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Reproduction of *Sphaerococcus coronopifolius* (Gigartinales, Rhodophyta) in natural populations of the Lazio coasts (central Italy) and in culture

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Abstract – As *Sphaerococcus coronopifolius* Stackhouse (Gigartinales, Rhodophyta) is a seaweed that has considerable potential for commercial uses, and as its life history in the various seas where it lives has not yet been thoroughly explored, we carried out a pilot study on the reproduction in nature and in vitro culture of field specimens established on the coasts of the Lazio region (Tyrrhenian Sea, central Italy). Spermatangia were not observed on the gametophyte (*S. coronopifolius*) and the tetrasporophyte of the species (*Haematocelis fissurata*) was not found either in the study site or in culture. As the carpospores developed directly into the gametophyte, we may conclude that this species reproduces itself, in both the study site and in culture, through a direct-type life history. It may thus be possible to use the variety of the clonal individuals growing on the coasts of Lazio for a future cultivation of *S. coronopifolius* on a large scale.

***Sphaerococcus coronopifolius* / reproduction / direct-type life history / field specimens / culture / Lazio coasts / central Italy**

Résumé – *Sphaerococcus coronopifolius* Stackhouse (Gigartinales, Rhodophyta) est une algue marine qui a un potentiel considérable d'utilisation commerciale mais dont le cycle de vie dans les diverses mers qu'elle colonise est encore imparfaitement connu. Pour cette raison nous avons étudié la reproduction, en nature et en culture, des populations naturelles de l'espèce établies sur nos côtes (Latium, Italie centrale). Nous n'avons pas observé de spermatanges produits par le gamétophyte (*S. coronopifolius*) ni des tétrasporophytes de l'espèce (*Haematocelis fissurata*), soit dans le site naturel, soit en culture. Les carpospores développent directement le gamétophyte en nature et en culture et l'espèce se reproduit donc sur nos côtes au moyen d'un type direct de cycle de vie. En conséquence il serait possible d'utiliser les exemplaires végétatifs qui croissent sur les côtes du Latium pour cultiver en masse *S. coronopifolius,* à des fins commerciales.

***Sphaerococcus coronopifolius* / reproduction / cycle de vie / spécimens récoltés / spécimens cultivés / côtes du Latium / Italie centrale**

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INTRODUCTION

Sphaerococcus coronopifolius Stackhouse (Sphaerococcaceae, Gigartinales, Rhodophyta) is a red alga that is common on the Atlantic and Mediterranean coasts and has been known since the 1980s (Caccamese *et al.,* 1981) to have significant antiviral, antimicrobial and antitumoral properties (Smyrniotopoulos *et al*., 2010a, 2010b, 2015; Rodrigues *et al*., 2015). A recent study has shown that aqueous extracts of the alga, collected from natural populations of the Lazio coasts (Tyrrhenian Sea, central Italy), may also be used for paper artwork restoration purposes, as is the non-European species of the genus *Gloiopeltis* (Endocladiaceae) (Fratini *et al*., 2016). The latter property is due to the water-soluble polysaccharides with agarose structure produced by *S. coronopifolius* (Bouhlal *et al*., 2011), *Gloiopeltis* and other red algae (Whyte *et al*., 1985; Craigie, 1990; Takano *et al*., 1995). Despite these properties, *S. coronopifolius* has not yet been exploited commercially nor cultivated on a large scale, probably because information on how this species develops in its various habitats is still scarce.

Searles (1968) was the last scholar to investigate the female reproductive system of *Sphaerococcus coronopifolius,* based on herbarium specimens collected in June 1954 at Banyuls (France) and kept at the University of California Herbarium. He described the female reproductive system (procarp) of the gametophyte as well as the cystocarps (*i.e.* the carposporophytes surrounded by a gametophytic tissue or pericarp found in this alga and in several other red algae; see Hommersand & Fredericq, 1990), but did not observe either spermatangia or tetrasporophytes (Searles, 1968). Spermatangia in *S. coronopifolius* have only been reported once before, in the nineteenth century, at the tips of thalli bearing cystocarps (Buffham, 1893). They developed from a small group of elongated cells (1-3) in depressions of the thallus surface that appeared as "minute spots, more lucid than the ordinary cortical cells" (Buffham, 1893: 293). The tetrasporophyte of *S. coronopifolius* was subsequently discovered to be, in culture, the crustose alga known as *Haematocelis fissurata* P. Crouan & H. Crouan (Maggs & Guiry, 1982; Maggs, 1990). The species thus has a heteromorphic trigenetic life cycle, with an alternation of a monoecious, erect and bushy gametophyte (*S. coronopifolius*), and a crustose tetrasporophyte (*H. fissurata*).

Since the composition and yield of polysaccharides in algae is notoriously affected by the nuclear phase of the life cycle, as well as by seasonal and site-specific variations (Craigie, 1990; Haslin *et al*., 2000), our group investigated both field specimens and carpospores grown in culture in order to understand how *Sphaerococcus coronopifolius* reproduces on the coasts of Lazio. This paper reports the results of these studies.

MATERIAL AND METHODS

Study site and morphological analysis

Specimens were collected seasonally from April 2013 to December 2015 at S. Marinella (41°47'21.95''N; 11°46'58.32"2 E) and Punta del Pecoraro (Civitavecchia, 42°04'10.66''N; 11°48'28.11''E) at depths ranging from 1 to 4 m. Some thalli were fixed in 4% Formalin/seawater for the morphological studies.

Cystocarps were separated from the thallus, softened by immersion in distilled water for 2-3 days, squashed and directly observed under a Zeiss microscope equipped with a Leica DFC 42 digital camera. Thin sections of cystocarps, isolated either from fresh material or from specimens conserved in 70% alcohol, were embedded in agar (5% w:v) and sectioned at 30-50 µm using a vibratome (TPI Series 1000, Portland, USA), as described in Valletta *et al*. (2013).

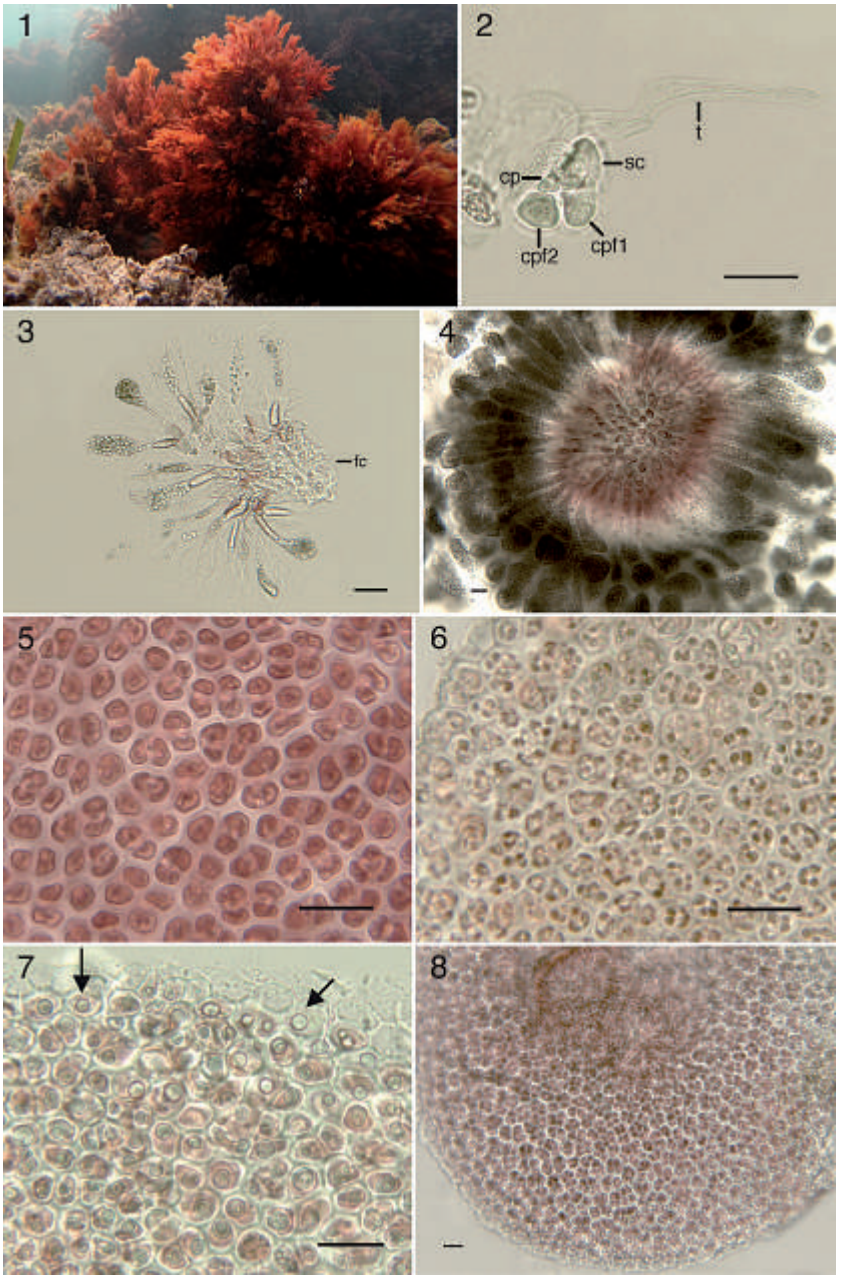
Culture experiments

Cystocarps detached from thalli collected on December 17, 2015 and slightly squashed, or single carpospores gathered with a Pasteur pipette after incision of the cystocarps with a scalpel, were grown in Enriched Natural Seawater Media (PES, Provasoli 1968) (Andersen *et al*., 2005), first in agar Petri plates, and subsequently after germination of the carpospores in liquid medium. Group of carpospores released from slightly squashed cystocarps (Fig. 12) developed better in culture than single carpospores gathered from incised cystocarps. Cultures were maintained until August 2016 at 15 ± 1°C, in a 12:12 h light/dark cycle and under a photon flux density of 5 µmol m^{-2} s^{-1} following Guiry & Maggs (1984). The thalli were moved periodically into clean 100 ml Pyrex glass dishes in order to avoid bacterial/fungal/algal proliferation. They were observed and photographed with both a stereomicroscope Nikon SMZ 2t and an Olympus B43 optical microscope equipped with an Olympus SC50 digital camera.

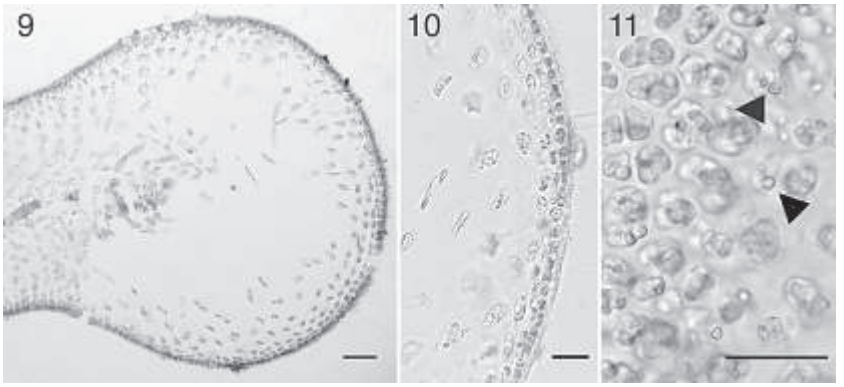
Abbreviations used in the figures are as follows: sc = supporting cell; cpf1 and cpf2 = first and second cell of the carpogonial branch; cp = carpogonium; t = trichogyne.

RESULTS

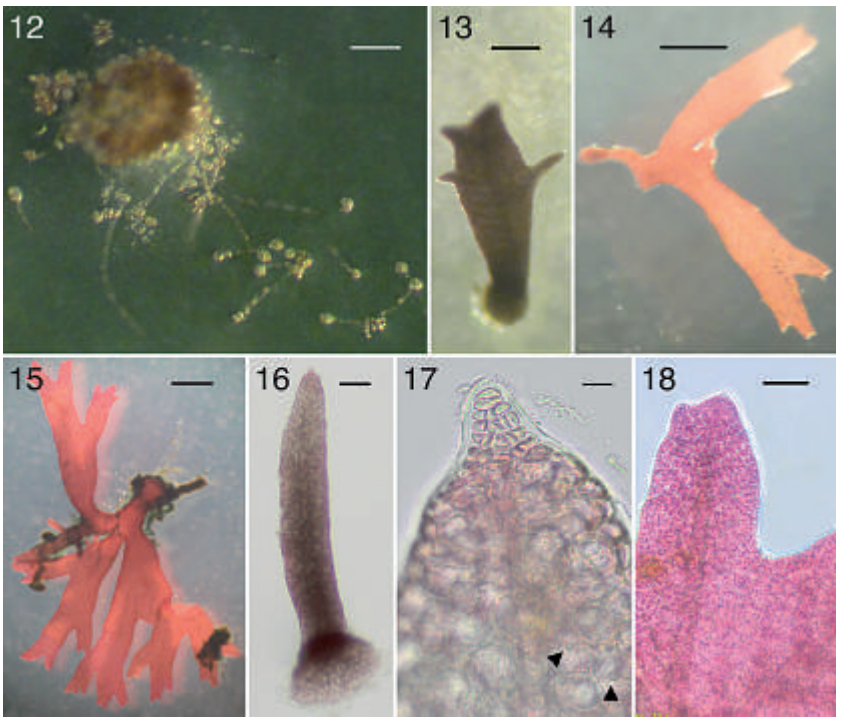
Gametophytes (Fig. 1) bearing stalked cystocarps were found throughout the year along the coasts of Lazio, with cystocarps being more numerous in the winter months. The procarp was observed throughout the year; it consists of a four-celled filament, *i.e.* the three-celled carpogonial branch and the supporting cell (Fig. 2), which, according to Kylin (1930 in Searles, 1968) functions as the generative auxiliary cell. The trichogyne was often observed to be coiled at the base (Fig. 2). Spermatia were not seen, either attached to the trichogyne or formed at the tips of the thallus. A large central fusion cell from which the gonimoblast filaments radiate was easily observed, persisting after the formation of the carpospores (Fig. 3). In mature cystocarps, each gonimoblast filament ended with chains of one or two carposporangia , with the carpospores densely packed with droplets of varying density (Fig. 4). A layer of small cortical photosynthetic cells (outer pericarp) surrounded the cystocarps (Fig. 5). Below were other layers (inner pericarp) of larger, closely packed, hyaline, polygonal cells 8-15(20) µm in length, and filled with the same droplets as those contained in the carpospore (Fig. 6). Many of the droplets they contain emerge on the surface of mature cystocarps, appearing as clear refractive droplets of different sizes (Figs 7-8). Those droplets can also be seen on thin transverse and tangential sections of cystocarps, which reveal the cuticle and the outer and inner pericarp tissues (Figs 9-11).



Figs 1-8. Development of field specimens of *Sphaerococcus coronopifolius* in nature. **1.** Habit of thalli *in situ*. **2.** Carpogonial branch with a trichogyne coiled at the base (cp = carpogonium; cpf1, cpf2 = first and second cell of the carpogonial branch; sc = supporting cell, t = trichogyne). **3.** Young squashed cystocarp showing the fusion cell (fc). **4.** Squashed mature cystocarp showing radiating gonimoblast filaments ending with one or two carposporangia in short chains. **5.** Outer pericarp cells of cystocarp, in surface view. **6.** Innermost pericarp cells filled with droplets, in surface view. **7-8.** Droplets emerging on the surface of mature cystocarps. (Fig. 1: Bar = 1 cm; Figs 2-7: Bar = 20 µm; Fig. 8: Bar = 50 µm).



Figs 9-11. Thin sections of *Sphaerococcus coronopifolius* cystocarp. **9-10.** Transverse sections showing the outer unmodified pericarp layer and the innermost layers composed of large cells filled with droplets. **11.** Tangential section. Note the emerging droplets (arrowheads). (Figs 9-11: Bar = 20 µm).



Figs 12-18. Development of carpospores of *Sphaerococcus coronopifolius* in culture. **12.** Germination of carpospores. **13-15.** Gametophytes developed from carpospores in culture, under the stereomicroscope (those photographs as the one in Fig. 12 were taken through the glass of culture dishes). **16-18.** The same thalli observed under the compound microscope. Note the uniaxial structure, the meristematic apical cell and in Figure 17 the emerging droplets (arrowheads). (Figs 12, 13, 16: Bar = 100 µm; Figs 14-15: Bar = 500 µm; Fig. 17: Bar= 10 µm; Fig. 18 Bar = 50 µm).

Carpospores grown in culture gave rise directly to erect gametophytes (*Sphaerococcus coronopifolius*, Figs 12-16). Refractive droplets, which may have been newly produced or have arisen from those present on cystocarps inserted in the plates, were seen on thalli grown in culture (Fig. 17, arrowheads). The young thalli continued to develop slowly in culture until epiphytic diatoms and green algae began to proliferate in the cultures (Figs 15, 18). They attained a size of 3-4 mm in height after 5 months of cultivation and are now placed under aeration to verify whether we can continue to keep them alive.

DISCUSSION

Field specimens of *Sphaerococcus coronopifolius* collected on the Lazio coasts displayed a marked production of refractive droplets that accumulate within the carpospores and emerge on the surface of mature cystocarps. Intercellular oil droplets were already observed on the thallus of *Haematocelis fissurata* (Maggs & Guiry, 1983); thus both the gametophyte and the tetrasporophyte of *S. coronopifolius* produced lipid droplets. The emerging droplets on cystocarps of *S. coronopifolius* were first mistakenly interpreted as spermatia since some Gigartinales (as *Gigartina pinnata* J. Agardh and *G. sonderi* Edyvane & Womersley, see Womersley, 1994) are known to produce spermatangial sori surrounding the cystocarps. Thin sections of mature cystocarps, however, did not reveal spermatangial sori developing from the outer cortical cells (pericarp), which were instead unmodified, nor were spermatangia observed at the tips of the thallus, as they had been by Buffham (1893). Moreover, the development of the carpospores directly into gametophytes in culture confirmed that spermatangia were not produced in the thalli collected from the coasts of Lazio. Although a careful search was conducted during the present surveys, *H. fissurata* was not found in the study site, nor was it ever encountered previously when we sampled down to depths of 35 m in various sites of the Lazio coasts to compile a flora of the marine algae of our region (Abdelahad *et al.,* 2002; D'Archino *et al*., 2004, 2005; available upon request). Reports of *H. fissurata* have also been rare in nature on both the Atlantic and Mediterranean coasts (for distributional data see Guiry & Guiry, 2015). In Italy *H. fissurata* has only been reported once (as *Ethelia fissurata* (P. Crouan & H. Crouan) Denizot), in Sicily, in deep water (Cinelli *et al*., 1979; see also Furnari *et al*., 2003) and not in association with *S. coronopifolius*. The results of this study suggest that our specimens from the Lazio coast reproduce without fertilisation through a direct-type life history (Maggs, 1988). Direct development of gametophytes from carpospores or of tetrasporophytes from tetraspores are not uncommon in red algae and may be more or less prevalent in populations according to their geographical distribution (Maggs, 1988; Hawkes, 1990). Asexual reproduction propagates clones and is an advantage when cultivating seaweeds for commercial purposes since it provides greater genetic uniformity in the thalli and consequently ensures that there is less variability in the composition and yield of the natural products synthesized by it (Waaland, 1983). Hence, it may be possible to use the individuals growing on the coasts of Lazio as the first variety for the cultivation of *S. coronopifolius* on a large scale. This will be one aim of our future researches. Furthermore, studies on the life cycle of populations living on other Mediterranean and Atlantic coasts, as well as studies on the quantity and quality of their natural products, are needed to find other cultivars that may be exploited for commercial purposes.

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