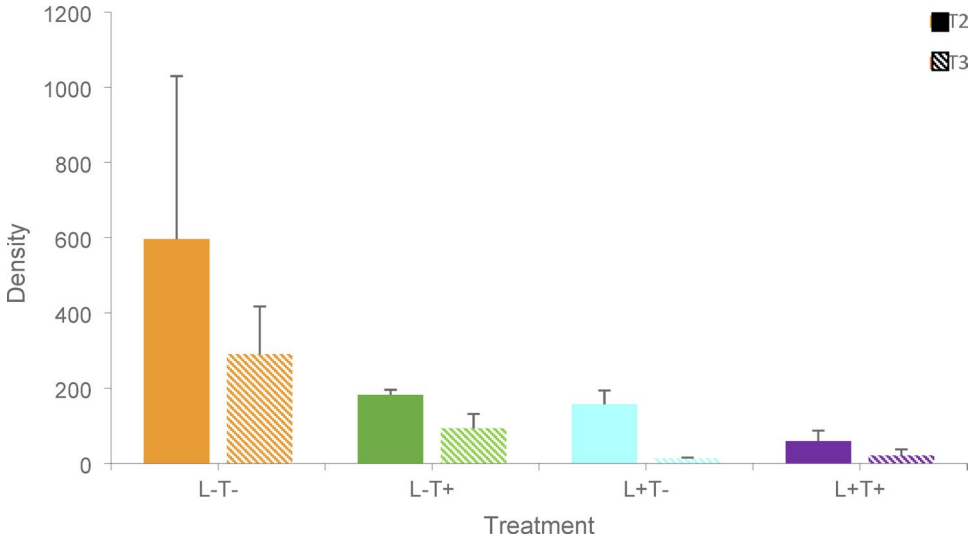

Fig. 4 Germination disc density. Number of germinative discs per slide (mean + SE $n = 3$) for each treatment at T2 and T3: $L^{-}T^{-}$: low light-low temperature, $L^{-}T^{+}$: low light-high temperature, $L^{+}T^{-}$: high light- low temperature, $L^{+}T^{+}$: high light-high temperature

Fig. 5 Developmental stages of *L. stictiforme* recruits during 14 day-culture: I- undivided spore, II- up to four-celled, III-up to eight-celled, IV- up to sixteen-celled, V- disc with meristematic peripheral cells, VI- disc with two rows of peripheral cells and VII- disc with three or more rows of peripheral cells

Table 4 Germination disc development. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on the percentage of germination disc at each stage of development at T1, T2 and T3 (A, B and C, respectively). Pair-Wise tests on the interaction Temperature x Light are provided at the bottom of each table. In bold the Pseudo-F corresponding to a significant result. Variance homogeneity was assessed by PERMDISP test (Table 8)

A Source of variation	df	Pseudo-F Stage 1	Pseudo-F Stage 2	Pseudo-F Stage 3	Pseudo-F Stage 4	Pseudo-F Stage 5	Pseudo-F Stage 6	Pseudo-F Stage 7
Temperature = Te	1	3.71	**7.12**	**15.072**	**68.15**			
Light = Li	1	2.28	**27.53**	**9.515**	**52.48**			
TexLi	1	0.39	**7.63**	0.004	**50.86**			
Residual	32							
Pair – Wise tests								
Temperature x Light								
			Stage 2					**Stage 4**
			L-T- >T+					L-T+>T-
			L+T- = T+					L+T+=T-
			T-L- = L+					T-L+=L-
			T+L- < L+					T+L+<L-

B Source of variation	df	Pseudo-F Stage 1	Pseudo-F Stage 2	Pseudo-F Stage 3	Pseudo-F Stage 4	Pseudo-F Stage 5	Pseudo-F Stage 6	Pseudo-F Stage 7
Temperature	1		4.57	0.68	0.27	**6.68**	**58.90**	
Light	1		**27.04**	**12.21**	7.51	**28.27**	**58.90**	
Te x Li	1		0.98	0.08	**7.62**	**12.71**	**58.90**	
Residual	32							
Pair – Wise tests								
Temperature x Light								
			Stage 4			**Stage 5**		**Stage 6**
			T- L- >L+			T- L- >L+		T- L- = L+
			T+L+=L-			T+L- = L+		T+L- >L+
			L- T- >T+			L-T- >T+		L-T+>T-
			L+T- =T+			L+T- = T+		L+T+=T-

C Source of variation	df	Pseudo-F Stage 1	Pseudo-F Stage 2	Pseudo-F Stage 3	Pseudo-F Stage 4	Pseudo-F Stage 5	Pseudo-F Stage 6	Pseudo-F Stage 7
Temperature = Te	1		**11.81**	0.19	0.002	**5.46**	**16.40**	**55.34**
Light = Li	1		2.11	**7.06**	1.739	**4.60**	**29.40**	**32.93**
Te x Li	1		0.63	0.29	1.052	**5.63**	**22.30**	**10.18**
Residual	32							
Pair – Wise tests								
Temperature x Light								
			Stage 5			**Stage 6**		**Stage 7**
			T- L- >L+			T-L- >L+		T-L- >L+
			T+L- =L+			T+L- =L+		T+L- >L+
			LT- >T+			L-T- >T+		L-T+>T-
			L+T- =T+			L+T- =T+		L+T+>T-

also found on the germination disc area, indicating a promoting effect of the low light condition on disc size (L^-T^- 9746.6±490.2, L^-T^+ 12622.2 ± 492.4) independently of the temperature (Fig. 7; Table 5).

At T3, the EDS analyses (Fig. 8; Table 6) showed a significant difference in Ca % content depending on light intensity: the highest value was measured at L^- independently on temperature. Depending on the portion of the germinal disc analyzed, different Ca contents were also determined. with the peripheral cells having a higher Ca content than the core area (L^-T^- 7.77 ± 0.37, L^-T^+ 5.94 ± 0.42) (Fig. 9B).

Treatments also affected the Mg/Ca ratio of the germination discs (Fig. 9C, D): a positive influence was found of both temperature and light intensity, but not in interaction (Table 7), with the core area having the highest Mg/Ca content (L^-T^- 0.12 ± 0.016, L^-T^+ 0.18 ± 0.02, L^+T^- 0.19 ± 0.02, L^+T^+ 0.26 ± 0.01).

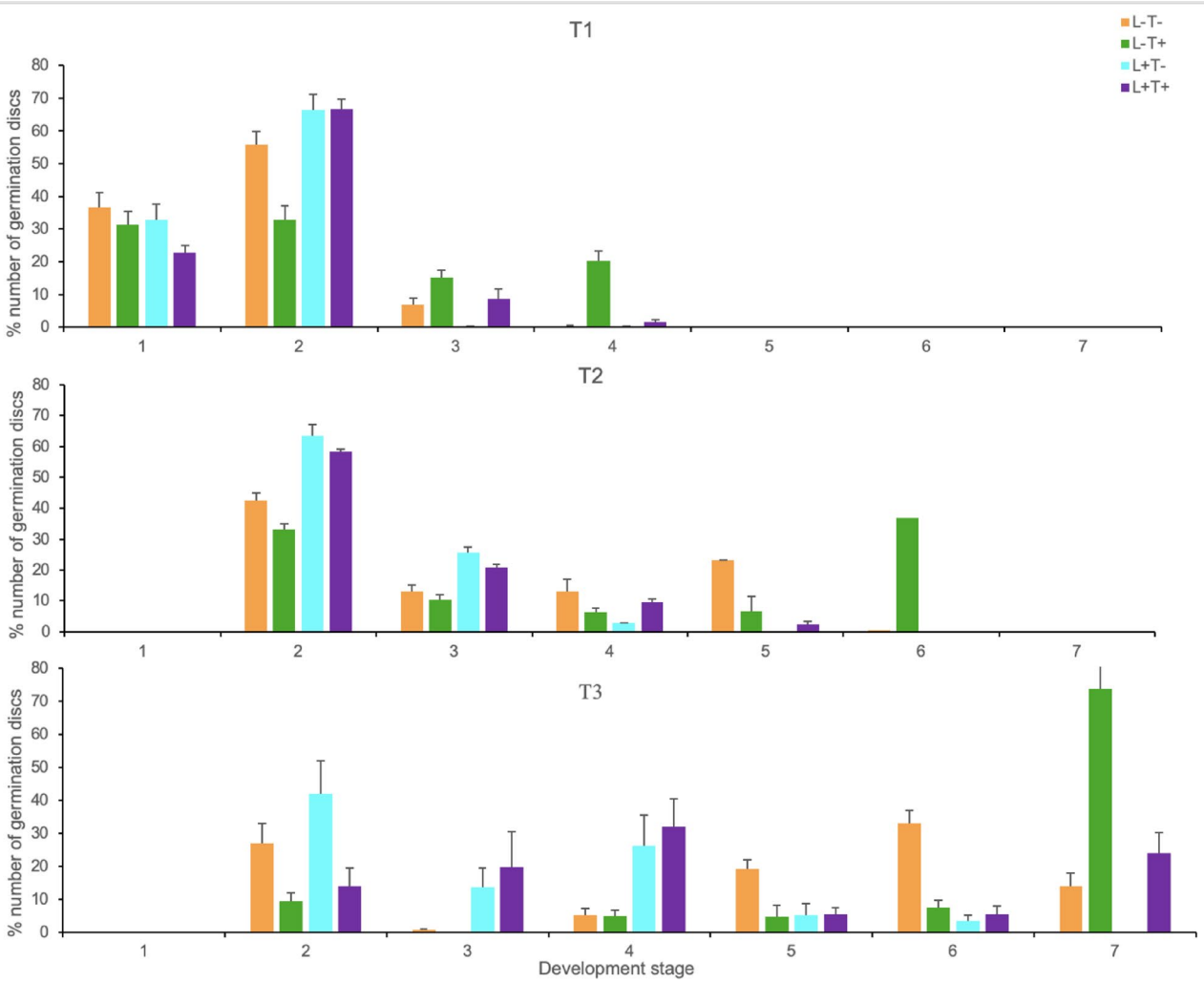

Fig. 6 Developmental stages of *L. stictiforme*. Percentage of live germination discs (mean+SE) at each developmental stage at T1, T2 and T3. L-T-: low light-low temperature, L-T+: low light- high temperature, L+T-: high light-low temperature, L+T+: high light-high temperature

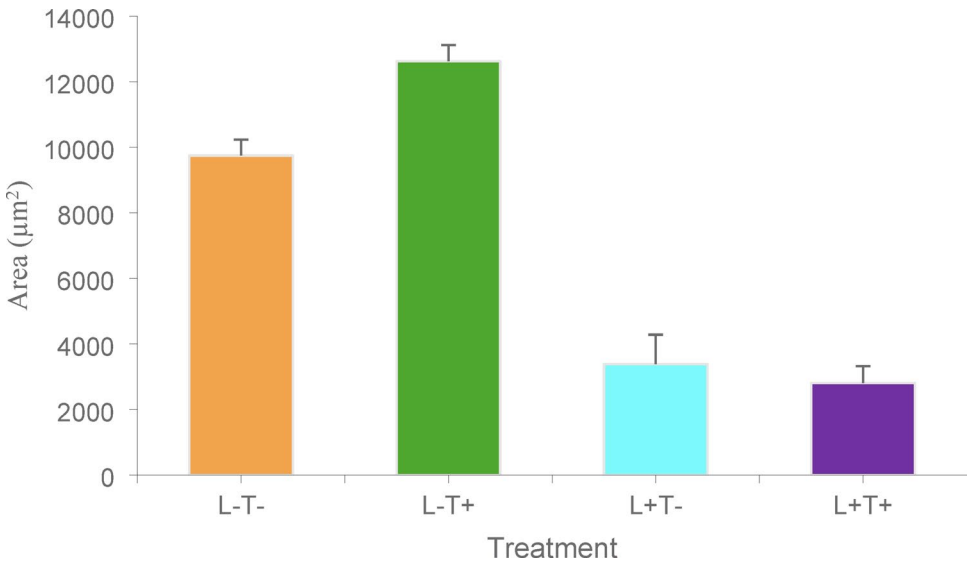

Fig. 7 Germination disc area. Mean (+SE) disc area (µm^2) for each treatment at T3 (14 days after spore release): L^+T^+: high light-high temperature, L^+T^-: high light-low temperature, L^-T^+: low light- high temperature, L^-T^-: low light-low temperature

Table 5 Germination disc area. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on the disc area at T3 (14 days AR). In bold the Pseudo-F corresponding to a significant result. Variance homogeneity was assessed by PERMDISP test (Table 8)

Source of variation	df	MS	Pseudo-F	*P*(perm)	perms
Temperature = Te	1	1048.6	1.12	0.330	987
Light = Li	1	6901.1	7.40	**0.005**	984
TeXLi	1	1669.1	1.79	0.213	986
Residual	8	932.3			

The results of the PERMDISP test, used to assess the homogeneity of variances for the previously mentioned response variable, are reported in Table 8.

Table 6 Calcification. Results of univariate PERMANOVA testing the effect of Temperature (Te), Light (Li) and Disc Portion (DP) on the Ca% content at T3 (14 days AR). In bold the Pseudo-F corresponding to a significant result. Variance homogeneity was assessed by PERMDISP test (Table 8)

Source of variation	df	MS	Pseudo-F	*P*(perm)	perms
Temperature = Te	1	1.60	0.52	0.455	996
Light = Li	1	21.42	6.99	**0.009**	998
Disc Portion = DP	1	104.03	33.95	**0.001**	994
TeXLi	1	0.07	0.02	0.871	998
LiXDP	1	2.28	0.74	0.408	997
TeXDP	1	6.43	2.10	0.164	993
LiXTeXDP	1	0.17	0.05	0.821	997
Residual	78	3.06			

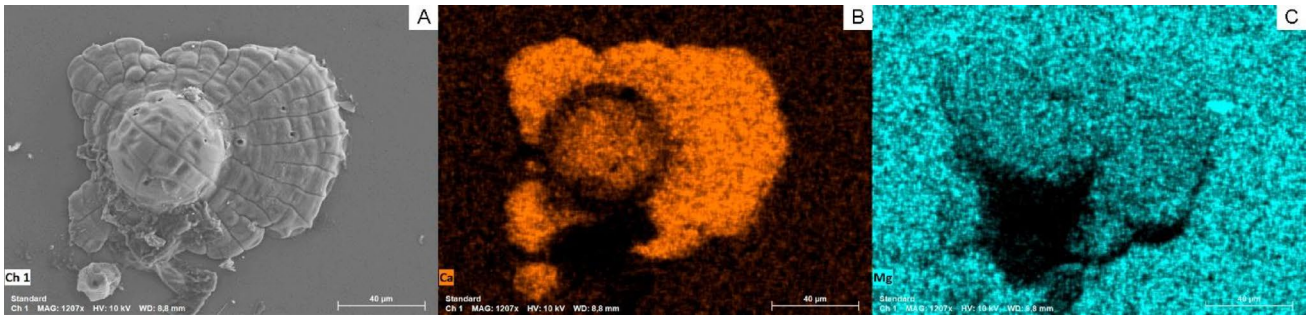

Fig. 8 *L. sicitiforme* germination disc at T3. **A** Scanning Electron Microscopy image. **B** EDS map of calcium.**C** EDS map of magnesium

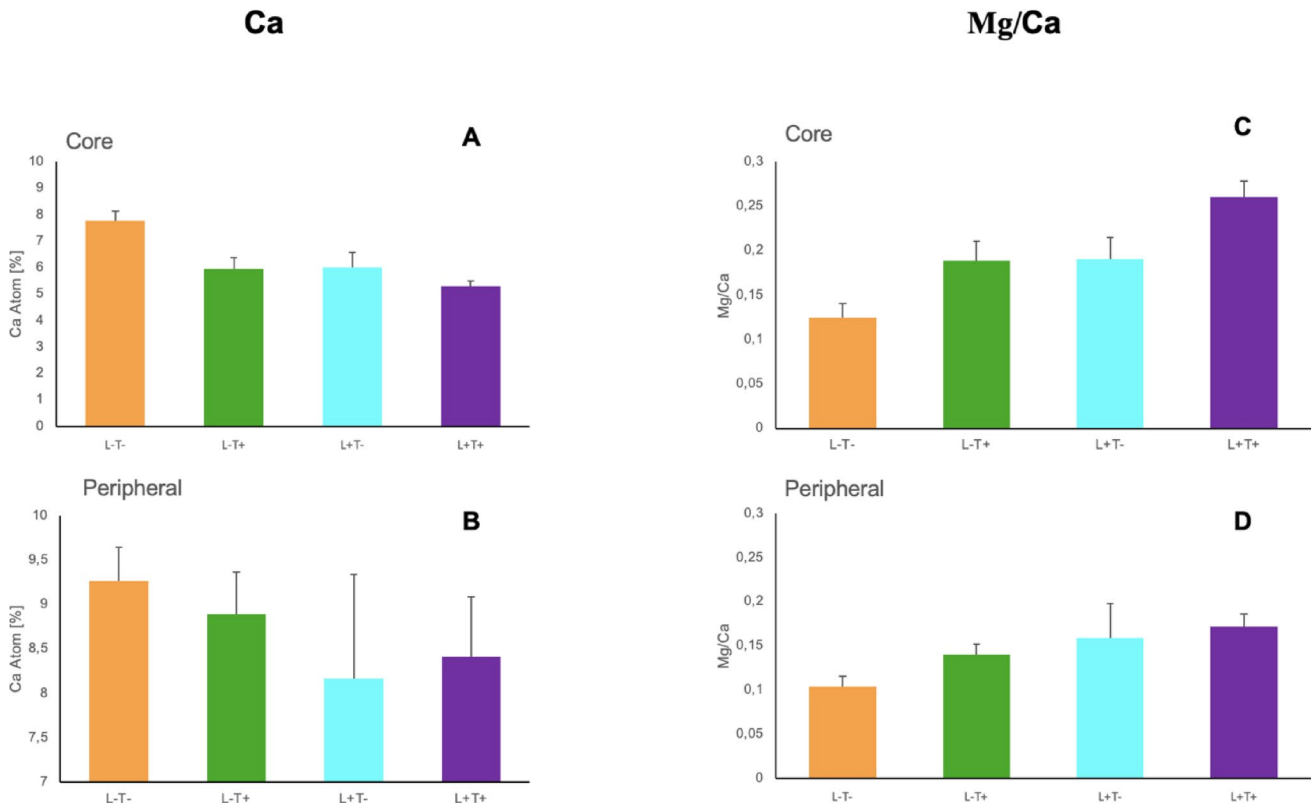

Fig. 9 Calcification disc distribution. Ca (%) content, measured over the core (A) and peripheral area (B) of the germination discs, Mg/Ca content, measured over the core (C) and peripheral area (D) of the germination discs from each treatment at T3 (14 days after spore release): $L^{+}T^{+}$: high light-high temperature, $L^{+}T^{-}$: high light-low temperature, $L^{-}T^{+}$: low light- high temperature, $L^{-}T^{-}$: low light-low temperature

Table 7 Calcification. Results of univariate PERMANOVA testing the effect of Temperature (Te), Light (Li) and Disc Portion (DP) on the Mg/Ca ratio at T3 (14 days AR). In bold the Pseudo-F corresponding to a significant result. Variance homogeneity was assessed by PERMDISP test (Table 8)

Source of variation	df	MS	Pseudo-F	*P*(perm)	perms
Temperature = Te	1	0.023	5.81	**0.024**	996
Light = Li	1	0.485	12.27	**0.003**	995
Disc Portion = DP	1	0.045	11.33	**0.001**	998
TeXLi	1	0.001	0.27	0.587	999
LiXDP	1	0.003	0.75	0.390	995
TeXDP	1	0.007	1.74	0.174	996
LiXTeXDP	1	0.002	0.49	0.487	998
Residual	78				

Discussion

This study is the first *ex-situ* experiment providing significant insights into the effects of the interaction of light and temperature on laboratory-developed recruits of *L. stictiforme*. Light and temperature conditions tested in the present study affected the germination success of *L. stictiforme*, influencing both density and growth of germination discs through time, as well as their elemental composition and the distribution of calcification after two weeks of culture.

In particular, light had a significant effect on the germination discs with density and growth promoted by lower light treatment. This is not surprising since *L. stictiforme* mainly occurs deeper than 20 m (Sartoretto et al. 1996; Ballesteros 2006; Pinna et al. 2022) and the L^{-} irradiance tested corresponds to the light intensity at the thalli collection depth. Furthermore, the results obtained indicate that the germination discs of *L. stictiforme* do not acclimate to higher irradiance which has reduced germination disc density and growth. This overall supports the evidence already provided for the growth of adult thalli of this species (Pinna et al. 2022). However, experiments carried out on early life stages of CCA, have produced heterogeneous results since the response to irradiance appears to be species-specific. For example, some evidence provided for *Lithophyllum yessoense* Foslie germination discs indicate their growth was favored by higher irradiance (i.e. 240µmol photons $m^{-2}s^{-1}$), although the species is a subtidal alga (Ichiki et al. 2000). Inconsistently, the fact that in intertidal CCAs, such as the *Lithophyllum okamurae* Foslie (Yoshioka et al. 2020), or in the coral reef builder *Porolithon onkodes* (Heydrich) Foslie (Ordoñez et al. 2017), germination disc growth rate was reduced at low irradiance suggests an adaptation to light condition specific of the environment where the species normally thrive.

A significant effect on the germination success of *L. stictiforme* discs was observed under high temperature treatment that promoted the germination of discs regardless of light intensities. In the location where adult thalli were collected for the experiment, the seawater temperature was approximately 14 °C (as usually at that time of year, Ceccherelli et al. 2020), the higher germination success was observed in laboratory raising the temperature to 20 °C, which has been simulated the thermocline deepening that naturally occurs

Table 8 PERMDISP test results

Response variable	F	df	*P*(perm)	Factor
Germination success	0.084	1,10	0.778	Temperature
Germination disc density T2	0.156	1,10	0.753	Light
Germination disc density T3	0.238	1,10	0.682	Light
Germination disc development (T1)	0.449	3,32	0.747	Interaction TexLi
Germination disc development (T2)	2.474	3,32	0.140	Interaction TexLi
Germination disc development (T3)	2.468	3,32	0.167	Interaction TexLi
Germination disc development (T1 stage 2)	0.595	1,34	0.644	Interaction TexLi
Germination disc development (T1 stage 3)	2.337	1,34	0.135	Temperature
Germination disc development (T1 stage 3)	0.001	1,34	0.972	Light
Germination disc development (T1 stage 4)	0.832	1,34	0.574	Interaction TexLi
Germination disc development (T2 stage 2)	0.079	1,34	0.780	Light
Germination disc development (T2 stage 3)	1.893	1,34	0.178	Light
Germination disc development (T2 stage 4)	1.364	1,34	0.532	Interaction TexLi
Germination disc development (T2 stage 5)	0.769	1,34	0.742	Interaction TexLi
Germination disc development (T2 stage 6)	0.456	1,34	0.801	Interaction TexLi
Germination disc development (T3 stage 2)	2.178	1,34	0.149	Temperature
Germination disc development (T3 stage 3)	03.147	1,34	0.085	Light
Germination disc development (T3 stage 5)	0.128	1,34	0.978	Interaction TexLi
Germination disc development (T3 stage 6)	0.742	1,34	0.679	Interaction TexLi
Germination disc development (T3 stage 7)	0.923	1,34	0.569	Interaction TexLi
Germination disc area	0.104	1,10	0.754	Light
Germination disc calcification (Ca)	2.389	1,84	0.126	Light
Germination disc calcification (Ca)	2.969	1,84	0.088	Disc Portion
Germination disc calcification (Mg/Ca)	1.076	1,84	0.303	Temperature
Germination disc calcification (Mg/Ca)	1.274	1,84	0.262	Light
Germination disc calcification (Mg/Ca)	2.183	1,84	0.143	Disc Portiona

in that area in August and September. These results are consistent with previous studies, showing that higher temperatures can enhance the metabolic processes that promote the germination disc growth in the laboratory (Ichiki et al. 2000; Ordoñez et al. 2017). Little is known about the effects of seawater warming on Mediterranean CCA and especially on the first life stages, although it is known that the calcification of *L. cabiochae*, is negatively affected by an increase in seawater temperature (Martin and Gattuso 2009).

Our study also highlights that an increase in temperature does not have a significant effect on the growth of germinative discs that, conversely, is only influenced by light. This is consistent with observations that adult thalli of *L. stictiforme* exhibited greater marginal growth in areas where the water temperature was lower (Pinna et al. 2022). However, no evidence was ever provided for recruits.

Valuable information was also provided by the interactive effect of light and temperature on the development time of *L. stictiforme* germination discs: it was predominantly observed that the segmentation of germination discs was accelerated when temperatures were high and irradiance low.

Optimal conditions do not only favor growth, but also mineralogical processes (Cornwall et al. 2017; Nash et al. 2019; McCoy et al. 2023). The EDS analysis performed in this study showed that higher Ca content was promoted at lower irradiance, conferring higher hardness and dissolution resistance, likely an advantage both in mesocosms and in the field (Ries 2006; Martin et al. 2013a, b; McCoy and Kamenos 2015; Ragazzola et al. 2016; Nash et al. 2019). This result supports the assumption that *L. stictiforme* is a low-light-adapted species, as higher irradiances than in the field would trigger the calcification suppression (Martin et al. 2013b; Egilsdottir et al. 2016). Apparently, not all the CCAs are low-light adapted, since in other species the calcification of adult thalli is light-enhanced (Borowitzka 1981; Borowitzka and Larkum 1987; Martin et al. 2013a).

Studies on adult thalli of CCAs have also found a close relationship between growth rate, degree of calcification and Mg content (Kamenos et al. 2008, 2009; Nash et al. 2019), with faster calcification being associated with a lower density of calcite and increased incorporation of Mg into the cell walls (Kamenos et al. 2009; Kamenos and Law 2010; Sletten et al. 2017; Krieger et al. 2023). In the present study, germination discs of *L. stictiforme* cultured under $L^{+}T^{+}$ had the slowest growth, but the Mg/Ca ratio was the highest among the treatments. A possible explanation for the higher Mg substitution could lie in the effect of both light and temperature, as a correlation between higher Mg/Ca and higher temperatures in adult CCAs is widely recognized (Halfar et al. 2008, 2013; Nash et al. 2016, 2017). It is likely that the germination discs from this treatment are more vulnerable to dissolution after outplanting, as dissolution is more pronounced with increasing Mg substitution in biogenic calcite (Andersson et al. 2008).

In general, CCAs control the mineralization process during the development of germination discs (de Carvalho et al. 2022): mineralization starts only in the innermost walls in 8-cell specimens and in 16-cell germination discs the outer part is not yet calcified (de Carvalho et al. 2022). In all treatments, the Ca content was higher in the peripheral portion of germination discs, which begins to grow once the marginal meristem has formed, allowing horizontal expansion of the young thallus. The studies conducted on adult thalli of CCAs indicate that their calcification is highest in the growth regions where most chloroplasts are located with a higher photosynthetic activity) (McCoy et al. 2023). This work corroborates the finding that CCAs germination discs can control their calcification process to a certain extent, however, further investigations are needed to elucidate the mechanisms involved in their biomineralization in relation to light intensity and temperature.

Overall, several questions remain to be answered about the response to the combination of these two conditions ex situ. Further studies should investigate the spatial consistency, by collecting thalli across Mediterranean sites, and temporal variability of our findings to investigate the importance of local and seasonal adaptations: for example, research is also necessary to understand if spores released from thalli collected below the thermocline during warm periods (summer) respond differently to a major temperature increase of 5–6 °C in terms of germination success, density, development and growth, exhibiting seasonal adaptations.

In conclusion, the present study expands upon the knowledge on development, growth and calcification of CCA germination discs. In particular, it assessed the feasibility and determined the optimal combination of light and temperature conditions for the *ex-situ* culture of *L. stictiforme*. Further experiments are needed to identify the best substrate (i.e. tile) for *L. stictiforme* recruitment to be used in mesocosm conditions, so that outplanting in the field the recruited tiles would be a technically possible and environmentally friendly action.

Authors' contribution Data collection was performed by AP and SK, while data analysis was conducted by AP and PS. SEM-EDS analyses were performed by DP and SK. The manuscript was authored by AP, following an initial draft written by AP and SK. Subsequent drafts were led by GC and AF. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP OGS D33C22000960007 and CUP UNISS J83C22000870007, Project title "National Biodiversity Future Center - NBFC". GC and AF also acknowledge the project RENOVATE "Ecosystem approach for compensation and mitigation actions in the coastal marine environment" funded by Autorità di Sistema Portuale del Mar Tirreno Centro Settentrionale, CUP UNISS: J34F22001920005.

Data availability All data produced by this study are presented within this manuscript.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval The authors have secured all the required permits from the appropriate authorities for conducting sampling and observational field studies.

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