

Research Article

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Early development of *Gongolaria montagnei* (Fucales, Phaeophyta) germlings under laboratory conditions, with a view to enhancing restoration potential in the Eastern Mediterranean

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Abstract: The loss of Mediterranean macroalgal populations dominated by *Cystoseira sensu lato* is driven by a multitude of pressures. In the eastern Mediterranean Sea, climate change and the establishment of Lessepsian herbivore species have further intensified the ongoing decline of these canopy-forming algae. Knowledge of the reproductive phenology, embryology and growth of *Cystoseira* species is the first step towards successful reforestation measures. *Gongolaria montagnei* is one of the most common canopy-forming algal species with a wide horizontal and vertical distribution along the Greek coasts. Mature receptacles were collected from Saronikos Gulf (Aegean Sea), and germlings were cultured in mesocosms for 34 days, reaching up to 1.30 mm in length. Divisions and early developmental stages of *G. montagnei* are described and possible implications for future restoration efforts are discussed. A new approach to growth rate modeling, based on surface measurements of embryos and germlings is presented.

1 Introduction

Mediterranean brown algae of the genera *Cystoseira* C. Agardh, *Gongolaria* Boehmer and *Ericaria* Stackhouse (Molinari Novoa and Guiry 2020; Orellana et al. 2019), hereafter referred to as *Cystoseira* s.l. (*sensu lato*), are perennial or semi-perennial, arborescent species that form dense and extensive stands with high species diversity and productivity. Such algal forests have long been considered as the climax community of pristine rocky coasts of the Mediterranean (Montesanto and Panayotidis 2001; Pérès and Picard 1964; Sala et al. 2012; Salomidi et al. 2016).

Due to their high vulnerability to natural and human disturbance (Mancuso et al. 2018; Orfanidis et al. 2021; Sales and Ballesteros 2009), *Cystoseira* s.l. species serve as indicators of Good Environmental Status in European (i.e., Marine Strategy Framework Directive 2008/56/EC, Water Framework Directive 2000/60/EC), and National Monitoring Programs. Moreover, healthy *Cystoseira* communities are crucial biotic elements for the assessment of “Reefs” (Habitat Type 1170), which need to be monitored, protected and restored under the EU Habitats Directive 92/43/EEC. Apart from *Cystoseira compressa* (Esper) Gerloff et Nizamuddin, all Mediterranean *Cystoseira* s.l. species are listed in Annex II (endangered and threatened species) of the Barcelona Convention, while “Photophilic communities with canopy-forming algae in Mediterranean infralittoral and upper circalittoral rock” were recently listed as endangered in the European Red List of Habitats (Gubbay et al. 2016).

In recent years, in the Greek Seas (Giakoumi et al. 2012; Panayotidis et al. 2004; Salomidi et al. 2016; Tsiamis et al. 2013; authors’ personal observations) and across the

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Mediterranean (Benedetti-Cecchi et al. 2001; Blanfuné et al. 2016; Eriksson 2002; Falace et al. 2010; Mangialajo et al. 2008; Perkol-Finkel and Airoidi 2010; Sala et al. 2011, 2012; Thibaut et al. 2005, 2015), a decline in *Cystoseira* s.l. populations has been observed, leading to a shift in the composition, structure and function of rocky reef ecosystems. At the same time, natural recovery of canopy forests is rare due to low growth rates (Orfanidis 1991) and the limited dispersal ability of large and heavy zygotes of *Cystoseira* s.l. species (Falace et al. 2018; Verdura et al. 2018).

Given the high potential of these species to produce zygotes under suitable temperature and light conditions, germling culture appears to be a viable option in initializing sustainable restoration efforts. In germling culture, only a small percentage of fertile apices is collected from natural populations. Thus, degraded *Cystoseira* forests are not further compromised, as is the case with other restoration approaches, such as transplantation that includes harvesting of adult thalli. Therefore, methods for outplanting lab-grown *Cystoseira* are increasingly being investigated (Cebrian et al. 2021). This process consists of two main steps: cultivation of germlings in mesocosms under controlled conditions (Falace et al. 2018; Savonitto et al. 2019), and subsequent outplanting onto natural reefs (De La Fuente et al. 2019; Orlando-Bonaca et al. 2021; Savonitto et al. 2021; Verdura et al. 2018).

Particularly pronounced effects of climate change and Lessepsian herbivore species are reported on local populations of *Cystoseira* s.l. (Katsanevakis et al. 2014; Lelieveld et al. 2012; Salomidi et al. 2016; Vergés et al. 2014). However, the reproductive timing and early life stages of these ecosystem engineers have not yet been studied in the eastern Mediterranean Sea, further limiting knowledge and the potential for effective interventions.

Gongolaria montagnei (J. Agardh) Kuntze (= *Cystoseira spinosa*) is a monopodial species growing in the littoral and sublittoral, under moderate to high hydrodynamic conditions. Following field surveys carried out to implement the WFD (2000/60/EC) and the Habitats Directives (92/43/EEC), *G. montagnei* was identified as one of the most common canopy-forming algal species along the Greek coasts. It typically occurs over a wide depth range, from the upper infralittoral (0–1 m) down to over 20 m, either in monospecific canopies or in association with one or more other fucoids, such as *C. compressa*, *Cystoseira corniculata* (Turner) Zanardini, *C. crinitophylla* Ercegovic, *C. foeniculacea* (Linnaeus) Greville, *Ericaria amentacea* (C. Agardh) Molinari et Guiry, *E. brachycarpa* (J. Agardh) Molinari et

Guiry, *E. crinita* (Duby) Molinari et Guiry, and *Sargassum vulgare* C. Agardh, *nom. illeg.*

Among these, *C. corniculata* and *G. montagnei* appear to be the most resistant to herbivore pressure; they even persist in eastern and south-eastern latitudes, where the Lessepsian rabbitfish *Siganus* spp. have long established high densities (Corsini-Foka and Economidis 2007; Giakoumi 2014; Salomidi et al. 2016; authors' unpublished data).

The embryology and zygote development of *G. montagnei* have not been described, not even in Guern's (1962) most comprehensive study on *Cystoseira* embryology. Against this background, we present the first attempt to describe the reproductive period and early developmental stages of *G. montagnei*, with the scope to provide basic insights for effective restoration of canopy algae.

2 Materials and methods

The reproductive phenology of *Gongolaria montagnei* was followed *in situ* at three sites from February to May 2021 (Figure 1). Fertile apices were detected from early April, but samples were collected in May 2021 in the upper infralittoral zone in Saronida, Attika, Saronikos Gulf, Aegean Sea (Figure 1). This site was selected due to the high coverage of *G. montagnei*. At the donor site, *G. montagnei* was found in the intertidal and upper sublittoral zones, at depths of 0–1 m, on semi-exposed to exposed rocky shores with gentle slopes. Sea surface temperature at the time of collection was 18 °C.

Five adult thalli were collected for morphological observation. Apical fronds ($n = 204$) of 2–3 cm, holding mature receptacles were carefully collected avoiding damaging the entire thallus (Falace et al. 2018). Samples were wrapped in aluminum foil and immediately (within 1 h) transferred to the Hellenic Center for Marine Research laboratory. Apical fronds were rinsed with sterile seawater to remove epiphytes and reduce bacterial and phytoplankton proliferation, placed in boxes without seawater, and then stored in dark and cold conditions (5 °C) for 24 h to induce zygote emission. The next day, mature apical fronds were placed on microscope slides in polypropylene tanks containing 2 l of 0.2 µm filtered and UV-sterilized natural seawater. The tanks were set in an environmentally controlled room, where temperature (19 °C), photoperiod (12:12) (PHILIPS LED tube lamps cool white, T8 14.5 W 1600 lm G13 230 V 4000 K) and irradiance ($125 \mu\text{mol m}^{-2} \text{s}^{-1}$) were adjusted to simulate seasonal conditions in the natural environment.

To minimize nutrient depletion during germling growth, UV-sterilized seawater was enriched with Von Stosch's solution (VSE), which was renewed every four days (Falace et al. 2018). Germanium dioxide (GeO_2) was added to prevent diatom growth (Falace et al. 2006) and air pumps were installed in the tanks to keep seawater aerated.

The experiment lasted 34 days, during which the different stages of zygote and germling development were observed with a light

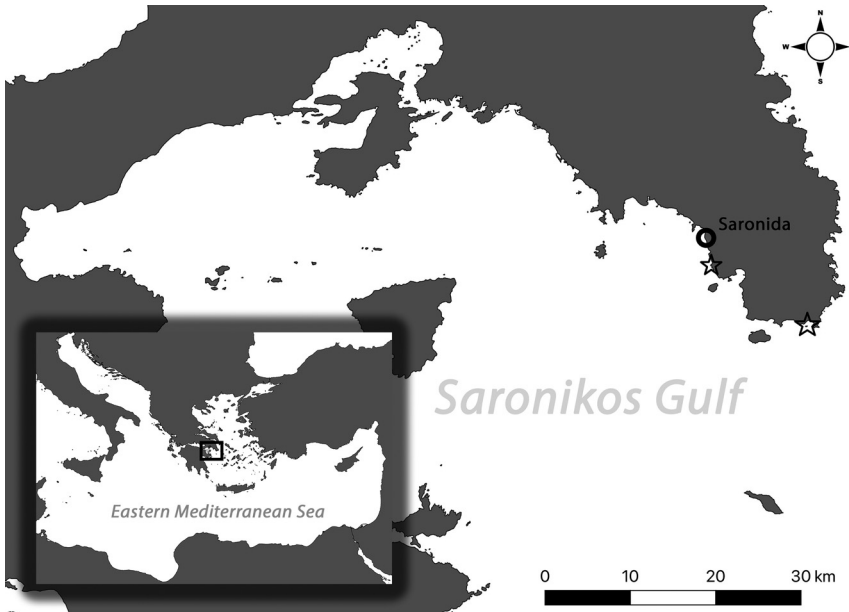


Figure 1: Map showing locations (marked with symbols ○ and ★) where the reproductive phenology of *Gongolaria montagnei* was followed *in situ* from February to May 2021. Mature receptacles were collected at Saronida (○).

microscope (OLYMPUS BX43, Japan). Measurements were made by carefully moving the slides with zygotes or germlings from the tank to the microscope twice daily for the first three days, once daily from day 4 to day 20 and every 3 or 4 days from day 20–34. The growth of germlings was calculated by processing the photographic data using Image Pro Premier 9.3. More specifically, the growth was estimated by fitting a second-order polynomial model to the measurements of the surface area covered by *G. montagnei* during its developmental stages using the relevant Microsoft Excel function. The relative growth rate (RGR) of *G. montagnei* zygotes and germlings was measured following the formula: $RGR = [\ln(SA_{t2}) - \ln(SA_{t1})]/\text{days}$, where SA_{t1} and SA_{t2} are germlings surface area at times $t1$ and $t2$.

3 Results

3.1 Reproductive morphology

During the reproductive season, adult thalli of *Gongolaria montagnei* were 8–13 cm high (Figure 2A). They had a tree-like habit, were non-caespitose, brown to yellow in color; the erect primary axis, usually unbranched, was attached to the substratum by a robust disk-shaped base; the apices of axes were spiny; tophules were numerous, spiny, and prominent; primary branches were cylindrical, ramified to secondary branches terete or flattened; spiny appendages (leaves) were present, robust, rigid, simple or bifid; cryptostomata on both branches and spinose appendages were evident; aerocysts were absent; receptacles were spiny, on apical part of branches (Figure 2B and C), and contained several conceptacles with antheridia and oogonia (Figure 3).

3.2 Segmentation and developmental stages

Zygote release occurred shortly (3–6 h) after the receptacles were placed on the slides (Figure 4A). Zygotes reached $106 \pm 19 \mu\text{m}$ (mean diameter \pm standard deviation; $n = 20$). In the following hours (18–24 h) after zygote release (AZR), the first equatorial division was observed (Figure 4B). Within 22–42 h AZR the second division was perpendicular to the first (Figure 4C), while in some zygotes the second division was parallel to the first (Figure 4D). From 36 h AZR, rhizoids began to develop (Figure 4E) and continued to increase in length over the following days (Figure 4F).

Up to this point, the embryos maintained their spherical shape; they were still surrounded by the fertilization membrane and their surface remained quite stable. From day 7 AZR, the fertilization membrane detached from the embryos (Figure 4G), they took on a more elongated shape and their surface gradually increased.

From day 12 AZR, hyaline hairs were observed growing from the apical region of the embryo (Figure 4H). On day 34, the length of the germlings ranged from 0.88 to 1.30 mm (Figure 4I), whereas the surface area of the germlings ranged from 201 to 582 mm^2 . In the fourth week of cultivation, a proliferation of microalgae belonging to the genus *Nannochloropsis* (Trebouxiophyceae) began in the tanks. In the fifth week, the mortality of *G. montagnei* germlings increased due to the *Nannochloropsis* outbreak, and at the end of the experiment (day 34) only 10 individuals had survived.

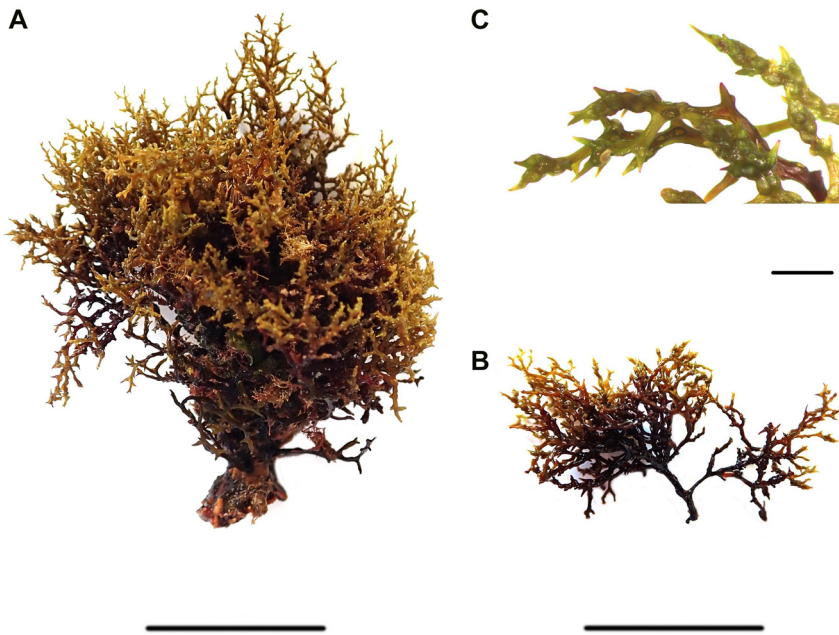


Figure 2: Thallus of *Gongolaria montagnei* during the reproductive period (A, scale bar = 3 cm), mature apical fronds (B, scale bar = 3 cm) and receptacles (C, scale bar = 2 mm).

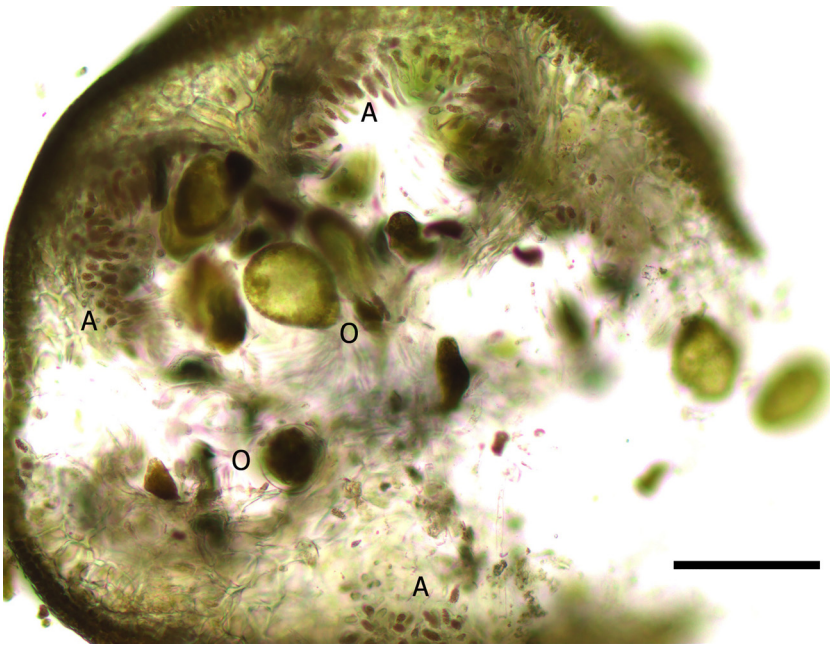


Figure 3: Cross section of a receptacle of *Gongolaria montagnei*, showing antheridia (A) and oogonia (O). Scale bar = 100 μm .

3.3 Growth and growth rates

The growth and growth rates of *G. montagnei* zygotes and germlings, are shown in Figure 5. The last day of the experiment (day 34) was not included in the calculations due to the high mortality of germlings.

Based on the surface area, the overall growth model (second order polynomial) was:

$$y = -0.0023x^2 + 0.1868x + 1.5092$$

with $R^2 = 0.9578$ ($p < 0.05$) where x = day of incubation and y = surface area of zygote or germling.

After a lag phase during the first week of incubation, the *G. montagnei* embryos started to grow exponentially with a mean RGR of 0.2 (day^{-1}) from day 8–15 (Figure 5). Germlings continued growing from day 21–31, yet at a slower rate of 0.1 day^{-1} (Figure 5).



Figure 4: Divisions and developmental stages of *Gongolaria montagnei*.

(A) Zygote after release from the conceptacle. (B) First division. (C) Second, most common, division. (D) Second, rare, division. (E) Development of the rhizoids (arrow). (F) Elongation of the rhizoids. (G) Fertilization membrane (arrow). (H) Hyaline apical hairs. (I) Germling after five weeks of culture. Scale bar = 100 μm .

4 Discussion

Guern (1962) divided the species of *Cystoseira* s.l. into three groups based mainly on zygote segmentation, oogonia shape and number of rhizoids. In Guern's embryonic classification, *Gongolaria montagnei* is placed in Group I. However, Guern mentions that the embryonic development of *G. montagnei* was not studied, as the species had completely disappeared from the sampling stations around Banyuls (France), where it was reported years ago by

Sauvageau (1912) and Feldmann (1937). From personal observations based on studying the embryonic classification of Guern (1962), *G. montagnei* follows Group I traits, as predicted. In particular, the first stages of segmentation, the spherical shape of the oogonia, and the number of primary rhizoids resemble those of *Ericaria mediterranea* (Sauvageau) Molinari et Guiry, *E. crinita* and *Gongolaria barbata* (Stackhouse) Kuntze, all of which are assigned to Group I (Guern 1962). Subsequent work has also supported the placement of *G. montagnei* in Group I. For example,

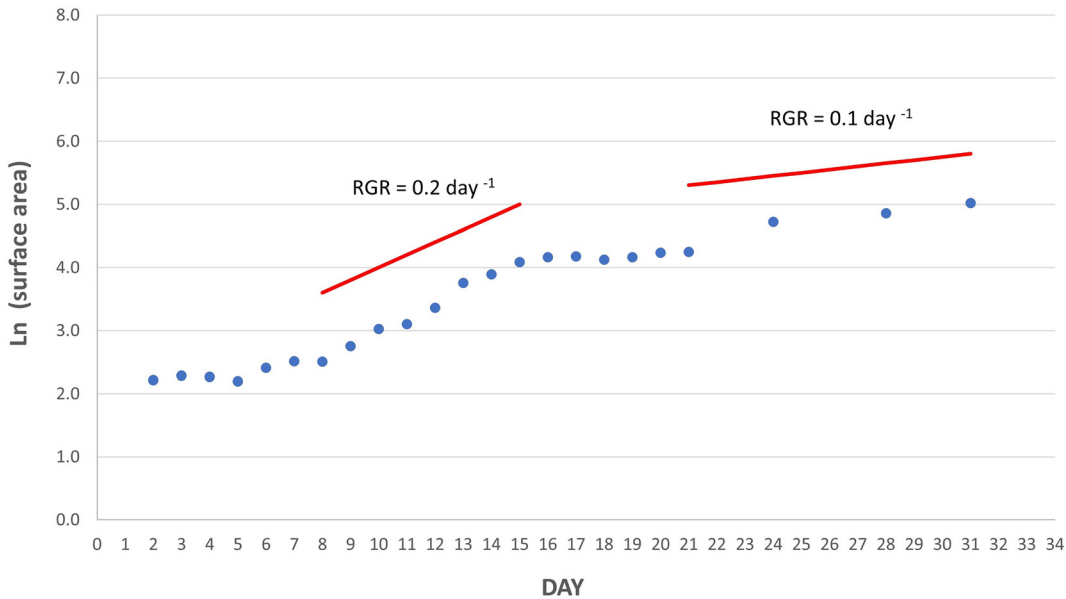


Figure 5: Growth of *Gongolaria montagnei* embryos and germlings during incubation (31 days). Increase in relative growth rates (RGR) was observed in two phases (red lines), from days 8–15 (0.2 day^{-1}) and days 21–31 (0.1 day^{-1}).

Colombo et al. (1982) identified morphological groups of *Cystoseira* s.l. based on embryo characteristics and classified *Cystoseira montagnei* in Group I, due to the spherical embryo morphology and the number of primary rhizoids (four). By combining morphological traits with phylogenetic traits, Bruno de Souza et al. (2019) also included *G. montagnei* (as *Cystoseira montagnei*, *Cystoseira*-IIA) in Group I.

In the present study, the final length of *G. montagnei* germlings (0.88–1.30 mm) after five weeks (34 days) of cultivation was quite small compared to other studies on different *Cystoseira* s.l. species. For example, the final length of *Ericaria barbatula* germlings after four weeks of culture ranged from 1.1 to 2.6 mm (Savonitto et al. 2019), while the length of *G. barbata* germlings after nearly three weeks (19 days) was 1.16 mm (Orlando-Bonaca et al. 2021) and 1.72–3.45 mm after four weeks (Savonitto et al. 2021), and the seedling length of *E. amentacea* was 1.38 mm after three weeks of culture (Falace et al. 2018). Although such differences in seedling lengths among *Cystoseira* s.l. species could be species-specific, this cannot be confirmed with certainty. In addition, microalgal proliferation after one month of cultivation may have prevented germlings from reaching their maximum potential length.

In previous studies, length or wet biomass measurements were used for the calculation of growth (Falace et al. 2018; Orfanidis 1991; Orlando-Bonaca et al. 2021; Savonitto et al. 2019, 2021). In the present study, a first attempt was made to calculate the growth of *G. montagnei* germlings from surface area measurements obtained by processing

digital images. Measuring the surface area rather than just the length was considered a more accurate approach because of the different shapes that embryos take during early developmental stages. Indeed, the growth model equation calculated for *G. montagnei* using surface area showed a high level of confidence in the present study ($R^2 = 0.9578$, $p < 0.05$) and could therefore be helpful in relevant studies to estimate the increase in zygote-germling surface area (mm^2) over time (days).

Based on the surface measurements (ln values) of the present study two distinct exponential growth phases were recognized (Figure 5). During the first phase (days 8–15), *G. montagnei* germlings elongated in shape and gradually increased in surface area with a higher mean RGR value (0.2 day^{-1}). In the second phase (days 21–31) the germlings continued to grow and started to form blades until the end of the incubation period, yet at a slower growth rate ($\text{RGR} = 0.1 \text{ day}^{-1}$). The observed decline in RGR with increasing size may be explained by the ontogenetic drift accompanied by changes in germling morphology, physiology, and resource allocation (Evans 1972; Hunt 1982). Indeed, in another study, the max RGR values of thalli of *G. barbata* were even lower (0.05 day^{-1} ; Orfanidis 1991).

Although we did not have bacterial or diatom outbreaks, as has been the case in other cultivation experiments (Orlando-Bonaca et al. 2021), microalgae covered *G. montagnei* germlings on week four, resulting in high germling mortality one week later. While natural mortality is to be expected (Falace et al. 2018), possible ways to prevent microalgal proliferation could be to thoroughly

rinse the freshly collected fertile apices and to shorten the overall duration of culture. Adding an intermediate step of moving the germlings to a suspended culture at sea prior to final outplanting (Orlando-Bonaca et al. 2021; Savonitto et al. 2021) would further mitigate the risk of bacterial or microalgal outbreaks that are common during prolonged culture in mesocosms.

Indeed, combining mesocosm and field cultivation could be advantageous because of the positive elements of both cultivation methods. Cultivation in mesocosms during the critical early life stages of zygotes provides an environment safe from grazers (e.g., sea urchins, gastropods, fish), competitors and extreme hydrodynamic events, thereby increasing settlement success and zygote numbers, and maximizing early survival rates. Transferring the germlings to floating structures in the sea after three to four weeks of mesocosm cultivation reduces the risk of contamination with microalgae and bacteria that have affected the late stages of this and other similar experiments. At the same time, this intermediate step allows germlings to reach a larger size, further increasing their chances of survival when they are outplanted at restoration targets.

The application and improvement of successful cultivation protocols and non-destructive restoration techniques is becoming increasingly urgent to halt the further decline of canopy-forming algae (De La Fuente et al. 2019; Falace et al. 2018; Orlando-Bonaca et al. 2021; Savonitto et al. 2019, 2021; Verdura et al. 2018). This process helps to build essential infrastructure, while also providing valuable insights into the early life-history stages and environmental requirements of target species, which may considerably vary at relatively small spatial scales (Buonomo et al. 2017). In a rapidly changing marine ecosystem, such as the Eastern Mediterranean Sea, understanding the reproductive phenology, embryology and growth of *G. montagnei* and other poorly studied *Cystoseira* s.l. species is a crucial first step towards establishing scientifically sound restoration techniques to reversing deteriorating trends in temperate reef ecosystem structure, function and services.

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