Tesi doctoral presentada per En/Na

Noemi SALVADOR SOLER

amb el títol

"El gènere Bonnemaisonia (Bonnemaisoniales, Rhodophyta) a la Península Ibèrica i les illes Balears: taxonomia, cicles vitals, corologia i aplicacions"

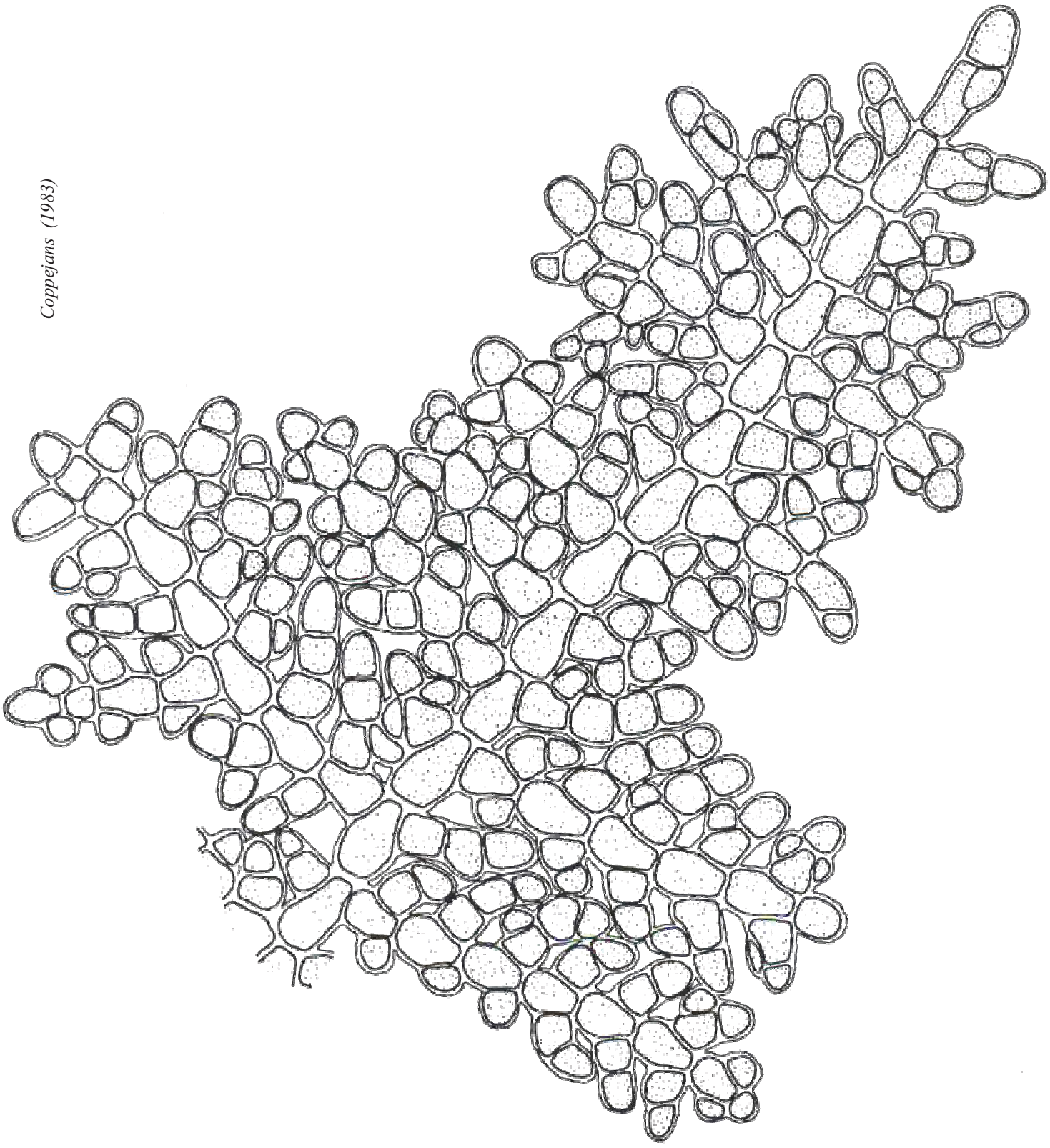
per a l'obtenció del títol de Doctor/a en

FARMÀCIA

Barcelona, 23 d'octubre de 2009.

Facultat deFarmàcia
Departament de Productes Naturals, Biologia Vegetal i Edafologia

UNIVERSITAT DE BARCELONA



Coppejans (1983)

ANNEX

1. Publicacions relacionades

Antimicrobial Activity of Iberian Macroalgae

N. Salvador, A. Gómez Garreta, L. Lavelli & M. A. Ribera. 2007.

Scientia Marina 71 (1): 101-113

SCIENTIA MARINA 71(1)
March 2007, 101-113, Barcelona (Spain)
ISSN: 0214-8358

Antimicrobial activity of Iberian macroalgae

NOEMÍ SALVADOR, AMELIA GÓMEZ GARRETA, LUCA LAVELLI
and MARÍA ANTONIA RIBERA

Laboratori de Botànica, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain.
E-mail: noasalvador@ub.edu

SUMMARY: The antibacterial and antifungal activity of 82 marine macroalgae (18 Chlorophyceae, 25 Phaeophyceae and 39 Rhodophyceae) was studied to evaluate their potential for being used as natural preservatives in the cosmetic industry. The bioactivity was analysed from crude extracts of fresh and lyophilised samples against three Gram-positive bacteria, two Gram-negative bacteria and one yeast using the agar diffusion technique. The samples were collected seasonally from Mediterranean and Atlantic coasts of the Iberian Peninsula. Of the macroalgae analysed, 67% were active against at least one of the six test microorganisms. The highest percentage of active taxa was found in Phaeophyceae (84%), followed by Rhodophyceae (67%) and Chlorophyceae (44%). Nevertheless, red algae had both the highest values and the broadest spectrum of bioactivity. In particular, *Bonnemaisonia asparagoides*, *Bonnemaisonia hamifera*, *Asparagopsis armata* and *Falkenbergia rufolanosa* (Bonnemaisoniales) were the most active taxa. *Bacillus cereus* was the most sensitive test microorganism and *Pseudomonas aeruginosa* was the most resistant. The highest percentages of active taxa from Phaeophyceae and Rhodophyceae were found in autumn, whereas they were found in summer for Chlorophyceae.

Keywords: antimicrobial activity, marine macroalgae, Bonnemaisoniales, agar diffusion technique, crude extracts, Iberian Peninsula.

RESUMEN: ACTIVIDAD ANTIMICROBIANA DE MACROALGAS MARINAS DE LA PENÍNSULA IBÉRICA. – Se analizó la actividad antibacteriana y antifúngica de 82 macroalgas marinas (18 *Chlorophyceae*, 25 *Phaeophyceae* y 39 *Rhodophyceae*) para valorar su potencial aplicación como conservantes naturales en la industria cosmética. Los extractos crudos de cada taxon, preparados tanto a partir de material fresco como liofilizado, fueron testados frente a tres bacterias Gram positivas, dos bacterias Gram negativas y una levadura, mediante la técnica de difusión en agar. Las muestras fueron recolectadas en diversas localidades de las costas mediterráneas o atlánticas de la Península Ibérica en distintas estaciones del año. El 67% de todas las macroalgas estudiadas mostraron actividad antimicrobiana frente al menos un microorganismo test de los seis utilizados. El mayor porcentaje de táxones activos lo presentó el grupo de las *Phaeophyceae* (84%) seguido por las *Rhodophyceae* (67%) y por las *Chlorophyceae* (44%). No obstante, las algas rojas fueron las que presentaron el mayor grado de actividad así como el espectro de acción más amplio y, dentro de este grupo, *Bonnemaisonia asparagoides*, *Bonnemaisonia hamifera*, *Asparagopsis armata* y *Falkenbergia rufolanosa* (*Bonnemaisoniales*) fueron los táxones más activos. En cuanto a los microorganismos, *Bacillus cereus* fue el más sensible y *Pseudomonas aeruginosa* el más resistente. Los tres grupos taxonómicos mostraron una variación estacional en la producción de sustancias antimicrobianas, siendo el otoño la estación con mayor porcentaje de táxones activos para las *Phaeophyceae* y *Rhodophyceae*, mientras que para las *Chlorophyceae* fue el verano.

Palabras clave: actividad antimicrobiana, macroalgas marinas, *Bonnemaisoniales*, técnica de difusión en agar, extractos crudos, Península Ibérica.

INTRODUCTION

Several marine organisms produce bioactive metabolites in response to ecological pressures such as competition for space, maintenance of unfouled surfaces, deterrence of predation and the ability to successfully reproduce (König *et al*., 1994). These bioactive compounds offer rich pharmacological potential (Muñoz, 1992).

There are numerous reports of macroalgae derived compounds that have a broad range of biological activities, such as antibiotic, antiviral, anti-

neoplastic, antifouling, anti-inflammatory, cytotoxic and antimitotic (Naqvi *et al*., 1980; Caccamese *et al*., 1981; Fenical and Paul, 1984; Hodgson, 1984; Ballesteros *et al*., 1992; Bhosale *et al*., 2002). Harder (1917) was the first to observe antimicrobial substances secreted by algae. However, it was not until the 1970s that large-scale screening of antimicrobial activity was carried out (Welch, 1962; Glombitza, 1970; Hornsey and Hide, 1974; Henríquez *et al*., 1977). In the past few decades, macroalgae have been widely recognised as producers of a broad range of bioactive metabolites (Caccamese *et al*., 1981; Fenical and Paul, 1984; Ma and Tang, 1984; Reichelt and Borowitzka, 1984; Hornsey and Hide, 1985; Rosell and Srivastava, 1987; Febles *et al*., 1995; Crasta *et al*., 1997; Melo *et al*., 1997; Centeno and Ballantine, 1999; Horikawa *et al*., 1999). However, the results obtained by the aforementioned authors suggest that the production of antimicrobial substances by the same species varies (Pesando, 1990). This intraspecific variability may be due to ecology, the stage of active growth or sexual maturity (Pratt *et al*., 1951; Chesters and Stott, 1956; Burkholder *et al*., 1960).

The purpose of this work was to evaluate the antibacterial and antifungal activity of Iberian marine macroalgae. To date, research on biologically active substances of Iberian seaweeds has been scarce (Serarols *et al*., 1982; Cabañes *et al*., 1984; Ballesteros *et al*., 1992). The relationships the geographical zone, sampling season and algal generation have with antimicrobial activity, as well as the influence of sample preparation methods on assay results, are of considerable interest and have scarcely been studied. This information could prove valuable for harvesting algae for industrial applications. In fact, the present study corresponds to the first experimental task of a European project aimed at evaluating using macroalgae as natural preservatives in the cosmetic industry.

MATERIAL AND METHODS

A total of 82 taxa (18 Chlorophyceae, 25 Phaeophyceae and 39 Rhodophyceae) were sampled at various sites along the northern Mediterranean (Llançà, Port de la Selva, Palamós, Begur, Lloret de Mar, Blanes and the Ebro Delta) and Atlantic (San Sebastián, Guetaria, Ondarreta, Zarauz, Ría de Vigo and Bayona) coasts of Spain. To evaluate the possible influence of sampling season on antimicrobial activity, the maximum possible number of these taxa in each season (winter, spring, summer and autumn) was collected. Seaweeds were collected by scuba diving or snorkelling and preserved on ice until further processing. Seaweed samples were manually cleansed of sand, epiphytes and animals, then rinsed in distilled water to remove salt. Samples from each taxon were prepared using two different treatments: freezing at - 40ºC (hereafter referred to as *fresh*) and lyophilisation. The bioactivities of the fresh and lyophilised samples were subsequently compared to determine any differences resulting from the respective preparation methods.

As the bioactivity of Bonnemaisoniales has previously been reported, we carried out complementary studies for some of the taxa present in the Iberian Peninsula (*Asparagopsis armata*, its tetrasporophyte *Falkenbergia rufolanosa*, *Bonnemaisonia asparagoides* and *Bonnemaisonia hamifera*). The Bonnemaisoniales present on both Atlantic and Mediterranean coasts were collected to assess the relationship between the geographical zone and bioactivity. To evaluate whether antimicrobial activity varies with life-cycle generations of algae, gametophytic and tetrasporophytic generations of *A. armata* were analysed.

The test microorganisms selected were spoiled microorganisms or common human pathogens, and were comprised of the three Gram-positive bacteria *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (identified strain by the CECT) and *Staphylococcus aureus* (ATCC 29213), the two Gram-negative bacteria *Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 9027), and the yeast *Candida albicans* (ATCC 48867). All cultures were kept on Brain Heart Infusion (BHI) agar plates and stored at 4ºC, except the initial stock cultures, which were stored at - 40ºC in BHI broth containing 20% glycerol.

Solid extracts from fresh and lyophilised material were prepared for all taxa following a modified version of the extraction method of Burkholder *et al*. (1960). The extracts were obtained by milling algal samples without solvent using a Waring blender and/or manually with a mortar. Due to the high bioactivity observed for the solid extracts of Bonnemaisoniales, we sought to prove this high bioactivity by also obtaining methanolic extracts. The extracts were prepared using a modified version of the method used by Caccamese *et al*. (1981) from

algal material (ca. 2 g of lyophilised or 6 g of fresh material) homogenised via a Polytron in 10 ml of methanol-toluene (3:1). The extracts were then centrifuged to remove insoluble material, the supernatants were evaporated at reduced pressure, and the solid residue was then dissolved in 1 ml of methanol.

Antimicrobial activity was evaluated by the agar diffusion method, which is the most widely used method for susceptibility testing and is simple, economical and reproducible (Álvarez Benito, 1990). Moreover, this standardised procedure is accepted for determining antimicrobial susceptibility by the National Committee for Clinical Laboratory Standards (NCCLS).

A liquid microorganism suspension corresponding to a 0.5 McFarland scale (standard suspension of barium sulfate which represents $1.5x10^8$ bacterial/ml) was applied to Mueller-Hinton plates using a cotton swab. After a few minutes, to allow complete absorption of the inoculum, the crude extracts were placed on the agar plates. The solid extracts (0.2 g), obtained from fresh and from lyophilised material of each taxon, were placed in 9.3 mm diameter wells made on the plates with a sterilised cork borer. The methanolic extracts from Bonnemaisoniales were absorbed onto non-impregnated discs (bioMérieux, 6 mm diameter), air-dried to eliminate residual solvent, and then placed onto the inoculated plates.

During overnight incubation at 37ºC, the yeast or bacterial lawn grew over the agar surface (Hodgson, 1984), except where it was inhibited by the radial diffusion of antimicrobial compounds of the extracts. The diameter of the inhibition halo is considered to be indicative of the bioactivity of the seaweed extract, and was measured (including the well or disc diameter) with a caliper. Mean diameter values were calculated from triplicate runs of each assay. Standardised values for diameters of the inhibition halo, expressed in mm, produced by the microorganisms against known antibiotics are listed in the literature (Álvarez Benito, 1990). Our results were interpreted according to these values, whereby a diameter less than 1 mm was interpreted as representing a taxon with trace activity, a diameter between 1 and 20 mm was interpreted as representing an active taxon, and a diameter larger than 20 mm was interpreted as representing a taxon with a level of bioactivity sufficient for antibiotic use (hereafter referred to as *high activity*).

The influence of algal treatment and sampling season on the results from solid extracts was assessed using variance analysis (ANOVA, Statgraphics Plus 5.1, Statistical Graphics Corp., 1994-2001). Both analyses were applied to the most sensitive microorganism, *Bacillus cereus*, and did not include the taxa belonging to Bonnemaisoniales.

Three analyses of variance were carried out for the Bonnemaisoniales taxa, including in this case all test microorganisms. Bonnemaisoniales from Atlantic and Mediterranean coasts were analysed for their season of maximum activity by two-way ANOVA with geographical zone and test microorganisms as factors. The difference in bioactivity between Mediterranean specimens of *A. armata* and its tetrasporophyte *F. rufolanosa* was assessed by two-way ANOVA using generations and test microorganisms as factors. To compare the bioactivities of fresh and lyophilised material from Bonnemaisoniales, solid and methanolic extracts were analysed by two-way ANOVA.

RESULTS

Antimicrobial activity of solid extracts for the whole taxa

The results for the solid extracts from each season are summarised in Tables 1, 2, 3 and 4. Of the 82 taxa analysed, 55 (67%) showed antimicrobial activity against at least one test microorganism (Fig. 1). Of these, one Phaeophyceae and five Rhodophyceae showed antimicrobial activity against all six test microorganisms: *Hapalospongidion macrocarpum*, *Asparagopsis armata*, its tetrasporophyte *Falkenbergia rufolanosa*, *Osmundea truncata*, *Plocamium cartilagineum* and *Rytiphlaea tinctoria* (Tables 1-4). The antimicrobial activities of six other red algae (*Bonnemaisonia asparagoides*, *Bonnemaisonia hamifera*, *Ceramium deslongchampsii*, *Jania rubens*, *Peyssonnelia rubra* and *Wrangelia penicillata)* and one brown alga (*Cystoseira mediterranea*) against five test microorganisms were among the highest (Tables 1-4). However, 18 taxa (22%) did not show antimicrobial activity against any microorganism assayed (Fig. 1), and nine (11%) only showed trace activity against at least one test microorganism (Fig. 1).

This work includes the first descriptions ever published of the bioactivities of 15 of the taxa studied (Tables 1-4). Of these taxa, seven were active against at least one microorganism: the green alga

TABLE 1. – Antimicrobial activity of solid extracts of Iberian macroalgae in winter. TG= Taxonomic Group (C: Chlorophyceae, P: Phaeophyceae, R: Rhodophyceae); C= Coast (M: Mediterranean, A: Atlantic); Bas= *Bacillus subtilis*; Bac= *B. cereus*; Sta= *Staphylococcus aureus*; Eco= *Escherichia coli*; Psa= *Pseudomonas aeruginosa*; Can= *Candida albicans*. Numbers indicate diameter of inhibition halo in mm; tr = trace; - = inactive.

			Fresh						Lyophilized					
			Gram +			Gram -		Yeast	Gram +			Gram -		Yeast
TG	C	Taxa	Bas	Bac	Sta	Eco	Psa	Can	Bas	Bac	Sta	Eco	Psa	Can
C	M	*Bryopsis muscosa* J.V. Lamouroux	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Chaetomorpha linum* (O.F. Muller) Kützing	-	-	-	-	tr	-						
	M	*Cladophora rupestris* (Linnaeus) Kützing	11.6	11.9	-	-	-	tr	12.6	13.2	tr	-	-	-
	M	*Codium bursa* (Linnaeus) C. Agardh	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Codium coralloides* (Kützing) P.C. Silva	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Codium vermilara* (Olivi) Delle Chiaje	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Flabellia petiolata* (Turra) Nizamuddin	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Halimeda tuna* (J. Ellis and Solander) J.V. Lamouroux	-	-	-	-	-	-						
	M	*Palmophyllum crassum* (Naccari) Rabenhorst	-	-	-	-	-	-						
	M	*Ulva rigida* C. Agardh	-	-	-	-	-	-						
	M	*Valonia macrophysa* Kützing	-	-	-	-	-	-						
P	M	*Colpomenia sinuosa* (Mertens ex Roth) Derbès and Solier	13.8	14.3	14.5	-	-	-	15.4	15.8	14.8	-	-	-
	M	*Cystoseira barbata* (Stackhouse) C. Agardh	10.1	11.9	tr	-		-						
	M	*Cystoseira brachycarpa* J. Agardh v. *balearica* (Sauvageau) Giaccone	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Cystoseira compressa* (Esper) Gerloff and Nizamuddin	13.4	13.4	14.1	-	-	-	14.1	14.9	15.2	-	-	-
	M	*Cystoseira mediterranea* Sauvageau	tr	tr	-	-	-	-	-	15.9	12.6	13.2	-	-
	A	*Cystoseira tamariscifolia* (Hudson) Papenfuss	11	13	tr	-		-	13.2	12.8	11.9	-	-	-
	M	*Dictyopteris polypodioides* (A.P. De Candolle) J.V. Lamouroux	-	-	-	-	-	-						
	M	*Dictyota dichotoma* (Hudson) J.V. Lamouroux var. dichotoma	tr	11.6	tr	-	-	-						
	M	*Dictyota dichotoma* (Hudson) J.V. Lamouroux var. intricata (C. Agardh) Greville	tr	11.6	-	-	-	-						
	M	*Dictyota spiralis* Montagne	12.8	11.8	tr	-	-	-	11.6	11.7	tr	-	-	-
	M	*Hapalospongidion macrocarpum* (Feldmann) León Álvarez & González González	17.2	18.7	17.5	12.6	12	13.3						
	M	*Padina pavonica* (Linnaeus) J.V. Lamouroux	13.5	12.4	11.3	-	-	-	13	13.5	14.1	-	-	-
	M	*Scytosiphon lomentaria* (Lyngbye) Link	-	-	-	-	-	-						
	M	*Stypocaulon scoparium* (Linnaeus) Kützing	-	-	-	-	-	-						
	M	*Taonia atomaria* (Woodward) J. Agardh	-	-	-	-	-	-	-	-	tr	-	-	12.3
	M	*Zanardinia typus* (Nardo) P.C. Silva	16.5	13.7	16.8	-	-	-	-	tr	-	-	12.9	tr
R	M	*Asparagopsis armata* Harvey	29.4	30.2	22.2	20.8	25.5	32	38.9	51.1	35.1	39.9	27.3	53.2
	M	*Bangia atropurpurea* (Roth) C. Agardh	-	tr	-	-	-	-	-	20.6	tr	-	-	tr
	A	*Bonnemaisonia hamifera* Hariot	46.8	34	40.5	12.5	-	52.6	52.1	56	41.4	15.4	tr	37.1
	M	*Bornetia secundiflora* (J. Agardh) Thuret	-	-	-	-	-	-						
	M	*Ceramium ciliatum* (J. Ellis) Ducluzeau	-	tr	tr	-	tr	15	-	-	-	-	-	-
	M	*Ceramium deslongchampsii* (Chauvin) ex Duby	13.9	14.9	16.5	13.7	14.1	-						
	M	*Corallina elongata* J. Ellis and Solander	-	-	-	tr	-	-	-	-	-	-	-	14.7
	A	*Falkenbergia rufolanosa* (Harvey) Schmitz				18.2			18	26.1	18.6	17.9	16.9	26.3
	M	*Falkenbergia rufolanosa* (Harvey) Schmitz	31.7	32.3	24.1	26.1	23.6	43.6	20.3	31.4	23.2	24.2	20.8	43.3
	M	*Gastroclonium clavatum* (Roth) Ardissone	-	-	-	-	-	-						
	M	*Gelidium spinosum* (S.G. Gmelin) P.C. Silva	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Gracilaria dura* (C. Agardh) J. Agardh	-	19.5	11.5	18.6		27.3	-	-	-	-		-
	M	*Grateloupia filicina* (J.V. Lamouroux) C. Agardh	-	tr	-	-	-	12.9						
	M	*Jania rubens* (Linnaeus) J.V. Lamouroux	tr	tr	-	-	-	13.4	-	-	12.8	-	-	15.1
	M	*Laurencia intricata* J.V. Lamouroux	tr	11.6	13.9	tr	-	12.4	-	tr	15	12	12.7	tr
	M	*Laurencia obtusa* (Hudson) J.V. Lamouroux	15.1	tr	-	tr	-	-						
	M	*Nemalion helminthoides* (Velley) Batters	-	-	-	-	-	-						
	M	*Osmundea truncata* (Kützing) K.W. Nam and Maggs	18.7	21.4	17.2	12.7	14.1	15.3	21.4	26.8	15.8	19	16.1	27.1
	M	*Peyssonnelia rubra* (Greville) J. Agardh	10.7	11.8	14.1	-	12.4	15.1						
	M	*Plocamium cartilagineum* (Linnaeus) P.S. Dixon	11	12.4	12	-	10.8	13.8						
	M	*Porphyra leucosticta* Thuret	-	-	-	tr	-	-						
	M	*Porphyra linearis* Greville	-	-	-	-	-	-						
	M	*Pterocladiella capillacea* (S.G. Gmelin) Santelices and Hommersand	tr	tr	-	-	-		-	-	-	-	-	-
	M	*Rissoella verruculosa* (A. Bertoloni) J. Agardh	-	-	-	-	-	-	-	-	-	-	-	-
	M	*Rytiphlaea tinctoria* (Clemente) C. Agardh	-	11.9	10.7	-	-	-	15.6	16.2	16.3	11.6	-	-
	M	*Schottera nicaeensis* (J.V. Lamouroux ex Duby) Guiry and Hollenberg	-	-	-	-	-	-						
	M	*Sphaerococcus coronopifolius* Stackhouse	12.1	14.3	12.7	tr	-	18.6	-	-	-	-	-	15.5

Enteromorpha muscoides, the brown algae *Fucus spiralis* var. *platycarpus* and *Spatoglossum solieri* and the red algae *Boergeseniella fruticulosa*, *Gracilaria dura*, *R. tinctoria*, *Schottera nicaeensis* and *Scinaia furcellata*. *G. dura* and *R. tinctoria* were notably active, the former against yeast and *Escherichia coli*,

TABLE 2. – Antimicrobial activity of solid extracts of Iberian macroalgae in spring. TG= Taxonomic Group (C: Chlorophyceae, P: Phaeophyceae, R: Rhodophyceae); C= Coast (M: Mediterranean, A: Atlantic); Bas= *Bacillus subtilis*; Bac= *B. cereus*; Sta= *Staphylococcus aureus*; Eco= *Escherichia coli*; Psa= *Pseudomonas aeruginosa*; Can= *Candida albicans*. Numbers indicate diameter of inhibition halo in mm; tr = trace; - = inactive.

			Fresh						Lyophilized					
			Gram +			Gram -		Yeast	Gram +			Gram -		Yeast
TG	C	Taxa	Bas	Bac	Sta	Eco	Psa	Can	Bas	Bac	Sta	Eco	Psa	Can
C	M	*Bryopsis muscosa* J.V. Lamouroux	-	12.2	tr	-		-	tr	23	27.1	-		-
	M	*Cladophora rupestris* (Linnaeus) Kützing	14	13.4	13.4	-		tr						
	A	*Codium fragile* (Suringar) Hariot subsp. *tomentosoides* (Goor) P.C. Silva	-	-	-	-		-						-
	M	*Enteromorpha intestinalis* (Linnaeus) Nees	-	-	-	-		-						
	M	*Flabellia petiolata* (Turra) Nizamuddin	-	-	-	-		-	-		-	-		-
	M	*Halimeda tuna* (J. Ellis and Solander) J.V. Lamouroux	-	-	-	-		-						
	M	*Ulva rigida* C. Agardh	-	tr	-	-		-	-	-	-	-		-
	M	*Valonia macrophysa* Kützing	-	-	-	-		-						
P	A	*Bifurcaria bifurcata* R. Ross	tr	tr	10.5	-		-	12.3	13	16.4	-		-
	M	*Cladostephus spongiosum* f. *verticillatum* (Lightfoot) Prud'homme van Reine	-	-	-	-		-	-	-	14.1	-		-
	M	*Colpomenia sinuosa* (Mertens *ex* Roth) Derbès and Solier	12.4	10.8	13.7	-		-	13.7	13.7	15.7	-		-
	M	*Cystoseira barbata* (Stackhouse) C. Agardh							11.6	10.9	17.3	-		-
	M	*Cystoseira brachycarpa* J. Agardh v. *balearica* (Sauvageau) Giaccone	tr	11	10.4	-		-	-	-	-	-		-
	M	*Cystoseira compressa* (Esper) Gerloff and Nizamuddin	12.9	12.8	14.2	-		-	13.5	13.6	23	tr		-
	M	*Cystoseira mediterranea* Sauvageau	10.8	12.8	12.4	10.6		-	12.1	12.4	13.5	11.6		-
	A	*Cystoseira tamariscifolia* (Hudson) Papenfuss	11.5	12.3	12	-		-	13.4	14.4	19.7	12.7		-
	M	*Dictyopteris polypodioides* (A.P. De Candolle) J.V. Lamouroux	-	-	-	-		-						
	M	*Dictyota dichotoma* (Hudson) J.V. Lamouroux var. *intricata* (C. Agardh) Greville	12.9	13.1	10.9	-		12.8	12.8	12	11.6	-		tr
	M	*Dictyota spiralis* Montagne	-	11.4	tr	-		-			tr	-		
	A	*Fucus spiralis* Linnaeus var. *platycarpus* Batters	10.8	11	11.6	-		-	12.6	12.2	13.3	-		-
	M	*Padina pavonica* (Linnaeus) J.V. Lamouroux	12.6	11.9	13.1	-		-						
	M	*Phyllariopsis brevipes* (C. Agardh) E.C. Henry and South	-	-	-	-		-						
	M	*Scytosiphon lomentaria* (Lyngbye) Link	-	-	tr	-		-						
	M	*Spatoglossum solieri* (Chauvin *ex* Montagne) Kützing	12.8	10.7	11	-		-	11.2	11.6	11.4	-		-
	M	*Sporochnus pedunculatus* (Hudson) C. Agardh	12.1	15.3	10.9	-		15	-	tr	-	-		14.5
	M	*Stypocaulon scoparium* (Linnaeus) Kützing	-	tr	-	-		-	-	-	12.4	-		-
	M	*Taonia atomaria* (Woodward) J. Agardh	-	-	-	-		-	tr	22.8	20.5	-		14.5
R	M	*Asparagopsis armata* Harvey	29.6	26.2	21.2	22.1		33.5						
	A	*Asparagopsis armata* Harvey	25.4	28.8	19.9	14		24.4	37.7	47.1	29.8	19.6		42.7
	M	*Boergeseniella fruticulosa* (Wulfen) Kylin	11.7	12.2	10.2	-		13.4						
	M	*Bonnemaisonia asparagoides* (Woodward) C. Agardh	55.2	82.9	68.5	18.1		49.7	31.5	41.2	27.3	18.9		33.5
	A	*Bonnemaisonia asparagoides* (Woodward) C. Agardh							69.9	78.3	70.5	22.7		68.2
	A	*Bonnemaisonia hamifera* Hariot	68.3	64.9	65.4	18.7		59.8						
	M	*Callithamnion granulatum* (Ducluzeau) C. Agardh	10.2	11.1	-	-		11.7						
	M	*Ceramium ciliatum* (J. Ellis) Ducluzeau							-	11.4	-	-		12.5
	M	*Ceramium rubrum* auctorum	-	10.8	-	-		11.1						
	M	*Corallina elongata* J. Ellis and Solander	-	-	-	-		14.3						
	M	*Falkenbergia rufolanosa* (Harvey) Schmitz	23	23.7	21.2	21.3		23.9	20.5	14	18.3	19.1		30.6
	M	*Gastroclonium clavatum* (Roth) Ardissone	-	tr	-	-		-	-	-	-	-		-
	M	*Gelidium spinosum* (S.G. Gmelin) P.C. Silva	-	-	-	-		-	-	-	-	-		-
	M	*Gracilaria dura* (C. Agardh) J. Agardh	-	19.7	11.8	19.7		25.5	-	-	-	-		-
	M	*Grateloupia filicina* (J.V. Lamouroux) C. Agardh	-	-	-	-		-	-	-	-	-		-
	M	*Jania rubens* (Linnaeus) J.V. Lamouroux	-	-	-	-		14.8						
	M	*Laurencia obtusa* (Hudson) J.V. Lamouroux	-	-	10.6	-		10.5						
	M	*Liagora viscida* (Forsskal) C. Agardh	-	-	-	-		-	-	-	-	-		-
	M	*Nemalion helminthoides* (Velley) Batters	-	-	-	-		-	-	-	-	-		-
	M	*Osmundea truncata* (Kützing) K.W. Nam and Maggs	15.6	17.2	11.5	12.7		21.7	15.2	13.6	13.4	tr		12.7
	M	*Pterocladiella capillacea* (S.G. Gmelin) Santelices and Hommersand	-	-	-	-		-	-	-	-	-		-
	M	*Rytiphlaea tinctoria* (Clemente) C. Agardh	15.8	16.3	19.6	11.4		15.2						
	M	*Schottera nicaeensis* (J.V. Lamouroux *ex* Duby) Guiry and Hollenberg	-	-	-	-		tr						
	M	*Sphaerococcus coronopifolius* Stackhouse	12.1	15.3	10.9	-		15	-	tr	-	-		14.5

TABLE 3. – Antimicrobial activity of solid extracts of Iberian macroalgae in summer. TG= Taxonomic Group (C: Chlorophyceae, P: Phaeophyceae, R: Rhodophyceae); C= Coast (M: Mediterranean, A: Atlantic); Bas= *Bacillus subtilis*; Bac= *B. cereus*; Sta= *Staphylococcus aureus*; Eco= *Escherichia coli*; Psa= *Pseudomonas aeruginosa*; Can= *Candida albicans*. Numbers indicate diameter of inhibition halo in mm; tr = trace; - = inactive.

			Fresh						Lyophilized				
			Gram +			Gram -		Yeast	Gram +			Gram -		Yeast
TG	C	Taxa	Bas	Bac	Sta	Eco	Psa	Can	Bas	Bac	Sta	Eco	Psa	Can
C	M	*Acetabularia acetabulum* (Linnaeus) P.C.Silva	-	-	-	-		-	-	-	-	-		-
	M	*Bryopsis corymbosa* J. Agardh	11.2	16.1	tr	-		tr	13.7	17.8	12.7	tr		13.5
	M	*Cladophora lehmannianna* (Linderberg) Kützing	-	-	-	-		-						
	M	*Codium bursa* (Linnaeus) C. Agardh	-	-	-	-		-	-	-	-	-		-
	M	*Codium coralloides* (Kützing) P.C. Silva	-	-	-	-		-	10.6	12.6	12.6	-		-
	A	*Codium tomentosum* Stackhouse	-	-	-	-		-						
	M	*Codium vermilara* (Olivi) Delle Chiaje							tr	11.9	11.9	-		-
	M	*Flabellia petiolata* (Turra) Nizamuddin	-	-	-	-		-	-	-	-	-		12.3
	M	*Halimeda tuna* (J. Ellis and Solander) J.V. Lamouroux	-	-	-	-		-	-	-	-	-		tr
	M	*Palmophyllum crassum* (Naccari) Rabenhorst	12.4	11.8	12.1	13		-						
	M	*Ulva rigida* C. Agardh	-	-	-	-		-	-	-	-	-		-
	M	*Valonia macrophysa* Kützing	-	-										
P	A	*Bifurcaria bifurcata* R. Ross	tr	-	tr	-		tr						
	M	*Cladostephus spongiosum* f. *verticillatum* (Lightfoot) Prud'homme van Reine	-	tr	-	-		-	-	-	-	-		-
	M	*Colpomenia sinuosa* (Mertens *ex* Roth) Derbès and Solier	15.3	15.9	17.1	-		-	14	14.8	15.4	tr		-
	M	*Cystoseira barbata* (Stackhouse) C. Agardh	12.1	12.1	12.5	-		-	11.8	12.4	18	-		-
	M	*Cystoseira brachycarpa* J. Agardh v. *balearica* (Sauvageau) Giaccone	-	-	-	-		-						
	M	*Cystoseira compressa* (Esper) Gerloff and Nizamuddin	10.3	11.7	11.8	-		-	11.8	12.8	16.2	-		-
	M	*Cystoseira mediterranea* Sauvageau	12.1	12	12.2	-		-	14	14.3	15.4	tr		-
	M	*Dictyopteris polypodioides* (A.P. De Candolle) J.V. Lamouroux	-	-	-	-		-	-	-	-	-		-
	M	*Dictyota dichotoma* (Hudson) J.V. Lamouroux var. *dichotoma*	-	tr	-	-		-	-	11	12.3	-		-
	M	*Dictyota dichotoma* (Hudson) J.V. Lamouroux var. *intricata* (C. Agardh) Greville	tr	tr	-	-		-	-	-	-	-		-
	M	*Hapalospongidion macrocarpum* (Feldmann) León Álvarez & González González	18.7	19.3	17.2	13		12.3						
	A	*Laminaria ochroleuca* La Pylaie	-	-	-	-		-						
	M	*Padina pavonica* (Linnaeus) J.V. Lamouroux	tr	tr	tr	-		-	-	-	12.4	-		-
	M	*Stypocaulon scoparium* (Linnaeus) Kützing	-	-	-	-		-						
	M	*Taonia atomaria* (Woodward) J. Agardh	-	-	-	-		-	-	-	-	-		-
	M	*Zanardinia typus (*Nardo) P.C. Silva	17.3	16.2	16.7	-		-	13.5	15.2	16.3	-		-
R	M	*Callithamnion granulatum* (Ducluzeau) C. Agardh	-	12.2	10.5	-		tr						
	M	*Ceramium ciliatum* (J. Ellis) Ducluzeau	-	-	-	-		11.9	-	12.1	11.7	-		tr
	M	*Corallina elongata* J. Ellis and Solander	tr	tr	-	tr		12.7	tr	10.5	13.2	11		13.7
	M	*Falkenbergia rufolanosa* (Harvey) Schmitz	17.6	17.8	19.4	14		20.8						
	A	*Gelidium corneum* (Hudson) J.V. Lamouroux	-	-	-	-		-						
	M	*Gelidium spinosum* (S.G. Gmelin) P.C. Silva	-	-	-	-		-	-	-	-	-		-
	M	*Grateloupia filicina* (J.V. Lamouroux) C. Agardh	-	13.9	tr	-		10.5						
	M	*Gymnogongrus crenulatus* (Turner) J. Agardh	13.8	17.4	tr	-		15.3						
	M	*Halymenia floresia* (Clemente) C. Agardh	tr	-	tr	tr		-						
	M	*Hypnea musciformis* (Wulfen) J.V. Lamouroux	-	-	-	-		-	-	-	14.3	-		-
	M	*Jania rubens* (Linnaeus) J.V. Lamouroux	11.2	12.2	11.4	10.9		14	-	11.4	-	-		15.1
	M	*Laurencia intricata* J.V. Lamouroux	10.4	12.8	tr	-		tr	tr	11.5	11.1	-		-
	M	*Laurencia obtusa* (Hudson) J.V. Lamouroux	17.9	19.3	17.3	-		tr	15.7	19.4	17.1	-		tr
	M	*Liagora tetrasporifera* Boergesen	-	-	-	-		-						
	M	*Liagora viscida* (Forsskal) C. Agardh	-	-	-	-		-	-	-	-	-		-
	M	*Nemalion helminthoides* (Velley) Batters	-	-	-	-		-	-	-	-	-		-
	M	*Plocamium cartilagineum* (Linnaeus) P.S. Dixon	15.1	16.7	14	10.6		14.2	12.6	14.8	14.2	13.1		tr
	M	*Pterocladiella capillacea* (S.G. Gmelin) Santelices and Hommersand	tr	11	tr	tr		10.8	-	-	-	-		-
	M	*Rissoella verruculosa* (A. Bertoloni) J. Agardh	-	-	-	-		-						
	M	*Schottera nicaeensis* (J.V. Lamouroux *ex* Duby) Guiry and Hollenberg	tr	tr	-	-		tr						
	M	*Scinaia complanata* (Collins) Cotton	-	-	-	-		-	-	tr	-	-		-
	M	*Scinaia furcellata* (Turner) J. Agardh	-	-	-	-		-						
	M	*Sphaerococcus coronopifolius* Stackhouse	-	-	-	-		-	-	-	-	-		-
	M	*Wrangelia penicillata* (C. Agardh) C. Agardh	11.2	13.1	12	10.6		14.1						

and the latter primarily against the Gram-positive bacteria. The remaining seven taxa, Chlorophyceae *Acetabularia acetabulum* and *Cladophora lehmanni-anna*, Phaeophyceae *Laminaria ochroleuca* and Rhodophyceae *Bornetia secundiflora*, *Gelidium corneum*, *Liagora tetrasporifera* and *Scinaia com-*

TABLE 4. – Antimicrobial activity of solid extracts of Iberian macroalgae in autumn. TG= Taxonomic Group (C: Chlorophyceae, P: Phaeophyceae, R: Rhodophyceae); C= Coast (M: Mediterranean, A: Atlantic); Bas= *Bacillus subtilis*; Bac= *B. cereus*; Sta= *Staphylococcus aureus*; Eco= *Escherichia coli*; Psa= *Pseudomonas aeruginosa*; Can= *Candida albicans*. Numbers indicate diameter of inhibition halo in mm; tr = trace; - = inactive.

			Fresh						Lyophilized					
				Gram +		Gram -		Yeast		Gram +		Gram -		Yeast
TG	C	Taxa	Bas	Bac	Sta	Eco	Psa	Can	Bas	Bac	Sta	Eco	Psa	Can
C	M	*Codium bursa* (Linnaeus) C. Agardh	-	-	-	tr		-	-	-	-	-	-	-
	M	*Codium coralloides* (Kützing) P.C. Silva	-	-	-	-		-	-	-	-	-	-	-
	M	*Codium vermilara* (Olivi) Delle Chiaje	-	-	-	-		-	-	24.7	-	-	-	-
	M	*Enteromorpha intestinalis* (Linnaeus) Nees	-	-	-	-		-	-	-	-	-	-	-
	M	*Enteromorpha muscoides (*Clemente) Cremades	-	10.8	-	-		-						
	M	*Flabellia petiolata* (Turra) Nizamuddin	-	-	-	-		-	-	-	-	-	-	tr
	M	*Halimeda tuna* (J. Ellis and Solander) J.V. Lamouroux	tr	-	-	-		-	-	tr	-	-	-	-
	M	*Palmophyllum crassum* (Naccari) Rabenhorst	-	10.5	tr	10.2		-	-	-	-	-	-	-
	M	*Ulva rigida* C. Agardh	-	tr	-	-		-	-	-	-	-	-	-
P	M	*Cladostephus spongiosum* f. *verticillatum* (Lightfoot) Prud'homme van Reine	-	11.9	-	-		12.3	tr	12.4	12.9	tr	-	14.9
	M	*Colpomenia sinuosa* (Mertens *ex* Roth) Derbès and Solier	15.5	14.1	15.9	-		-	14.9	11.8	11.7	-	-	-
	M	*Cystoseira barbata* (Stackhouse) C. Agardh	-	-	-	-		-	tr	tr	14.5	-		-
	M	*Cystoseira compressa* (Esper) Gerloff and Nizamuddin	13.7	13.5	14.4	-		-	13.1	13.4	13.6	tr	-	-
	M	*Cystoseira mediterranea* Sauvageau	11.3	10.6	10.3	-		-	13.5	16.2	15.1	17.1	14.8	-
	M	*Dictyota fasciola* (Roth) J.V. Lamouroux var. *repens* (J. Agardh) Ardissone							11.6	tr	-	-	-	-
	M	*Dictyota spiralis* Montagne	12.9	11.4	11.4	-		-						
	M	*Halopteris filicina* (Grateloup) Kützing	tr	11.6	-	-		tr	-	tr	13.1	-	-	14.2
	M	*Hapalospongidion macrocarpum* (Feldmann) León Álvarez & González González	17.9	20.2	18.2	14.4		13.4						
	M	*Padina pavonica* (Linnaeus) J.V. Lamouroux	11.5	11.7	12.1	-		-	tr	-	-	-	-	-
	M	*Scytosiphon lomentaria* (Lyngbye) Link		tr	-	-		-						
	M	*Stypocaulon scoparium* (Linnaeus) Kützing	-	-	-	-		11.9	-	-	tr	-	-	-
R	M	*Bangia atropurpurea* (Roth) C. Agardh	-	-	-	-		-	-	-	-	-	-	-
	M	*Bornetia secundiflora* (J. Agardh) Thuret	-	-	-	-		-						
	M	*Corallina elongata* J. Ellis and Solander	tr	tr	-	-		12.2	tr	tr	11.5	-	tr	13.8
	M	*Falkenbergia rufolanosa* (Harvey) Schmitz	30.0	38.5	27.4	27.8		37.8	24.4	30.5	21	25.7	16.2	41.7
	M	*Gelidium spinosum* (S.G. Gmelin) P.C. Silva	-	-	-	-		-	-	-	-	-	-	-
	M	*Gracilaria dura* (C. Agardh) J. Agardh	-	11.6	-	12.2		19.5	-	-	-	-		-
	M	*Jania rubens* (Linnaeus) J.V. Lamouroux	-	-	-	-		13.9	-	-	-	-	-	14.7
	M	*Laurencia intricata* J.V. Lamouroux	10.4	12.6	11.6	-		tr	11.2	12.7	12.3	-	-	12.9
	M	*Osmundea truncata* (Kützing) K.W. Nam and Maggs	21.5	24.7	16.7	19.0		21	26.1	28.8	14.7	16.5	12.6	27.9
	M	*Peyssonnelia rubra* (Greville) J. Agardh	tr	tr	10.3	-		tr	-	tr	16.4	tr	13.5	12.3
	M	*Porphyra linearis* Greville	13.9	20.2	13.7	11.0		21.2	14.9	16.8	15.6	11.7	14.2	18.7
	M	*Rissoella verruculosa* (A. Bertoloni) J. Agardh	-	-	-	-		-						
	M	*Rytiphlaea tinctoria* (Clemente) C. Agardh	17.0	17.7	19.3	12.3		11.0	17.9	21.6	22.4	15.4	16.4	10.9
	M	*Scinaia furcellata* (Turner) J. Agardh	-	-	-	-		13.2						
	M	*Sphaerococcus coronopifolius* Stackhouse	tr	13.5	tr	-		15.1	-	tr	tr	-	-	15.4

planata, did not demonstrate any activity against the test microorganisms.

The sensitivity of the test microorganisms was in the following decreasing order: *Bacillus cereus* (inhibited by 57% of tested taxa), *Staphylococcus aureus* (55%), *Candida albicans* (44%), *Bacillus subtilis* (43%), *E. coli* (22%) and *Pseudomonas aeruginosa* (21%).

Antimicrobial activity of solid extracts according to taxonomic group

Chlorophyceae had the lowest percentage of active taxa (44%, Fig. 1), with low bioactivity and a narrow spectrum of action that was generally limited to Gram-positive bacteria. Nevertheless, two taxa

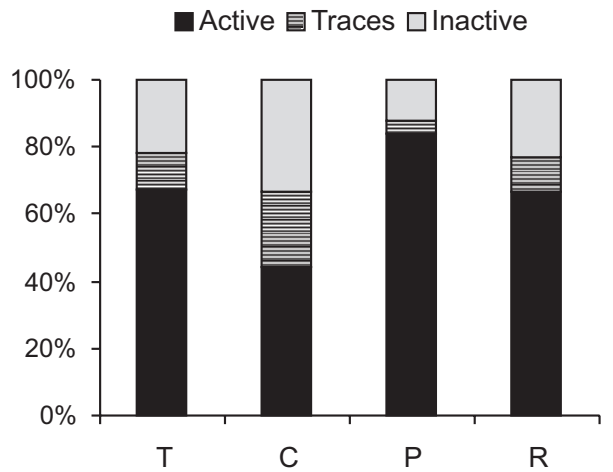


FIG. 1. – Percentage of inactive, trace active, active taxa (solid extracts). T, total taxa; C, Chlorophyceae; P, Phaeophyceae; R, Rhodophyceae.

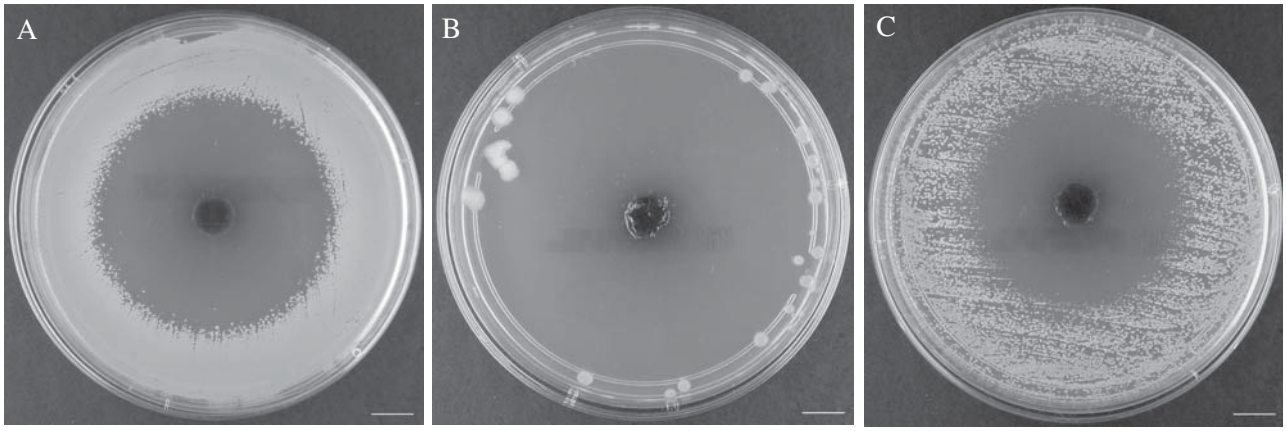


FIG. 2. – *Bonnemaisonia asparagoides* bioassays (solid extracts). A, against *Bacillus subtilis*; B, against *Bacillus cereus*; C, against *Candida albicans*. Scale bar = 10 mm.

were active against yeast, and *Palmophyllum crassum* was the only Chlorophyceae active against *Escherichia coli* (Tables 3-4). The largest number of active Chlorophyceae taxa belonged to the genus *Bryopsis*, with *Bryopsis muscosa* and *B. corymbosa* showing the highest values and the broadest spectrum respectively.

Phaeophyceae had the highest percentage of active taxa (84%, Fig. 1), although these did not exhibit the highest antimicrobial activity among the taxa tested. The action spectrum of Phaeophyceae was broader than that of Chlorophyceae, as some taxa were active against the yeast or the Gram-negative bacteria (Tables 1-4). The highest activities for Phaeophyceae were observed for taxa from the genera *Cystoseira*, *Dictyota* and *Taonia*. Although all of these genera were active against the Gram-positive bacteria, *Cystoseira* was also active against the Gram-negative bacteria whereas *Dictyota* and *Taonia* showed antifungal action. Finally, among all of the brown algae tested, *Hapalospongidion macrocarpum* showed the highest antimicrobial activity.

Rhodophyceae demonstrated the highest antimicrobial activity and the highest number of taxa active against Gram-negative bacteria and yeast, thus it was the group with the broadest spectrum of action. However, the percentage of active Rhodophyceae (67%) was lower than that of Phaeophyceae (Fig. 1). Within this group, Ceramiales and Gigartinales had noteworthy antimicrobial activity, and Bonnemaisoniales was the order that had the highest bioactivity (Fig. 2).

Antimicrobial activity of the Bonnemaisoniales

Both solid and methanolic extracts from *B. hamifera* and *B. asparagoides* of the genus *Bonnemaisonia* showed a broad spectrum and high bioactivity; although the values for inhibition obtained from methanolic extracts were lower than those from solid extracts (Fig. 3). The microorganisms most inhibited by these taxa were the Gram-positive bacteria: *Bacillus subtilis* for *B. hamifera*, and *Bacillus cereus* and *Staphylococcus aureus* for *B. asparagoides* (Tables 1, 2).

The solid and methanolic extracts of *Asparagopsis armata* and its tetrasporophyte *Falkenbergia rufolanosa* also exhibited a broad spectrum and high activity, although as above, methanolic extracts were

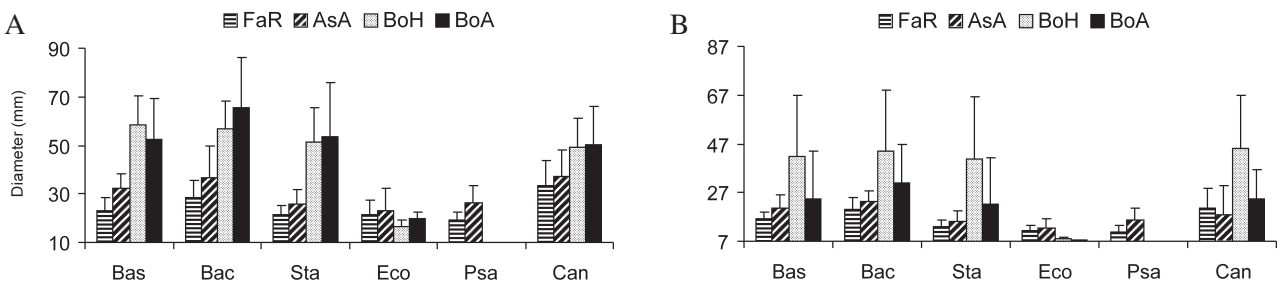


FIG. 3. – Average diameter (±SE) of inhibition halo of *Falkenbergia rufolanosa* (FaR), *Asparagopsis armata* (AsA), *Bonnemaisonia hamifera* (BoH), *Bonnemaisonia asparagoides* (BoA) against each test microorganism. BaS, *Bacillus subtilis*; Bac, *B. cereus*; Sta, *Staphylococcus aureus*; Eco, *Escherichia coli*; Psa, *Pseudomonas aeruginosa*; Can, *Candida albicans*. A, solid extracts; B, methanolic extracts.

somewhat less active than solid extracts (Fig. 3). For both generations the most inhibited microorganism was *Candida albicans* (Tables 1-4). The variation in antimicrobial activity between the algal generations was assessed by means of two-way ANOVA, using the test microorganisms and the generations as factors. Significant differences were observed for the generation ($F_{1,54}$= 8.89, p<0.01), *A. armata* was more active than *F. rufolanosa*, and for the microorganisms ($F_{5,54}$= 10.29, p<0.001), *C. albicans* was the most sensitive. The interaction term was not significant ($F_{5,54}$= 0.79, p>0.05), which indicates that the action spectrum did not vary with the generation. Three average groups were obtained in relation to the Tukey comparisons: one group comprised of *C. albicans* (the most inhibited microorganism), another group with all the Gram-positive bacteria and *E. coli* and finally one made up of the most resistant microorganism *P. aeruginosa*.

Out of all tested taxa, the Bonnemaisoniales had the maximum activity against all microorganisms: *A. armata* showed the maximum bioactivity against Gram-negative bacteria whereas the *Bonnemaisonia* species presented the maximum bioactivity against Gram-positive bacteria and yeast (Fig. 3).

Effects of sample preparation on antimicrobial activity

For each season, the antimicrobial activity between taxa and different algal treatment (fresh and lyophilised material) was compared by two-way ANOVA. No significant differences between treatments were found (winter: $F_{1,28}$= 1.23, p>0.05; spring: $F_{1,49}$= 1.85, p>0.05; summer: $F_{1,41}$= 2.30, p>0.05; autumn: $F_{1,35}$= 2.79, p>0.05), although the mean values of the lyophilised material were higher than those of the fresh material for all seasons except autumn. In contrast, significant differences among taxa were detected (winter: $F_{14,28}$= 11.78, p<0.001; spring: $F_{17,49}$= 7.60, p<0.001; summer: $F_{11,41}$= 26.03, p<0.001; autumn: $F_{15,35}$= 14.31, p<0.001). Furthermore, the interaction term was significant in all seasons except autumn (winter: $F_{14,28}$ = 4.52, p<0.05; spring: $F_{17,49}$= 11.5, p<0.001; summer: $F_{11,41}$= 2.64, p<0.05; autumn: $F_{15,35}$= 1.38, p>0.05), which indicates that effects of sample preparation on observed activities varied with taxa. Nevertheless, the bioactivity observed for lyophilised samples suggests that lyophilisation may provide better extraction of compounds.

For the order Bonnemaisoniales, the two treatments (fresh and lyophilised) were compared across taxa by two-way ANOVA for solid and for methanolic extracts. The differences between treatments were significant for methanolic extracts ($F_{1,173}$ = 55.91, p<0.001), but not for solid extracts ($F_{1,255}$ = 0.1, p>0.05). Significant differences between species were found for both solid ($F_{3,255}$= 39.62, p<0.001) and methanolic extracts ($F_{3,173}$= 13.5, p<0.001). The interaction term was also significant for both solid ($F_{3,255}$= 9.03, p<0.001) and methanolic extracts ($F_{3,173}$= 18.7, p<0.001). For *Asparagopsis armata*, lyophilisation was the most effective treatment for both types of extracts, whereas for its tetrasporophyte *Falkenbergia rufolanosa*, similar results were obtained from fresh and lyophilised material. For *Bonnemaisonia* species, the results varied according to the extracts: solid extracts from fresh samples had higher bioactivities than those from lyophilised samples, whereas the results were the opposite for the methanolic extracts.

Seasonal variation of antimicrobial activity

Autumn and spring were the seasons with the highest percentage of active taxa against at least one test microorganism (69% and 67% respectively), followed by winter (56%) and summer (50%) (Fig. 4). At a taxonomic group level, for Phaeophyceae and Rhodophyceae the highest percentage of active taxa was also in autumn; however, for Chlorophyceae it was in summer (Fig. 4). In contrast, bioactivity was not significantly different between seasons for any group (one-way ANOVA):

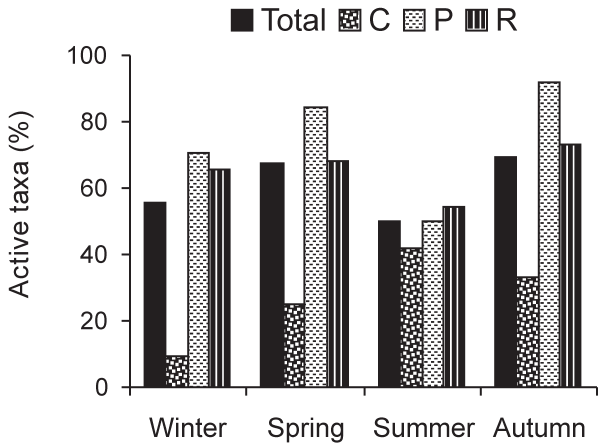


FIG. 4. – Seasonal variation of the percentage of active taxa (solid extracts). T, total taxa; C, Chlorophyceae; P, Phaeophyceae; R, Rhodophyceae.

TABLE 5. – Taxa for which a broader spectrum of action was observed compared to previous studies on non-Mediterranean and Mediterranean samples. Bas= *Bacillus subtilis*; Bac= *B. cereus*; Sta= *Staphylococcus aureus*; Eco= *Escherichia coli*; Psa= *Pseudomonas aeruginosa*; Can= *Candida albicans*.

non-Mediterranean samples	Mediterranean samples
Asparagopsis armata (*Bac*, *Psa*, *Can*)	*Bangia atropurpurea* (*Sta*, *Eco*)
Bangia atropurpurea (*Sta*, *Can*)	*Bryopsis corymbosa* (*Bas*, *Bac*, *Sta*, *Eco*, *Can*)
Callithamnion granulatum (*Bas*, *Bac*, *Sta*, *Can*)	*Codium vermilara* (*Sta*)
Ceramium ciliatum (*Bac*, *Sta*, *Can*)	*Falkenbergia rufolanosa* (*Bac*)
Cladophora rupestris (*Bas*, *Bac*, *Sta*)	*Jania rubens* (*Eco*)
Codium coralloides (*Bac*, *Sta*)	*Plocamium cartilagineum* (*Psa*, *Can*)
Codium vermilara (*Bas*, *Bac*)	*Wrangelia penicillata* (*Bas*, *Psa*)
Corallina elongata (*Bas*, *Bac*, *Eco*, *Psa*)	
Halopteris filicina (*Bas*, *Bac*, *Sta*)	
Hapalospongidion macrocarpum (*Bac*, *Can*)	
Jania rubens (*Bac*, *Eco*)	
Peyssonnelia rubra (*Bas*, *Bac*, *Sta*, *Eco*, *Psa*, *Can*)	
Plocamium cartilagineum (*Bac*)	
Sphaerococcus coronopifolius (*Bac*, *Sta*, *Eco*)	
Taonia atomaria (*Bac*, *Sta*, *Can*)	

Chlorophyceae ($F_{3, 33}$=2.51, p>0.05), Phaeophyceae ($F_{3, 162}$= 1.29, p>0.05), Rhodophyceae ($F_{3, 134}$= 2.55, p>0.05).

The bioactivity values of the taxa were evaluated by two-way ANOVA with taxonomic group and season as factors. No significant differences between seasons ($F_{3, 330}$= 0.68, p>0.05) were found, whilst they were found among taxonomic groups ($F_{2, 330}$= 9.63, p<0.05). The significant interaction term found ($F_{6, 330}$= 3.32, p<0.05) reflected the different variations in bioactivity of the taxonomic groups according to season: Chlorophyceae and Rhodophyceae demonstrated maximum activity during spring and autumn-winter respectively, and Phaeophyceae had constant activity throughout the year.

The greatest antimicrobial activity observed for the order Bonnemaisoniales against all test microorganisms was in winter for *Asparagopsis armata* and in spring for *Bonnemaisonia hamifera* (Tables 1-4). However, it must be taken into account that these taxa were only available during the winter-spring period due to their own seasonal dynamic. *Falkenbergia rufolanosa*, which was present all year-round, showed the greatest activity in autumn-winter.

Geographical variation of antimicrobial activity

Since the species *Asparagopsis armata, Falkenbergia rufolanosa* and *Bonnemaisonia asparagoides* were available from both Atlantic and Mediterranean coasts, their bioactivities were evaluated as a function of geographical zone by two-way ANOVA. The Mediterranean populations of *A. armata* and *F. rufolanosa* were significantly more active than those from the Atlantic ($F_{1, 20}$= 15.65, p<0.05; $F_{1, 24}$= 31.64, p<0.001 respectively), whereas the opposite trend was observed for *B. asparagoides* ($F_{1, 20}$= 31.44, p<0.001). Significant differences among the microorganisms assayed were found for the three taxa (*A. armata*: $F_{4, 20}$= 18.31, p<0.001; *F. rufolanosa*: $F_{5, 24}$= 20.31, p<0.001; *B. asparagoides*: $F_{4, 20}$= 55.21, p<0.001). The analysis of the interaction terms showed that the differences were always significant for the three Bonnemaisoniales (*A. armata*: $F_{4, 20}$= 4.58, p<0.05; *F. rufolanosa*: $F_{5, 24}$= 3.43, p<0.05; *B. asparagoides*: $F_{4, 20}$= 15.62, p<0.001), therefore the bioactivity of each species against each microorganism varied with their geographic location.

DISCUSSION AND CONCLUSIONS

The antimicrobial activities of several of the algae assayed differed from those previously reported. An extended spectrum of action was observed for sixteen taxa compared to studies performed with non-Mediterranean (Baker, 1984; Espeche *et al*., 1984; Reichelt and Borowitzka, 1984; Usmanghani *et al*., 1984; Hornsey and Hide, 1985; Ballantine *et al*., 1987; Navarro *et al*., 1990; Padmakumar and Ayyakkannu, 1997) as well as Mediterranean samples (Caccamese *et al*., 1980, 1981, 1985; Serarols *et al*., 1982; Moreau *et al*., 1984; Ballesteros *et al*., 1992) (Table 5). *Dictyopteris polypodioides*, *Halimeda tuna* and *Hypnea musciformis*, three taxa reported as active in other surveys of non-Mediterranean samples (Hornsey and Hide, 1974; Sreenivasa Rao and Parekh, 1981; Usmanghani *et*

al., 1984; Campos-Takaki *et al*., 1988; Navarro *et al*., 1990; Pérez *et al*., 1990; Padmakumar and Ayyakkannu, 1997) and Mediterranean samples (Ballesteros *et al*., 1992), were inactive in our study. Finally, *Padina pavonica*, reported as an inactive taxa in previous Indian (Padmakumar and Ayyakkannu, 1997) and Mediterranean studies (Khaleafa *et al*., 1975; Ballesteros *et al*., 1992), showed antibiotic activity for the first time in our work, namely against *Bacillus subtilis*, *B. cereus* and *Staphylococcus aureus*.

The aforementioned observations and the differences in bioactivity between Mediterranean and Atlantic specimens of the Bonnemaisoniales observed in this work suggest that the bioactivity of the same taxon can vary with the geographical sampling zone. Martí *et al*. (2004) pointed out that these differences could depend on ecological parameters such as irradiance and nutrients.

Our observations of the effects of the sample preparation method/algal treatment (i.e., fresh or lyophilised) on bioactivity revealed that lyophilisation generally allows greater compound extraction. However, as the differences with the fresh material were not significant, it was not possible to determine the most universally efficient treatment. Previous studies that compared different treatments are scarce and were carried out on only a few taxa. Campos-Takaki *et al*. (1988) and Padmini Sreenivasa Rao *et al*. (1986) compared fresh and dried algal material; their results also showed lower activity in extracts from fresh tissue than in extracts from dried material. This is probably due to a higher dilution of the bioactive metabolites in the fresh material because of the higher water content. Only Della Pietà *et al*. (1996) employed lyophilised material, among other materials, but they did not compare their results. Nevertheless, we can conclude from their results that, as in our study, the lyophilised material showed the highest values of antimicrobial activity.

As regards seasonal variation of bioactivity, for all of the taxa tested, autumn was the season with the highest percentage of active taxa against at least one test microorganism, followed by spring. These results agree with those obtained from Indian samples by Sreenivasa Rao and Parekh (1981), and Arun Kumar and Rengasamy (2000), and from Mediterranean samples by Martí *et al*. (2004). In contrast, in the study carried out by Hornsey and Hide (1974) using Atlantic samples, the most active season was spring. In relation to taxonomic groups, the season with the highest percentage of active taxa was autumn for Phaeophyceae and Rhodophyceae, and summer for Chlorophyceae. However, the results observed for Chlorophyceae did not concur with the constant production of active compounds by this group throughout the year reported by Padmakumar and Ayyakkannu (1997). Some authors have associated peak activity with physiological phenomena; however, the peaks observed in the present work could not be attributed to a single biological process. In some taxa, such as *Bonnemaisonia hamifera* and *Falkenbergia rufolanosa*, the peak of bioactivity observed in our study may be related to the reproductive or growth period, as reported by some authors (Hornsey and Hide, 1974; Moreau *et al*., 1984; Muñoz, 1992). However, in other cases, such as for *Osmundea truncata*, the peak of bioactivity (autumn-winter) occurred after the reproductive period (spring-summer). This finding was in agreement with that of Martí *et al*. (2004), who stated that peak bioactivity may be related to processes of ageing and allocation of resources from growth or reproduction to production of toxic compounds.

Of all the Iberian taxa screened, the highest antimicrobial activity was observed for Rhodophyceae, among which the order Bonnemaisoniales was the most active. Among the taxa tested in the present work, *Bonnemaisonia asparagoides* and *B. hamifera* had the highest degree of antimicrobial action against Gram-positive bacteria and yeast. Likewise, *A. armata, F. rufolanosa,* and its tetrasporophyte, which have been highlighted by other authors previously (Serarols *et al*., 1982; Cabañes *et al*., 1984; Pesando and Caram, 1984; Ballesteros *et al*., 1992), presented the highest activity against Gram-negative bacteria out of all the taxa tested in the present article. Comparing both generations, the gametophyte exhibited a broader spectrum and higher degree of antimicrobial action than its tetrasporophyte. Literature data about differences in antimicrobial activity between generations of the same species are scarce. In contrast to our results, Hornsey and Hide (1985) reported higher activity for the tetrasporophyte (*Trailliella intricata*) than for the gametophyte (*B. hamifera*).

Preservatives are described as substances that guarantee microbiologically safe products. After

perfumes, preservatives are the cosmetic ingredients that cause the most skin irritations, allergies and atopic reactions. Based on the results of this paper, we suggest that the taxa *B. asparagoides*, *B. hamifera* and *F. rufolanosa* may have potential as industrial preservatives, analogous to the currently used *A. armata* (Seguin *et al*., 1995; Algues et Mer 2002). Out of these, *F. rufolanosa* could be the most suitable taxon for use as a natural preservative due to its year-round presence and its easiness to culture. Nevertheless, due to the high bioactivity obtained against Gram-positive bacteria and yeast, for the two *Bonnemaisonia* species, and against Gram-negative bacteria for *F. rufolanosa*, we propose a mixture of their active extracts to obtain a preservative with a broad spectrum of action. Moreover, these taxa merit further studies both with the aim of isolating their active metabolites and for assaying culture methods for supplying algal biomass for industry. We suggest analysing other taxa of this order for which antimicrobial activity is unknown but probably notable, such as *B. clavata*, which has never been tested before because it is generally misidentified (Salvador *et al*., 2006) with *B. asparagoides*.

ACKNOWLEDGEMENTS

The authors thank Dr. J. Rull and Mr. A. Manghisi (University of Barcelona) for their help in the field work and their comments; Dr. F. Cinelli and his research group (University of Pisa) for their collaboration in the beginning of this study and their valuable suggestions; Dr. S. Senesi (University of Pisa) for providing the microorganism cultures; Dr. A. Marqués (University of Barcelona) for providing the *Pseudomonas aeruginosa* culture and for her helpful comments; Dr. C. Casares and Dr. J. A. Seoane Camba (University of Barcelona) for providing Atlantic samples; Mr. J. A. Navas, Mr. R. Bou, Mr. M. Mumbrú and Dr. C. Codina and his research group (University of Barcelona) for their technical assistance in obtaining methanolic extracts; the Drug Development Service at the University of Barcelona for lyophilisation of the samples; and Mrs A. Henderson and Mr. Gregory Y. Qushair for reviewing the English text.

This study was supported by the project NATURE (CRAFT-2001-70571).

REFERENCES

Algues et Mer SARL. – 2002. Method of obtaining an antibacterial and/or antifungal extract from the algae, Bonnemaisoniaceae. Inventor: J-Y. Moigne. *Off. gaz. U.S. Pat. Trademark Off. Pat*., invention patent, US 6346252B1.

Álvarez Benito, Ma. V. – 1990. *Manual de técnicas en microbiología clínica*. Asociación Española de Farmacéuticos Analistas, San Sebastian.

Arun Kumar, K. and R. Rengasamy. – 2000. Evaluation of antibacterial potential of seaweeds occurring along the coast of Tamil Nadu, India against the plant pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Dye. *Bot. Mar*., 43: 409-415.

Baker, J.T. – 1984. Seaweeds in pharmaceutical studies and applications. *Hydrobiologia*, 116/117: 29-40.

Ballantine, D.L., W.H. Gerwick, S.M. Velez, E. Alexander and P. Guevara. – 1987. Antibiotic activity of lipid-soluble extracts from Caribbean marine algae. *Hydrobiologia*, 151/152: 463-469.

Ballesteros, E., D. Martín and M.J. Uriz. – 1992. Biological activity of extracts from some Mediterranean macrophytes. *Bot. Mar*., 35: 481-485.

Bhosale, S.H., V.L. Nagle and T.G. Jagtap. – 2002. Antifouling potential of some marine organisms from India species of *Bacillus* and *Pseudomonas*. *Mar. Biotechnol*., 4: 111-118.

Burkholder, P.R., L.M. Burkholder and L.R. Almodóvar. – 1960. Antibiotic activity of some marine algae of Puerto Rico. *Bot. Mar*., 2: 149-156.

Cabañes, F.J., L. Abarca, M.A. Calvo and J.A. Seoane. – 1984. Determinación de la capacidad antifúngica de algas mediterráneas. *2ª Reunión conjunta de micología. Resúmenes de ponencias y comunicaciones*, p.171.

Caccamese, S., R. Azzolina, G. Furnari, M. Cormaci and S. Grasso. – 1980. Antimicrobial and antiviral activities of extracts from Mediterranean algae. *Bot. Mar*., 23: 285-288.

Caccamese, S., R. Azzolina, G. Furnari, M. Cormaci and S. Grasso. – 1981. Antimicrobial and antiviral activities of some marine algae from eastern Sicily. *Bot. Mar*., 24: 365-367.

Caccamese, S., R.M. Toscano, G. Furnari and M. Cormaci. – 1985. Antimicrobial activities of red and brown algae from southern Italy coast. *Bot. Mar*., 28: 505-507.

Campos-Takaki, G.M., M.B.S. Diu, M.L. Koening and E.C. Pereira. – 1988. Screening of marine algae from Brazilian northeastern coast for antimicrobial activity. *Bot. Mar*., 31: 375-377.

Centeno, P.O.R. and D.L. Ballantine. – 1999. Effects of culture conditions on production of antibiotically active metabolites by the marine alga *Spyridia filamentosa*. *J. Appl. Phycol*., 10: 453-460.

Chesters, C.G. and J.A. Stott. – 1956. Production of antibiotic substances by seaweeds. *Proc. Int. Seaweed Symp*., 2: 49-54.

Crasta, P.J., N.S. Raviraja and K.R. Sridhar. – 1997. Antimicrobial activity of some marine algae of southwest coast of India. *Indian J. Mar. Sci*., 26: 201-205.

Della Pietà, F., L. Panizzi, P.L. Cioni, F. Cinelli and I. Morelli. – 1996. Attività antimicrobica di estratti di *Caulerpa taxifolia* su batteri e micetti terrestri. In: M.A. Ribera, E. Ballesteros, C.F. Boudouresque, A. Gómez and V. Gravez (eds.). *Second International Workshop on* Caulerpa taxifolia, pp. 261-264. Publ. Univ. Barcelona, Barcelona.

Espeche, M.E., E.R. Fraile and A.M.S. Mayer. – 1984. Screening of Argentine marine algae for antimicrobial activity. *Hydrobiologia*, 116/117: 525-528.

Febles, C.I., A. Arias, A. Hardisson, A. Sierra López and M.C. Gil-Rodríguez. – 1995. Antimicrobial activity of extracts from some Canary species of Phaeophyta and Chlorophyta. *Phytotherapy Res*., 9: 385-387.

Fenical, W. and V.J. Paul. – 1984. Antibiotic and cytotoxic terpenoids from tropical green algae of the family Udoteaceae. *Hidrobiologia*, 116/117: 137-140.

Glombitza, K.W. – 1970. Antimicrobial constituents in algae. Quantitative determination of acrylic acid in sea-algae. *Planta Med*., 18: 210-221.

Harder, R. – 1917. Ernährungsphysiologische Untersuchungen an Cyanophyceen, hauptsächlich am endophytischen *Nostoc punctiforme*. *Z. Bot*., 9: 145.

Henríquez, P., R. Zemelman, M.A. Moncada and I.L. Benoit. –

1977. Propiedades antibióticas de algas marinas. *Bol. Soc. Biol. Concepción*, 11: 119-122.
Hodgson, L.M. – 1984. Antimicrobial and antineoplastic activity in some south Florida seaweeds. *Bot. Mar.*, 27: 387-390.
Horikawa, M., T. Noro and Y. Kamel. – 1999. *In vitro* anti-methicillin-resistant *Staphylococcus aureus*. Activity found in extracts of marine algae indigenous to the coastline of Japan. *J. Antibiot.*, 52: 186-189.
Hornsey, I.S. and D. Hide. – 1974. The production of antimicrobial compounds by British marine algae. I. Antibiotic-producing marine algae. *Br. Phycol. J.*, 9: 353-361.
Hornsey, I.S. and D. Hide. – 1985. The production of antimicrobial compounds by British marine algae. IV. Variation of antimicrobial activity with algal generation. *Br. Phycol. J.*, 20: 21-25.
Khaleafa, A.F., M.A.M. Kharboush, A. Metwalli, A.F. Mohsen and A. Serwi. – 1975. Antibiotic (fungicidal) action from extracts of some seaweeds. *Bot. Mar.*, 18: 163-165.
König, G.M., A.D. Wright, O. Stiche, C.K. Angerhofer and J.M. Pezzuto. – 1994. Biological activities of selected marine natural products. *Planta Med.*, 60: 532-537.
Ma, J.W. and W.C. Tang. – 1984. Screening for antimicrobial activities in marine algae from the Qingdao coast, China. *Hydrobiologia*, 116/117: 517-520.
Martí, R., M.J. Uriz and X. Turon. – 2004. Seasonal and spatial variation of species toxicity in Mediterranean seaweed communities: correlation to biotic and abiotic factors. *Mar. Ecol. Prog. Ser.*, 282: 73-85.
Melo, V.M.M., D.A. Medeiros, F.J.B. Rios, L.I.M. Castelar and A. de F.F.U. Carvalho. –1997. Antifungal properties of proteins (agglutinins) from the red alga *Hypnea musciformis* (Wulfen) Lamouroux. *Bot. Mar.*, 40: 281-284.
Moreau, J., D. Pesando, and B. Caram. – 1984. Antifungal and antibacterial screening of Dictyotales from the French Mediterranean coast. *Hydrobiologia*, 116/117: 521-524.
Muñoz, A. – 1992. *Drogas del mar. Sustancias biomédicas de algas marinas*. Servicio de Publicaciones e Intercambio Científico, Universidad de Santiago de Compostela, Santiago de Compostela.
Naqvi, S.W.A., S.Y. Kamat, L. Fernandes, C.V.G. Reddy. – 1980. Screening of some marine plants from the Indian coast for biological activity. *Bot. Mar.*, 24: 51-55.
Navarro, J.N., A. Rodríguez, N. Vásquez, A. Sánchez and M.A. Rivera. – 1990. Efectos antibióticos de las algas marinas de Puerto Rico. *Rev. Fac. Ocean. Pesq. Cs. Alimentarias*, 2: 165-173.
Padmakumar, K. and K. Ayyakkannu. – 1997. Seasonal variation of antibacterial and antifungal activities of the extracts of marine algae from southern coasts of India. *Bot. Mar.*, 40: 507-515.
Padmini Sreenivasa Rao, P., P. Sreenivasa Rao and S.M. Karmarkar. – 1986. Antibacterial substances from brown algae. II. Efficiency of solvents in the evaluation of antibacterial substances from *Sargassum johnstonii* Setchell et Gardner. *Bot. Mar.*, 29: 503-507.
Pérez, G.R.M., A.J.G. Avila, G.S. Pérez, C.A. Martínez and C.G. Martínez. – 1990. Antimicrobial activity of some American algae. *J. Ethnopharmacol.*, 29: 111-116.
Pesando, D. – 1990. Antibacterial and antifungal activities of marine algae. In: I. Akatsuka (ed.), *Introduction to Applied Phycology*, pp. 3-26. SPB Academic Publishing, The Hague.
Pesando, D. and B. Caram. – 1984. Screening of marine algae from the French Mediterranean coast for antibacterial and antifungal activity. *Bot. Mar.*, 27: 381-386.
Pratt, R., R.H. Mautner, G.M. Gardner, Y. Sha and F. Dufrenoy. – 1951. Report on the antibiotic activity of seaweed extracts. *J. Amer. Pharm. Assoc. Sci. Edn.*, 40: 575-579.
Reichelt, J.L. and M.A. Borowitzka. – 1984. Antimicrobial activity from marine algae: results of a large-scale screening programme. *Hydrobiologia*, 116/117: 158-168.
Rosell, K.G. and L.M. Srivastava. – 1987. Fatty acids as antimicrobial substances in brown algae. *Hydrobiologia*, 151/152: 471-475.
Salvador Soler, N., A. Gómez Garreta, and M.A. Ribera Siguan. – 2006. Mapas de distribución de algas marinas de la Península Ibérica y las islas Baleares. *Bonnemaisonia* (Bonnemaisoniaceae, Rhodophyta). *Bot. Complut.*, 30: 159-164.
Seguin, M.C., A. Franco, E. Feno, J.Y. Moigne and F. Bresdin. – 1995. Extraction de composés organiques de silicium biologiquement actifs d'origine algale. *Brevet* nº FR 2 732 022.
Serarols, M.D., M.C. Hernández and J.A. Seoane. – 1982. Sobre la actividad antibiótica de ciertas especies de algas del Mediterráneo. *Collect. Bot.* (*Barcelona*), 13: 919-927.
Sreenivasa Rao, P. and K.S. Parekh. – 1981. Antibacterial activity of Indian seaweed extracts. *Bot. Mar.*, 24: 577-582.
Usmanghani, K., M. Shameel, M. Sualeh, K.H. Khan and Z.A. Mahmood. – 1984. Antibacterial and antifungal activities of marine algae from Karachi seashore of Pakistan. *Fitoterapia*, 55: 73-77.
Welch, A.M. – 1962. Preliminary survey fungistatic properties of marine algae. *J. Bacteriol.*, 83: 97-99.

Received June 16, 2006. Accepted July 21, 2006.
Scient. ed.: I. Uriz.
Published online February 26, 2007.

Polysiphonia perforans Cormaci, G. Furnari, Pizzuto & Serio and Bonnemaisonia hamifera Hariot, new records for the Mediterranean Spanish coast.

José Silva, N. Salvador & J. Rull Lluch. 2009.

Proceedings of the 1st Mediterranean symposium on the conservation of the coralligenous and other calcareous bio-concretions: 255-258

José SILVA, N. SALVADOR, J. RULL LLUCH
Laboratori de Botànica. Facultat de Farmàcia. Universitat de Barcelona. Av. Joan XXIII s/n. 08028 - Barcelona. Spain.
e-mail: joseamsilva@yahoo.com

POLYSIPHONIA PERFORANS CORMACI, G. FURNARI, PIZZUTO & SERIO AND *BONNEMAISONIA HAMIFERA* HARIOT, NEW RECORDS FOR THE MEDITERRANEAN SPANISH COAST.

Abstract
While we were studying the community of Lithophyllum stictaeforme *throughout Catalonian coast, two scarcely cited species in the Mediterranean Sea were identified. We provide a description of these species, which represent new records for the Mediterranean coasts of the Iberian Peninsula.* Polysiphonia perforans *(Ceramiales, Rhodophyta) described by Cormaci et al. (1998) from Catania (Italy) and until now only found throughout Italian coasts, and* Bonnemaisonia hamifera *(Bonnemaisoniales, Rhodophyta) a native species of Japan widely distributed in the Atlantic and Pacific Oceans (Guiry & Guiry 2008), but only cited as Trailliella phase in the Mediterranean Sea (Cormaci et al., 2004). Although both generations were initially cited from the Mediterranean coasts by Conde et al. (1996) and Furnari et al. (2003), in the revision of the Mediterranean alien species by Cormaci et al. (2004) only the sporophytic generation was confirmed in the Mediterranean.*

Key-words: *Lithophyllum stictaeforme* community, coralligen, *Polysiphonia perforans, Bonnemaisonia hamifera,* new records.

Introduction

Studying some samples from the coralligenous community of *Lithophyllum stictaeforme* of the coasts of Catalonia (NE of the Iberian Peninsula) we identified two species that until now have been scarcely cited in the Mediterranean and represent new records for the Mediterranean coasts of the Iberian Peninsula: *Polysiphonia perforans* Cormaci, G. Furnari, Pizzuto & Serio and *Bonnemaisonia hamifera* Hariot. The first was described by Cormaci *et al*. (1998) from Catania (Italia) and until now its distribution had been restricted to the Italian coasts (Rindi *et al*., 2002; Cormaci *et al*., 2004). *Bonnemaisonia hamifera* is a Japanese species with a heteromorphic life history that is widespread in both Atlantic and Pacific oceans (Guiry & Guiry, 2008). Both generations of this species have been cited for the Mediterranean Sea (Conde *et al*., 1996; Furnari *et al*., 2003) but in the review of the Mediterranean alien species, Cormaci *et al*. (2004) indicate that only the sporophyte (Trailliella phase) occurs in the Mediterranean. We present a morphological and anatomical description of our specimens and we compare it with available information.

Materials and methods

The specimens were collected in two localities of the Catalonian coasts [Arenys de Mar (Barcelona) and Hospitalet de l'Infant (Tarragona)] in the coralligenous *Lithophyllum stictaeforme* community. Other collection details are given in the species accounts. Specimens were preserved in 4% formalin-seawater and deposited BCN-Phyc (the Herbarium of the Plant Biodiversity Documentation Centre of the University of Barcelona). Cells and other anatomical features were measured with an ocular micrometric and expressed as a variation interval. Some morphological and anatomical features were drawn with a *camera lucida* or photographed.

Results and discussion

Polysiphonia perforans Cornaci, G. Furnari, Pizzuto & Serio

Plant dark red in colour, consisting of prostrate and erect axes; axes polysiphonous, ecorticated and composed of an axial cell (6 µm in diameter) and four periaxial cells (Fig. 1e). Prostrate axes 27-30 µm diameter, with segments 1.2-1.6 times longer than broad (30-42 x 21-25 µm), occurring under the blades of *Peyssonnelia bornetii* Boudouresque & Denizot (Fig. 1b) and attached to it through dorsal rhizoids (Fig. 1d); rhizoids unicellular, ending in a digitate attachment disc, arising from the periaxial cells and remaining in open connection with them. Erect axes up to 3 mm high, scarcely branched, originated from the prostrated ones and crossing the blades of *Peyssonnelia*, often forming a more or less right

angle at the exit site in the upper face of the blade (Fig. 1c); erect axes 30-40 µm in diameter in its median part (26-30 µm at the apical zone and up to 50 µm at the base) with segments 0.8-0.9 times longer than broad (36-42 x 21-25 µm); segments of the upper part of the erect axes 0.5-0.6 times longer than broad (15-18 x 27-30 µm). Branching endogenous (Fig. 1f). Trichoblasts or scar-cells lacking. Tetrasporangia not completely developed, located in straight series in the upper part of the branches, one in every segment (Fig. 1a); other reproductive structures not seen.

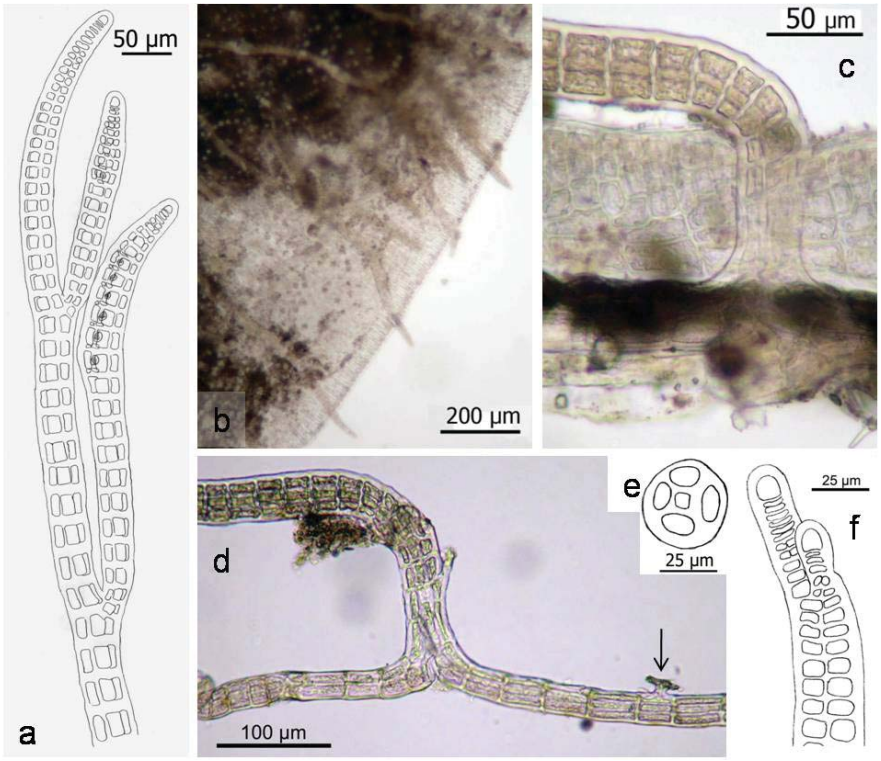


Fig. 1. *Polysiphonia perforans*. a: upper part of an erect axis with tetrasporangia; b: prostrate axes under *Peyssonnelia* blade; c: erect axis crossing the *Peyssonnelia* blade; d: prostrate axis with a dorsal rhizoid (arrow) and an erect axis; e: axis cross section; f: endogenous branching.

Habitat: Growing on *Peyssonnelia bornetii* in the *Lithophyllum stictaeforme* community, at 30 m depth.
Studied specimens: Wamgarrós (Arenys de Mar, Barcelona), 28/04/2006, BCN-Phyc 3233.
Distribution: Until now only known from the Italian coasts (Cormaci *et al*., 1998; Rindi *et al*., 2002).
Remarks: *Polysiphonia perforans* was described by Cormaci *et al*. (1998) on the basis of material from Catania (Italy) collected at 25 m depth on *Peyssonnelia rubra* (Greville) J. Agarth. At the same time, these authors also report this species from Tremiti Islands, in the Adriatic coast of Italy. Subsequently, *P. perforans* only has been cited from the Toscana, in the north western Italy (Rindi *et al*., 2002). Therefore, the specimens here described represent the third record of *P. perforans* for the Mediterranean Sea and a new species for the flora of the Iberian Peninsula. Our specimens agree very well with the description of *P. perforans* provided by Cormaci *et al*. (1998), although they are smaller (3 mm high in comparison with 5-10 mm in Italian specimens) and present shorter segments (0.8-1.6 times longer than broad in comparison with 1.5-2 times in Italian material).

Bonnemaisonia hamifera Hariot
Gametophyte erect, 2 cm high, consisting of a much branched main axis, 740-860 µm in diameter. Branching opposite and spirally arranged, with unequal development of the two components of each pair; the longer branch of 0.9-1.7 mm in length and 130-170 µm in diameter, with thorny cells at the apical zone (Fig. 2a); the shorter branch is a small protuberance, some of them replaced by an indeterminate axes, other modified to form hook branches (Fig. 2c) and one converted into a cystocarp. Axes of uniaxial structure; axial cells long, 265-305 x 20-40 µm (Fig. 2e), bearing two opposite periaxial cells; cortex composed of three cell-layers, the innermost with cells more or less isodiametric, 80-90 µm in diameter, and the outer with cells ovoid or polygonal in shape (10-20 µm in greater diameter) forming a continuous layer (Fig. 2b); vesicle cells of 11-20 µm in diameter scattered among outer cortical cells. A single cystocarp (490 x 400 µm) without carposporangia was found (Fig, 2d). Sporophyte not seen.
Habitat: Growing in the community of *Lithophyllum stictaeforme*, at 28 m depth.

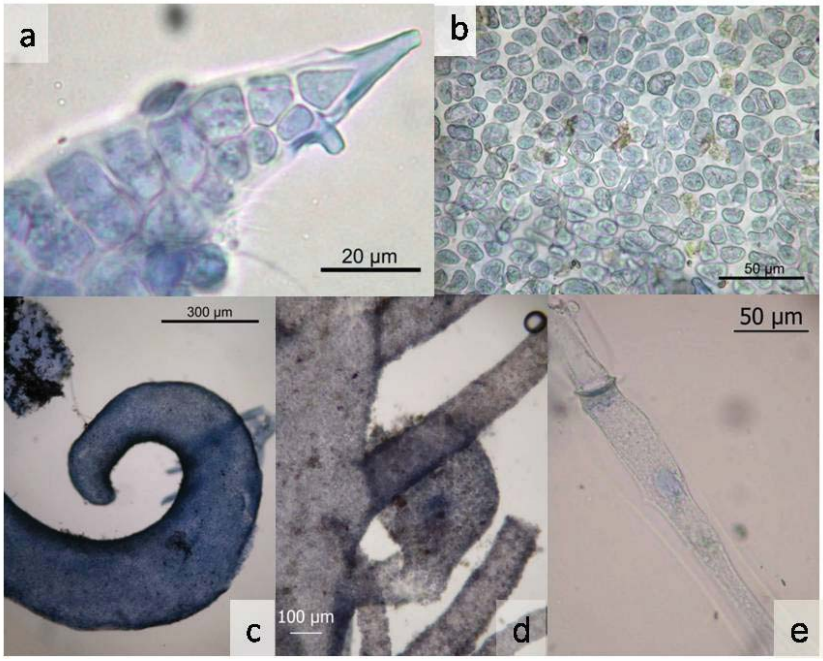


Fig. 2. *Bonnemaisonia hamifera*. a: apex of a long branch; b: outer cortical cells in surface view; c: hook branch; d: cystocarp; e: axial filament fragment.

Studied specimens: Hospitalet de L´Infant (Tarragona), 06/06/2007, BCN-Phyc 3234.

Distribution: Widespread in the Atlantic and Pacific oceans (Guiry & Guiry, 2008). In the Mediterranean Sea, only the presence of Trailliella phase has been confirmed until now (Cormaci *et al*., 2004).

Remarks: Our specimens are compatible with the available descriptions of *B.hamifera* gametophyte, showing thorny cells at the apical zone of the long branches and the typical hook branches. Likewise, other typical features of the genus *Bonnemaisonia* were also observed, such as the presence of vesicle cells and the endophyte *Colaconema asparagopsis* Chemin among the cortical cells. Despite the collected specimen had little dimensions, it presented a cystocarp, although without carposporangia. The wide distribution of *B. hamifera* contrasts with the absence of its gametophyte in the Mediterranean Sea, where up to date this species has been only reported as Trailliella phase (Cormaci *et al*., 2004). In fact, previously the gametophyte was cited from Sicily and the Italian Peninsula (Furnari *et al*., 2003) but in the review on the Mediterranean alien species of Cormaci *et al.* (2004) these cites were excluded. Comparing the gametophyte and sporophyte distributions of *B. hamifera*, McLachlan *et al*. (1969) and Breeman *et al*. (1988) noted that the Trailliella phase shows a wider distribution than the gametophyte. Breeman *et al*. (1988) pointed out that in *B. hamifera* both sporophyte and gametophyte show different temperature tolerance in relation to growth, survival and reproduction, being Trailliella the generation more resilient. This fact would explain its wider distribution.

Acknowledgments

This work was supported by the project CGL2004- 05556-C02-02/BOS of the Spanish Government.

Bibliography

BREEMAN A.M., MEULENHOFF E.J.S., GUIRY M.D. (1988) - Life history regulation and phenology of the red alga *Bonnemaisonia hamifera*. *Helgoländer Meeresuntersuchungen* 42: 535-551.

CONDE F., FLORES-MOYA A., SOTO J., ALTAMIRANO M., SÁNCHEZ A. (1996) - Check-list of Andalusia (S. Spain)seaweeds. III. Rhodophyceae. *Acta Bot. Malacitana*, 21: 7-33.

CORMACI M., FURNARI G., GIACCONE G., SERIO D. (2004) - Alien macrophytes in the Mediterranean Sea: a review. *Recent Research Developments in Environmental Biology*, 1: 153-202.

CORMACI M., FURNARI G., PIZZUTO F., SERIO D. (1998) - *Polysiphonia perforans* sp. nova (Ceramiales, Rhodophyta) from the Mediterranean Sea. *Plant Biosystems*, 132: 77-81.

FURNARI G., GIACCONE G., CORMACI M., ALONGI G., SERIO D. (2003) - Biodiveristà marina delle coste italiane: catalogo del macrofitobenthos. *Biol. Mar. Medit*., 10: 1-482.

GUIRY M.D., GUIRY G.M. (2008) - *AlgaeBase version 4.2*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; [07 August 2008].

MCLACHLAN J., CHEN L.C.-M., EDELSTEIN, T. (1969) - Distribution and life history of *Bonnemaisonia hamifera* Hariot. *Proceedings of the International Seaweed Symposium* 6: 245-249.

RINDI F., SARTONI G., CINELLI F. (2002) - A floristic account of the benthic marine algae of Tuscany (Western Mediterranean Sea). *Nova Hedwigia*, 74: 201-250.

Fucales (Phaeophyceae) from Spain characterized by large scale discontinuous nuclear DNA contents consistent with ancestral cryptopolyploidy.

Amelia Gómez Garreta, Mª Antonia Ribera Siguan, Noemi Salvador Soler, Jordi Rull Lluch & Donald F. Kapraun. 2009.

Phycologia. (En premsa)

Phycologia (2009) Volume 48 (6), 000–000 Published XXX XXX 2009

Fucales (Phaeophyceae) from Spain characterized by large-scale discontinuous nuclear DNA contents consistent with ancestral cryptopolyploidy

Amelia Gómez Garreta[1]*, Ma Antonia Ribera Siguan[1], Noemi Salvador Soler[1], Jordi Rull Lluch[1] and Donald F. Kapraun[2]

[1]*Laboratori de Botànica, Facultat de Farmàcia, Universitat de Barcelona. Av. Joan XXIII s/n, 08028 Barcelona, Spain*
[2]*Department of Biology & Marine Biology, University of North Carolina Wilmington, 601 South College Road, Wilmington, North Carolina 28403-3915, USA*

Garreta A.G., Siguan M.A.R., Soler N.S., Lluch J.R. and Kapraun D.F. 2009. Fucales (Phaeophyceae) from Spain characterized by large-scale discontinuous nuclear DNA contents consistent with ancestral cryptopolyploidy. *Phycologia* 48: 000–000. DOI: 10.2216/09-14.1

2

The DNA-localizing fluorochrome 4′,6-diamidino-2-phenylindole and chicken erythrocytes standard were used with image analysis and static microspectrophotometry to estimate nuclear DNA contents (I_f) in 19 species and varieties of Fucales from the Atlantic and Mediterranean coasts of Spain. Negligible differences were found between specimens fixed in Carnoy's solution (EtOH) and methanol-Carnoy's (methacarn). Present and previously published nuclear DNA content estimates expand our database to include 23 species and varieties representing nine genera with a 2C range of 0.4–0.8 pg in Sargassaceae and 1.1–2.2 pg in Fucaceae and Himanthaliaceae, excluding polyploid isolates. Intraplant variation was observed in most isolates and 8C nuclei were quantified in seven taxa and isolates. In 11 taxa, I_f levels in 2C male gamete nuclei were found to closely approximate 50% of 4C values in vegetative cells of mature plants, consistent with meiosis and a sexual life history in these haplobiontic algae. Availability of consensus higher-level phylogenetic trees for Fucales has opened the way for determining evolutionary trends in DNA amounts. The largest genome sizes were observed in cold-water species of Fucaceae. Both estimated genome sizes and published chromosome numbers for Fucales suggest a large-scale, discontinuous distribution of discrete values that can be explained in terms of ancestral cryptopolyploidy events.

Key Words: DNA C-values, Fucales, Nuclear genome size, Phaeophyceae

INTRODUCTION

In the last decade, DNA sequence data, especially from studies utilizing ribosomal (r)DNA internal transcribed spacer (Peters *et al.* 1997) and ribulose 1,5-bisphosphate carboxylase/oxygenase spacer sequences (Kraan & Guiry 2000), have shown that classic brown algal phylogenies based on a species sequence of simple/primitive to complex/advanced were more apparent than real (Rousseau & de Reviers 1999a; Phillips *et al.* 2008). A comprehensive phylogeny of the Phaeophyceae developed from DNA sequence data (*rbc*L, small-subunit and large-subunit rDNA) resolves several monophyletic early lineages, with the remaining brown algae forming two groups: Dictyotales and Sphacelariales, among others, and a crown group that includes the Fucales (Rousseau *et al.* 2001; Phillips *et al.* 2008).

The Fucales (Phaeophyceae), which includes about 40 genera (Clayton 1984), evolved and diversified in southern Australia and is now widely distributed throughout the world (Clayton 1988). The monotypic Notheiaceae is the most basal family in this order and the other fucalean families are divided over two well-supported groups (Saunders & Kraft 1995; Horiguchi & Yoshida 1998, Leclerc *et al.* 1998, Rousseau & de Reviers 1999b; Cho *et al.* 2006, Harvey & Goff 2006, Phillips *et al.* 2008): Group I is composed of Fucaceae, Himanthaliaceae, Hormosiraceae, Seirococcaceae, Durvillaeaceae, and the recently erected Bifurcariopsidaceae and Xiphophoraceae. Group II is composed of an expanded Sargassaceae that includes the Cystoseiraceae, which were previously treated as a distinct family.

Group I has a bipolar distribution, with the Fucaceae and Himanthaliaceae being restricted to the Northern Hemisphere and the other families restricted to the Southern Hemisphere (Clayton 1984, 1994). Molecular data support a large divergence time between these Northern and Southern Hemisphere taxa (Serrão *et al.* 1999). In group II, recent molecular studies indicate that Atlantic and Pacific genera *Cystoseira* and *Halidrys* are not monophyletic and include species that appear to have arrived at similar morphologies independently (Harvey & Goff 2006). The relationship of European populations of *Cystoseira* and *Halidrys* to their North American congeneric counterparts remains poorly understood. The Fucaceae are restricted to cold-temperate water environments, whereas the Sargassaceae have primarily tropical and warm-temperate distributions (Phillips & Fredericq 2000). Unfortunately, in group I, there are no published nucleotype data for any Southern Hemisphere taxa, and data for only a few species in the North Atlantic (Kapraun 2005). Published nucleotype data for the Sargassaceae (group II) are limited to three species of *Sargassum* and one of *Turbinaria* (Kapraun 2005). The cold-water genera of

* Corresponding author (ameliagomez@ub.edu).

0

Table 1. Nuclear DNA contents of Fucales taxa from Spain. Data standardized to the DNA level of chicken erythrocytes (RBC) = 2.4 pg. Empty cells refer to the same voucher specimen as in the cell(s) above (but separate measurements).

Taxon	Collection location Voucher number	Fixation/ cell type	No. slides	No. nuclei	2C	4C	8C	M[1]
Fucaceae								
Ascophyllym nodosum (Linnaeus) Le Jolis	A Coruña BCN-Phyc 5684	Et/♂	2	72	3.6 ± 0.5			MI
		Et/veg	1	21		6.8 ± 1.3		MI
		Me/veg	3	103		6.0 ± 0.8		MI
		Me/♂	1	9	3.5 ± 0.4			MI
Fucus ceranoides Linnaeus	Zumaya BCN-Phyc 2775	Et/♂	4	151	1.8 ± 0.3			MI
		Me/♂	4	121	1.8 ± 0.2			MI
		Et/♂	1	16	1.8 ± 0.3			MI
		Et/veg	1	37		3.5 ± 0.7		MI
		Et/veg	1	30			7.3 ± 0.8	MI
Fucus serratus Linnaeus	A Coruña BCN-Phyc 5673	Et/veg	4	107		3.7 ± 0.7		MI
		Me/veg	1	27			7.2 ± 1.0	MI
Fucus serratus	A Coruña BCN-Phyc 5674	Me/veg	3	114			6.7 ± 1.0	MI
Fucus spiralis Linnaeus	A Coruña BCN-Phyc 5675	Et/veg	1	5			5.8 ± 0.2	MI
		Et/♂	2	21	1.6 ± 0.2			MI
		Et/veg	2	205		3.0 ± 0.4		MI
		Me/veg	3	160		3.0 ± 0.5		MI
Fucus spiralis	A Coruña BCN-Phyc 5676	Me/veg	1	15		3.0 ± 0.3		MI
Fucus vesiculosus Linnaeus var. *vesiculosus*	A Coruña BCN-Phyc 5703	Et/♂	1	16	2.2 ± 0.2			MI
		Et/veg	1	12		4.3 ± 0.6		MI
		Et/veg	1	30			8.3 ± 1.5	MI
Fucus vesiculosus var. *compressus* Kjellman	A Coruña BCN-Phyc 5659	Et/♂	2	36	2.0 ± 0.2			MI
		Et/veg	2	93		4.2 ± 0.4		MI
		Et/veg	1	32			8.3 ± 0.9	MI
		Me/♂	2	51	2.0 ± 0.3			MI
		Me/veg	3	89		4.0 ± 0.4		MI
Pelvetia canaliculata (Linnaeus) Decaisne & Thuret	A Coruña BCN-Phyc 5662	Me/veg	3	128		2.9 ± 0.4		MI
Himanthaliaceae								
Himanthalia elongata (Linnaeus) S.F.Gray	A Coruña BCN-Phyc 5661	Et/veg	1	20		3.1 ± 0.7		MI
		Et/veg	1	38			6.5 ± 1.0	MI
		Me/veg	1	18		3.5 ± 0.6		MI
		Me/veg	1	57			6.8 ± 1.3	MI
Sargassaceae								
Bifurcaria bifurcata R. Ross	Zumaya BCN-Phyc 5660	Et/♂	4	158	0.7 ± 0.1			MI
		Et/veg				1.5 ± 0.3		
		Me/♂	3	160	0.8 ± 0.2			MI
Cystoseira baccata (S.G. Gmelin) P.C. Silva	Zumaya BCN-Phyc 5649	Et/♂	4	67	0.7 ± 0.2			MI
		Me/veg	3	91		1.3 ± 0.2		MI
Cystoseira brachycarpa var. *balearica* (Sauvageau) Giaccone	Cadaqués BCN-Phyc 2767	Et/♂	3	72	0.8 ± 0.1			MI
		Et/veg	3	61		1.4 ± 0.3		MI
		Me/veg	2	58		1.5 ± 0.3		MI
Cystoseira compressa (Esper) Gerloff & Nizamuddin	Calella BCN-Phyc 2768	Et/veg	2	109		1.5 ± 0.3		IA[7]
		Me/veg	2	38		1.6 ± 0.2		IA
		Et/veg	2	11		1.4 ± 0.2		MI
		Et/♂	3	23	0.6 ± 0.1			MI
Cystoseira foeniculacea (Linnaeus) Greville	Foz SANT-Algae19567	Me/veg	2	62		1.1 ± 0.2		MI
		Et/veg	2	50		1.1 ± 0.2		MI
		Et/veg	1	14			2.2 ± 0.2	MI
Cystoseira mediterranea Sauvageau	Calella BCN-Phyc 2770	Et/veg	2	62		2.5 ± 0.4		MI
		Me/veg	5	137		2.5 ± 0.3		MI

Table 1. Continued

Taxon	Collection location Voucher number	Fixation/ cell type	No. slides	No. nuclei	2C	4C	8C	M[1]
Cystoseira nodicaulis (Withering) M. Roberts	Candás SANT-Algae19553	Me/veg	4	97		5.2 ± 0.8		MI
		Et/veg	2	22		5.2 ± 1.0		MI
		Et/veg	4	166		4.6 ± 0.5		MI
Cystoseira tamariscifolia (Hudson) Papenfuss	Ondarreta BCN-Phyc 2771	Et/veg	4	190		1.7 ± 0.3		MI
		Et/♂	2	64	0.7 ± 0.1			MI
		Me/veg	3	127		1.5 ± 0.4		MI
		Me/♂	3	70	0.6 ± 0.1			MI
Cystoseira usneoides (Linnaeus) M. Roberts	Lorbé SANT-Algae19449	Et/♂	4	153	0.4 ± 0.1			MI
		Et/veg	2	35		0.8 ± 0.1		MI
Halidrys siliquosa (Linnaeus) Lyngbye	Zumaya BCN-Phyc 2772	Me/veg	5	251		1.3 ± 0.2		MI
Halidrys siliquosa	Zumaya BCN-Phyc 2773	Me/veg	2	50		1.2 ± 0.2		MI
Sargassum muticum (Yendo) Fensholt	A Coruña BCN-Phyc 5659	Et/veg	1	20		0.9 ± 0.2		IA

[1] M, DNA estimation method; Et, ethanol fixation; ♂, male gamete; MI, microspectrophotometry (UNCW); veg, vegetative cells; Me, methanol fixation; IA, image analysis (Barcelona).

Fucaceae (i.e. *Ascophyllum* and *Fucus*) have larger nuclear genomes than do the warmer-water genera of Sargassaceae (i.e. *Sargassum* and *Turbinaria*) (Peters *et al*. 2004; Kapraun 2005). However, there is no indication that genome size and habitat temperature are correlated as for other cold-temperate brown algae; both high and low genome sizes are reported (Peters *et al*. 2004).

The present investigation was initiated to provide nuclear DNA content estimates (I_f) for additional taxa of Fucales occurring on the Atlantic and Mediterranean coasts of Spain and to determine the extent of inter- and intraspecific nuclear DNA content variation, to correlate genome sizes with emerging patterns of evolution and phylogeny, to determine if DNA contents are diagnostic and represent synapomorphies and to corroborate an alternation of haploid and diploid nuclear DNA contents in gametes and adult plants, respectively.

MATERIAL AND METHODS

Source of specimens

In Spanish coasts, 19 taxa of Fucales (Fucaceae, Himanthaliaceae and Sargassaceae) were collected from the Mediterranean [Calella and Cadaqués (Girona)] and Atlantic [A Coruña, Lorbé (A Coruña), Foz (Lugo), Candás (Asturias), Ondarreta and Zumaya (Guipúzcoa)] (Table 1). All the specimens studied are held at the BCN-Phyc herbarium (Centre de Documentació de Biodiversitat Vegetal, University of Barcelona) and at the Sant-Algae herbarium (University of Santiago de Compostela) (Table 1).

Assignment of ploidy level

Assignment of estimated nuclear DNA contents to specific C-values in the present study is presumptive in that no karyological investigations were conducted on the algal samples used for nuclear DNA content estimates. Members of Fucales are characterized by a haplobiontic life history and macroscopic gametophytes are assumed to be diploid with 4C nuclei in replicated vegetative cells (Le Gall *et al*. 1993). Male gametangia undergo meiosis in the production of male gametes (sperm), which are assumed to have replicated haploid (2C) nuclear genomes.

Nuclear DNA content estimates

For each species, one or two specimens were studied and from each one, several samples were analyzed. To obtain vegetative cell values, we examined apical parts to avoid thick cell walls and external epiphytes. When we found fertile specimens we analyzed the sperm nuclei to obtain the minimum DNA values.

Algal material was fixed in Carnoy's solution (Kapraun 2005) and in methacarn (methanol-Carnoy) to avoid reported staining inhibition associated with intracellular phenolic compounds (Puchtler *et al*. 1970a, b). Samples were stored in 70% ethanol at 4°C, rehydrated in water and softened in 5% w/v EDTA (Goff & Coleman 1990) for 12–48 h. Algal specimens were transferred to coverslips treated with subbing solution and then air dried and stained with 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, MO 63178) as previously described (Goff & Coleman 1990; Kapraun & Nguyen 1994). Nuclear DNA contents were estimated from both microspectrophotometry and image analysis. The estimates on the basis of microspectrophotometry with DAPI followed procedures specified previously (Kapraun & Nguyen 1994; Kapraun 1994) using a protocol modified after Goff & Coleman (1990). Nuclear DNA content estimates on the basis of image analysis of DAPI-stained specimens followed a procedure modified from Kapraun & Dunwoody (2002) and Choi *et al*. (1994) using a cooled CCD Miramax RTE 782-Y high-performance digital camera placed on a Leica DMRB fluorescence microscope and analyzed using MetaMorph software (Molecular Devices, Toronto, ON, Canada). For a comprehensive

review of theory and practice of DNA quantification by densitometry, see Hardie *et al.* (2002).

Nuclear DNA contents of Fucales specimens were estimated by comparing their I_f values with those of chicken erythrocytes (RBCs; Kapraun 1994; Kapraun & Dunwoody 2002), which have a DNA content of 2.4 pg (Clowes *et al.*1983). DAPI binds by a nonintercalative mechanism to adenine- and thymine-rich regions of DNA that contain at least four A-T base pairs (Portugal & Waring 1988). Consequently, RBCs can be used directly as standards for determining amounts of DNA only when the A-T contents of both standard and experimental DNA are equivalent (Coleman *et al.* 1981). Chicken has a nuclear DNA base composition of 42–43 mol % G + C (Marmur & Doty 1962). Limited published data for the Phaeophyceae indicate values in the range of 38–43 mol % G + C (Olsen *et al.* 1987; Stam *et al.* 1988; Le Gall *et al.* 1993). Members of the Phaeophyceae investigated in this study are assumed to have a similar range of base pair compositions, and the linearity is accepted between DAPI-DNA binding in both RBC and algal samples (Le Gall *et al.* 1993). The number of algal nuclei examined in each sample is recorded in Table 1.

Nuclear DNA content data for these and other brown algae were incorporated into a database of plant genome sizes (Kapraun 2005; Gregory *et al.* 2007) hosted by the Royal Botanic Gardens Kew web page (http://www.rbgKew.org.uk/cval/homepage.html).

RESULTS

Nuclear genome size estimates (pg) ± SD were obtained for 19 Fucales taxa studied from the Atlantic and Mediterranean coasts of Spain. These data are recorded in Table 1. DAPI staining with the protocol modified from Goff & Coleman (1990) yielded reproducible, stable nuclear fluorescence with little apparent interference from autofluorescence, nonspecific binding or other cellular material. Algal material fixed in Carnoy's solution and methanol-Carnoy's (methacarn) resulted in similar I_f values. Estimated nuclear DNA content variation between fixation samples and within samples of the same fixation was typically less than 10% (Table 1).

Comparison of I_f values for species of the Fucales with I_f values for RBCs permitted estimation of nuclear DNA contents for taxa investigated in this study. Our results reveal that the members of the Fucaceae are characterized by discrete ranges of 2C nuclear genome sizes of 1.1–2.2 pg, excluding *Ascophyllum nodosum* (Linnaeus) Le Jolis (3.6 pg). *Himanthalia elongata* (Linnaeus) S.F. Gray, the only species of the family Himanthaliaceae, has a 2C value of 1.7 pg The members of the Sargassaceae are characterized by discrete ranges of 2C nuclear genome sizes of 0.4–0.8 pg excluding *Cystoseira mediterranea* Sauvageau (1.3 pg) and *Cystoseira nodicaulis* (Withering) M. Roberts (2.4 pg) (Table 2, Fig. 1).

In 11 of the Spanish Fucales investigated, 2C nuclear DNA levels in replicated haploid male gametes were found to closely approximate 50% of the 4C values in diploid vegetative gametophyte nuclei (Table 2). Polyploid nuclei were observed in most samples of Fucales investigated and 8C nuclei were quantified in vegetative cells of *H. elongata, Cystoseira foeniculacea* (Linnaeus) Greville, *Fucus ceranoides* Linnaeus, *Fucus serratus* Linnaeus, *Fucus spiralis* Linnaeus, *Fucus vesiculosus* Linnaeus var. *vesiculosus* and *F. vesiculosus* var. *compressus* Kjellman (Table 1).

Table 2. Mean values of nuclear DNA content estimates (pg) for Spanish Fucales and some previous published data.[1]

Taxon	2C (male gametes)	2C (50% of 4C)	4C (vegetative cells)
Fucaceae			
Ascophyllum nodosum	3.6	3.2	6.4
Ascophyllum nodosum[1]	1.7	1.7	3.3
Fucus ceranoides	1.8	1.8	3.5
Fucus serratus	n.d.[2]	1.9	3.7
Fucus spiralis	1.6	1.5	3.0
Fucus vesiculosus var. vesiculosus[1]	1.1	1.1	2.2
Fucus vesiculosus var. *vesiculosus*	2.2	2.2	4.3
Fucus vesiculosus var. *compressus*	2.0	2.1	4.1
Pelvetia canaliculata	n.d.	1.5	2.9
Himanthaliaceae			
Himanthalia elongata	n.d.	1.8	3.3
Sargassaceae			
Bifurcaria bifurcata	0.8	0.8	1.5
Cystoseira baccata	0.7	0.7	1.3
Cystoseira brachycarpa var. *balearica*	0.8	0.8	1.5
Cystoseira compressa	0.6	0.8	1.5
Cystoseira foeniculacea	n.d.	0.6	1.1
Cystoseira mediterranea	n.d.	1.3	2.5
Cystoseira nodicaulis	n.d.	2.5	5.0
Cystoseira tamariscifolia	0.7	0.8	1.6
Cystoseira usneoides	0.4	0.4	0.8
Halidrys siliquosa	n.d.	0.7	1.3
Sargassum echinocarpum[1]	n.d.	0.7	1.3
Sargassum filipendula[1]	n.d.	0.4	0.8
Sargassum fluitans[1]	n.d.	0.4	0.8
Sargassum muticum	n.d.	0.5	0.9
Turbinaria turbinata[1]	n.d.	0.4	0.8

[1] Data from non-Spanish coasts (Kapraun 2005).
[2] n.d., not determined.

DISCUSSION

EtOH vs methanol

Brown algae are generally polysaccharide and polyphenol rich, making DNA extraction and quantification problematic (Lewis *et al.* 1993; Phillips *et al.* 2001). Methacarn fixative (Puchtler *et al.* 1970a, b) substitution for Carnoy's is recommended to enhance DNA-localizing fluorochrome performance. In the present study, similar DNA content estimates were obtained from samples following both fixation protocols.

DNA and phylogeny

Estimated nuclear genome sizes for Spanish specimens expand our database for Fucales to include 23 taxa (Peters

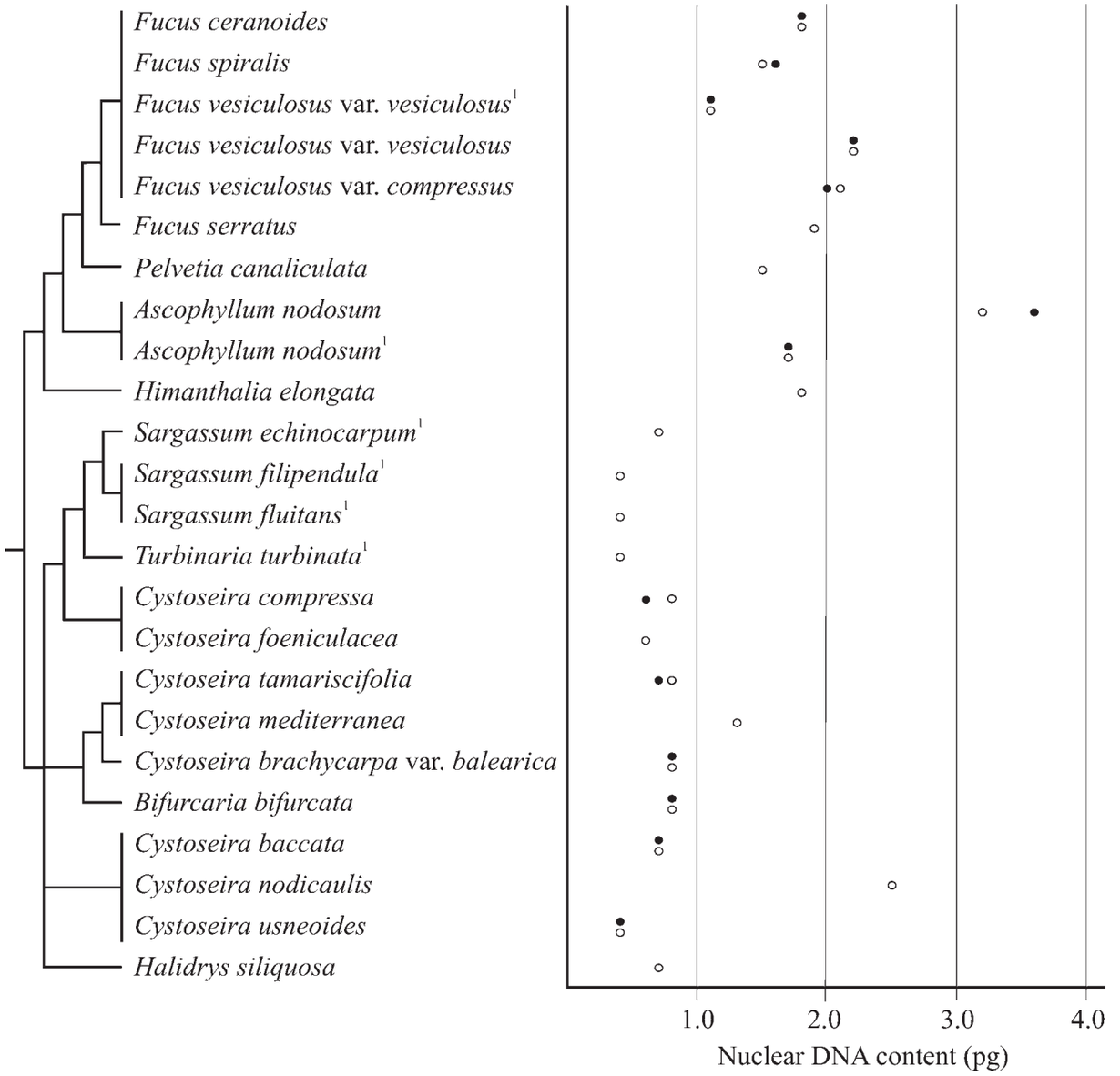


Fig. 1. 2C nuclear DNA contents superimposed on a consensus molecular phylogenetic tree for Fucales on the basis of supported clades in published phylogenies (Rousseau *et al.* 1997; Rousseau & de Reviers 1999b; Serrão *et al.* 1999; Phillips & Fredericq 2000; Stiger *et al*. 2000, 2003; Cho *et al*. 2006; Coyer *et al*. 2006b, Harvey & Goff 2006; Susini 2006; Phillips *et al*. 2008). (•) 2C nuclear DNA contents estimated from I_f values of replicated haploid male gametes; (○) 2C nuclear DNA contents extrapolated from 50% of the 4C values in diploid vegetative nuclei.

et al. 2004; Kapraun 2005). Members of the Sargassaceae, Fucaceae, and Himanthaliaceae are characterized by discrete ranges of 2C nuclear genome sizes of 0.4–0.8 pg, 1.1–2.2 pg, and 1.7 pg respectively (Table 2). The sole exceptions to this generalization, *C. mediterranea, C. nodicaulis* and *A. nodosum,* almost certainly represent polyploid isolates. These results are consistent with recent taxonomic delineations that include the Fucaceae and Himanthaliaceae in group I and recognize the Sargassaceae a distinct taxon in group II. Specifically, the ranges of nuclear genome sizes that characterize these families (Fig. 1) are consistent with consensus molecular phylogenetic trees for Fucales that support the monophyly of these families (Phillips *et al*. 2008) and suggest that DNA contents can be indicative for these families (Kapraun 2005).

It has been previously noted that brown algae including the Fucales that are characterized by oogamy and large female gametes have the largest nuclear genomes (Kapraun 2005). It is tempting to speculate that in the Fucaceae and Himanthaliaceae, extreme cold tolerance is enhanced by an increased genome load (Kapraun 2005). It is recognized that although nuclear genome size is highly correlated with many cellular and ecological parameters, 'correlation' and

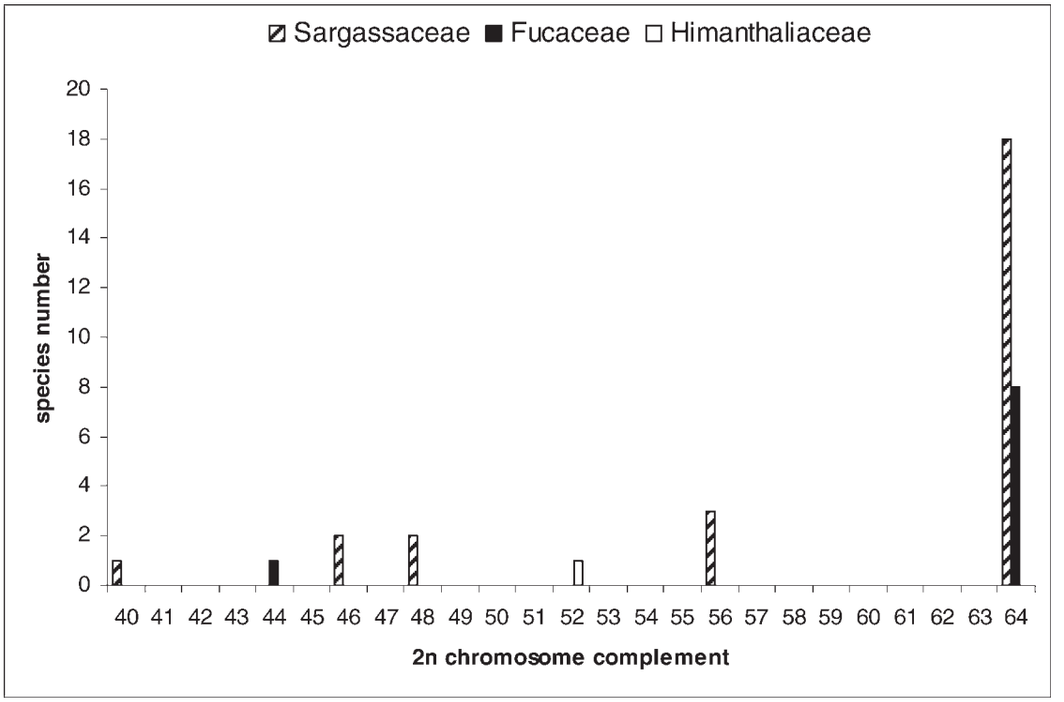


Fig. 2. Diploid chromosome numbers reported in species of Fucaceae, Himanthaliaceae, Sargassaceae (Lewis 1996).

'causation' are far from interchangeable (Gregory 2005). The many complex causal factors behind our observations in Fucales remain obscure.

Intraplant DNA variation

Intraplant nuclear DNA content variation from 2C to 16C has been documented previously in vegetative cells of other brown algae (Laminariales) (Gall *et al.* 1996; Garbary & Clarke 2002). In our study, intraplant variations from 2C to 8C were observed in five taxa of group I (*F. ceranoides*, *F. serratus*, *F. vesiculosus* var. *vesiculosus*, *F. vesiculosus* var. *compressus* and *H. elongata*) and in only one of group II (*C. foeniculacea*), a fact that corroborates the separation of both groups (Table 1). Higher nuclear DNA levels typically correlate with changes in cell size. This relationship is well documented in both green (Kapraun & Nguyen 1994; Kapraun & Buratti 1998) and red algae (Kapraun & Dunwoody 2002; Kapraun 2005), with nuclear DNA variation of over two orders of magnitude within the same thallus reported in the latter (Goff & Coleman 1990). In contrast, elevated nuclear DNA levels in brown algae show little relationship to cell size, apparently because cells retain a relatively constant cytoplasmic volume by changing their vacuolar volume (Garbary & Clarke 2002).

DNA and life history

Microspectrophotometry has been used previously to demonstrate life cycle-associated DNA content variation in *Fucus distichus* Linnaeus (Motomura 1995). In the present study, 2C nuclear DNA levels in replicated haploid male gametes for 11 taxa were found to closely approximate 50% of the 4C values in diploid vegetative gametophyte nuclei (Table 1), consistent with a haplobiontic life history and gametic meiosis (Bell 1997).

Chromosome number and DNA content

Reported $2n$ chromosome numbers in the Fucales range from 4 to 64, but the lower numbers are primarily from the early literature and almost certainly are incorrect (Lewis 1996). The most common chromosome complement in the Fucales is $2n = 64$ (Fig. 2), which could represent $4\times$ of a basic ancestral number of $n = 8$ (Cole 1967; Lewis 1996). Chromosome complements for these species in Spain are unknown, but Fucales are generally characterized by conservation of chromosome numbers. Previous karyological studies (Lewis 1996) include data for eight of the species included in the present investigation. These species, representing Fucaceae and Himanthaliaceae as well as Sargassaceae, share a similar small range of chromosome complements ($2n = c.$ 55–64) but a substantial range of 1.3–6.9 pg 4C (diploid) nuclear DNA contents (Fig. 3).

The most parsimonious explanation for this large-scale, discontinuous variation in discrete DNA contents is to assume that the common ancestor was characterized by a smaller chromosome complement, possibly $2n = 16$, derived from a basic number of $n = 8$ (Lewis 1996), and a haploid nuclear genome size of about 0.6 pg. This value was extrapolated from the smallest unreplicated postmeiotic gamete genome size in extant species. The derived polyploid chromosome complement was conserved in the three families studied. The Sargassaceae are characterized by only modest nuclear genome size increase. By contrast, the evolution and radiation of the Fucaceae was accompa-

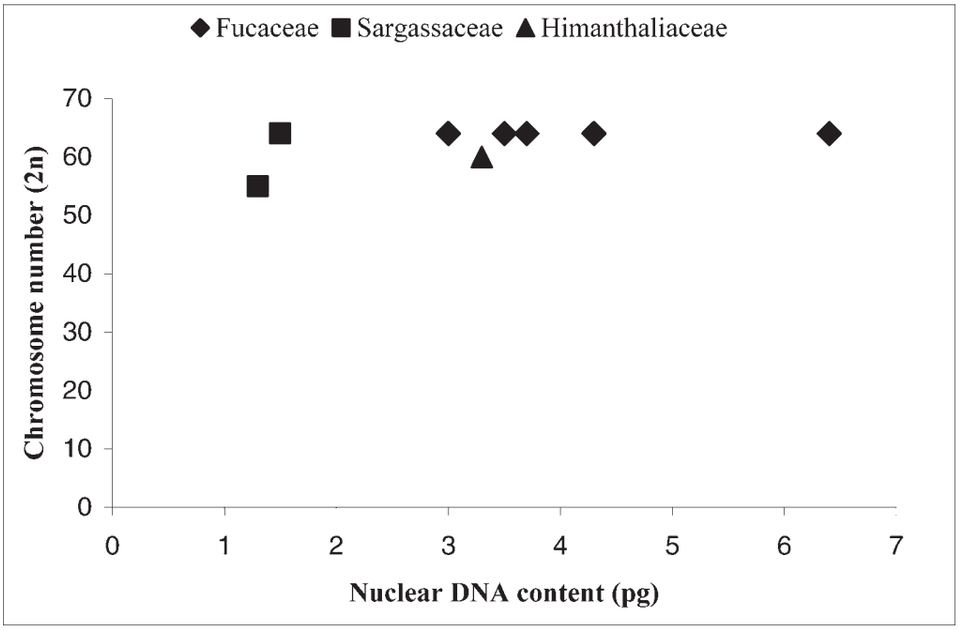


Fig. 3. Comparison of 4C nuclear DNA contents from the present study and Kapraun (2005), and 2*n* chromosome complements (Cole 1967; Lewis 1996) for eight species of Fucales.

nied by large-scale nuclear genome size increase without apparent increase in chromosome numbers (Fig. 3).

The poor correlation between chromosome number and DNA contents can be attributed to 'cryptoploidy' indicating large-scale accumulation of redundant DNA (Sparrow & Nauman 1973) that results in nuclear DNA content magnification without chromosome number increase (Kapraun & Martin 1987; Kapraun *et al*. 1988; Kapraun 1993). Such organisms in which DNA increases in approximate multiples of all or part of the basic genome are considered to be the genetic equivalent of autopolyploids (Stebbins 1971). For contemporary reviews of polyploidy effects on genomic plasticity and phenotypic variation in plant systems see Chen (2007) and Leitch & Leitch (2008).

The many complex causal factors behind large-scale duplications have been discussed by some authors (Wenzel & Hemleben 1982; Pichersky 1990; Bennetzen 2002). Although it is not understood how these processes influence patterns of evolution, it has been suggested that redundant genomes are not necessarily useless (Bennetzen & Kellogg 1997; Bennetzen 2002). Specifically, one of the genome copies is freed from functional constraints and is more amenable to mutations that could result in novel genes with new and fortuitous functions (Lynch & Conery 2000). For example, pronounced genome size increase in the Fucaceae could be associated with enhanced cold tolerance.

Intraspecific polyploid races

Polyploidy has been widely reported in Phaeophyceae (Lewis 1996), especially in the genera *Laminaria* (Lewis *et al*. 1993) and *Fucus* (Coyer *et al.* 2006a). Members of the Ectocarpales are notorious for development of polyploid populations, with 'haploid, diploid and tetraploid plants connected with each other in a complex system of meiosis, heteroblasty and spontaneous increase in chromosome numbers' (Müller 1967, 1970). Chromosome numbers have confirmed polyploidy in the Fucales. For example, tetraploid chromosome numbers were reported in populations of *Sargassum confusum* C. Agardh (Yabu & Yasui 1983) and *Sargassum horneri* (Turner) C. Agardh (Lewis 1996).

In the present study, elevated nuclear DNA contents in *C. nodicaulis* (2C = 2.4 pg) and *C. mediterranea* (2C = 1.2 pg) are approximately 3–6× and 1.5–3×, respectively, the 2C values for other Sargassaceae (Table 2). DNA content values for Spanish isolates of *A. nodosum* (2C = 3.2–3.6 pg) are double a previous 2C estimate of 1.7 pg for this species in New England (Kapraun 2005). In the same way, Spanish isolates of *F. vesiculosus* var. *vesiculosus* of 2C = 2.2 pg are double a previous estimate of 2C = 1.1 pg for an isolate of *F. vesiculosus* from New England (Kapraun 2005) (Table 2). It is probable that Spanish isolates with elevated nuclear DNA contents are true polyploids with an even multiple number (2×) of their basic chromosome complements (Kapraun & Buratti 1998; Kapraun 2005, Kapraun *et al.* 2007). These conclusions must be considered tentative, and assignment of ploidy status for these species will require determination of their chromosome complements.

ACKNOWLEDGEMENTS

We are grateful to Ignacio Barbara, Coro Casares and Angela Noguerol for providing material from the Atlantic, Marius Mumbrú for his technical support in the laboratory, Raquel García for her assistance in the image analysis and Joan Vallès for his comments. This study was supported by project CGL2005-02330/BOS of the Spanish Government.

REFERENCES

BELL G. 1997. The evolution of the life cycle of brown seaweeds. *Biological Journal of the Linnean Society* 60: 21–38.

BENNETZEN J.L. 2002. Mechanisms and rates of genome expansion and contraction in flowering plants. *Genetica* 115: 29–36.

BENNETZEN J.L. & KELLOGG E.A. 1997. Do plants have a one-way ticket to genomic obesity? *The Plant Cell* 9: 1509–1514.

CHEN Z.J. 2007. Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. *Annual Review in Plant Biology* 58: 377–406.

CHO G.Y., ROUSSEAU F., DE REVIERS B. & BOO S.M. 2006. Phylogenetic relationships within the Fucales (Phaeophyceae) assessed by the photosystem I coding *psa*A sequences. *Phycologia* 45: 512–519.

CHOI H.G., LEE Y.K. & LEE I.K. 1994. Measurement of DAPI-stained DNA in *Dasysiphonia chejuensis* Lee et West (Rhodophyta) by a Video Interfaced Digital Image processor. *The Korean Journal of Phycology* 9: 21–28.

CLAYTON M.N. 1984. Evolution of the Phaeophyta with particular reference to the Fucales. In: *Progress in phycological research,* vol. 3 (Ed. by F.E. Round & D.J. Chapman), pp. 11–46. Biopress, Bristol, UK.

CLAYTON M.N. 1988. Evolution and life histories of brown algae. *Botanica Marina* 31: 379–387.

CLAYTON M.N. 1994. Circumscription and phylogenetic relationships of the Southern Hemisphere family Seirococcaceae. *Botanica Marina* 37: 213–220.

CLOWES A.W., REIDY M.A. & CLOWES M.M. 1983. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in absence of endothelium. *Laboratory Investigations* 49: 327–333.

COLE K. 1967. Chromosome numbers in the Phaeophyceae. *Canadian Journal of Genetics and Cytology* 9: 519–530.

COLEMAN A.W., MAGUIRE M.J. & COLEMAN J.R. 1981. Mithramycin- and 4′-6 diamidino-2-phenolindole (DAPI)-staining for fluorescence microspectrophotometric measurement of DNA in nuclei, plastids, and virus particles. *Journal of Histochemistry and Cytochemistry* 29: 959–968.

COYER J.A., HOARAU G., PEARSON G.A., SERRAO E.A., STAM W.T. & OLSEN J.L. 2006a. Convergent adaptation to a marginal habitat by homoploid hybrids and polyploidy ecads in the seaweed genus *Fucus*. *Biology Letters* 2: 405–408.

COYER J.A., HOARAU G., OUDOT-LE SECQ M.-P., STAM W.T. & OLSEN J.L. 2006b. A mtDNA-based phylogeny of the brown algal genus *Fucus* (Heterokontophyta; Phaeophyta). *Molecular Systematics and Evolution* 39: 209–222.

GALL E.A., ASENSI A., MARIE D. & KLOAREG B. 1996. Parthenogenesis and apospory in the Laminariales: a flow cytometry analysis. *European Journal of Phycology* 31: 369–380.

GARBARY D.J. & CLARKE B. 2002. Intraplant variation in nuclear DNA content in *Laminaria saccharina* and *Alaria esculenta* (Phaeophyceae). *Botanica Marina* 45: 211–216.

GOFF L.J. & COLEMAN A.W. 1990. DNA: microspectrofluorometric studies. In: *Biology of the red algae* (Ed. by K.M. Cole & R.G. Sheath), pp. 43–72. Cambridge University Press, New York.

GREGORY T.R. 2005. The C-value enigma in plants and animals: a review of parallels and an appeal for partnership. *Annals of Botany* 95: 133–146.

GREGORY T.R., NICOL J.A., TAMM H., KULLMAN B., KULLMAN K., LEITCH I.J., MURRAY B.G., KAPRAUN D.F., GREILHUBER J. & BENNETT M.D. 2007. Eukaryotic genome size databases. *Nucleic Acids Research* 35: D 332–338.

HARDIE D.C., GREGORY T.R. & HEBERT P.D.N. 2002. From pixels to picograms: a beginners' guide to genome quantification by Feulgen image analysis densitometry. *Journal of Histochemistry and Cytochemistry* 50: 735–749.

HARVEY J.B.J. & GOFF L.J. 2006. A reassessment of species boundaries in *Cystoseira* and *Halidrys* (Phaeophyceae, Fucales) along the North American West Coast. *Journal of Phycology* 42: 707–720.

HORIGUCHI T. & YOSHIDA T. 1998. The phylogenetic affinities of *Myagropsis myagroides* (Fucales, Phaeohyceae) as determined from 18S rDNA sequences. *Phycologia* 37: 237–245.

KAPRAUN D.F. 1993. Karyology of marine green algae. *Phycologia* 32: 1–21.

KAPRAUN D.F. 1994. Cytophotometric estimation of nuclear DNA contents in thirteen species of the Caulerpales (Chlorophyta). *Cryptogamic Botany* 4: 410–418.

KAPRAUN D.F. 2005. Nuclear DNA content estimates in multicellular green, red and brown algae: phylogenetic considerations. *Annals of Botany* 95: 7–44.

KAPRAUN D.F. & BURATTI J.R. 1998. Evolution of genome size in the Dasycladales (Chlorophyta) as detemined by DAPI cytometry. *Phycologia* 37: 176–183.

KAPRAUN D.F. & DUNWOODY J.T. 2002. Relationship of nuclear genome size to some reproductive cell parameters in the Florideophycidae (Rhodophyta). *Phycologia* 41: 507–516.

KAPRAUN D.F. & MARTIN D.J. 1987. Karyological studies of three species of *Codium* (Codiales, Chlorophyta) from coastal North Carolina. *Phycologia* 26: 228–234.

KAPRAUN D.F. & NGUYEN M.N. 1994. Karyology, nuclear DNA quantification and nucleus cytoplasmic domain variations in some nultinucleate green algae. *Phycologia* 33: 42–52.

KAPRAUN D.F., GARGIULO G.M. & TRIPODI G. 1988. Nuclear DNA and karyotype variation in species of *Codium* (Codiales, Chlorophyta) from the North Atlantic. *Phycologia* 27: 273–282.

KAPRAUN D.F., BRALY K. & FRESHWATER D.W. 2007. Nuclear DNA content variation in the freshwater red algal orders Batrachospermales and Thoreales (Florideophyceae, Nemaliophycidae). *Phycologia* 46: 54–62.

KRAAN S. & GUIRY M.D. 2000. Molecular and morphological character inheritance in hybrids of *Alaria esculenta* and *A. praelonga* (Alariaceae, Phaeophyceae). *Phycologia* 39: 554–559.

LECLERC M.C., BARRIEL V., LECOINTRE G. & DE REVIERS B. 1998. Low divergence in rDNA ITS sequences among five species of *Fucus* (Phaeophyceae) suggests a very recent radiation. *Journal of Molecular Evolution* 46: 115–120.

LE GALL Y., BROWN S., MARIE D., MEJJAD M. & KLOAREG B. 1993. Quantification of nuclear DNA and G-C content in marine macroalgae by flow cytometry of isolated nuclei. *Protoplasma* 173: 123–132.

LEITCH A.R. & LEITCH I.J. 2008. Genomic plasticity and the diversity of polyploidy plants. *Science* 320: 481–483.

LEWIS R.J. 1996. Chromosomes of the brown algae. *Phycologia* 35: 19–40.

LEWIS R.J., JIANG B.Y., NEUSHUL M. & FEI X.G. 1993. Haploid parthenogenetic sporophytes of *Laminaria japonica* (Phaeophyceae). *Journal of Phycology* 29: 363–369.

LYNCH M. & CONERY J.S. 2000. The evolutionary fate and consequences of duplicate genes. *Science* 290: 1151–1155.

MARMUR J. & DOTY P. 1962. Determination of the base composition of desoxyribonucleic acid from its thermal denaturation temperature. *Journal of Molecular Biology* 5: 109–118.

MOTOMURA T. 1995. Premature chromosome condensation of the karyogamy-blocked sperm pronucleus in the fertilization of *Fucus distichus* (Fucales, Phaeophyceae). *Journal of Phycology* 31: 108–113.

MÜLLER D.G. 1967. Generationswechsel, Kernphasenwechsel und Sexualität der Braunalge *Ectocarpus siliculosus* im Kulturversuch. *Planta* 75: 39–54.

MÜLLER D.G. 1970. Diploide, heterozygote Gametophyten beider Braunalgae *Ectocarpus siliculosus*. *Naturwissenschaften* 57: 357–358.

OLSEN J.L., STAM W.T., BOT P.V.M. & VAN DEN HOEK C. 1987. ScDNA-DNA hybridization studies in Pacific and Caribbean isolates of *Dictyosphaeria cavernosa* (Chlorophyta) indicate a long divergence. *Helgoländer Meeresuntersuchungen* 41: 377–383.

PETERS A.F., VAN O.M.J.H., WIENCKE C., STAM W.T. & OLSEN J.L. 1997. Phylogeny and historical ecology of the Desmarestiaceae (Phaeophyceae) support a Southern Hemisphere origin. *Journal of Phycology* 33: 294–309.

PETERS A.F., MARIE D., SCORNET D., KLOAREG B. & COCK J.M. 2004. Proposal of *Ectocarpus siliculosus* (Ectocarpales, Phaeophyceae) as a model organism for brown algal genetics and genomics. *Journal of Phycology* 40: 1079–1088.

PHILLIPS N. & FREDERICQ S. 2000. Biogeographic and phylogenetic investigations of the pantropical genus *Sargassum* (Fucales, Phaeophyceae) with respect to Gulf of Mexico species. *Gulf of Mexico Science* 18: 77–87.

PHILLIPS N., SMITH C.M. & MORDEN C.W. 2001. An effective DNA extraction protocol for brown algae. *Phycological Research* 49: 97–102.

PHILLIPS N., BURROWS R., ROUSSEAU F., dE REVIERS B. & SAUNDERS G.W. 2008. Resolving evolutionary relationships among the brown algae using chloroplast and nuclear genes. *Journal of Phycology* 44: 394–405.

PICHERSKY E. 1990. Nomad DNA – a model for movement and duplication of DNA sequences in plant genomes. *Plant Molecular Biology* 15: 437–448.

PORTUGAL J. & WARING M. 1988. Assignment of DNA binding sites for DAPI and bisbenzimide (Hoeschst 33258). Comparative footprinting study. *Biochimica et Biophysica Acta* 949: 158–168.

PUCHTLER H., WALDROP F.S., MELOAN S.N., TERRY M.S. & CONNER H.M. 1970a. Methacarn (methanol-Carnoy) fixation: practical and theoretical considerations. *Histochemistry and Cell Biology* 21: 97–116.

PUCHTLER H., WALDROP F.S., LELOAN S.N., TERRY M.S. & CONNER H.M. 1970b. Methacarn (methanol-Carnoy) fixation. *Histochemie* 21: 97–116.

ROUSSEAU F. & DE REVIERS B. 1999a. Circumscription of the order Ectocarpales (Phaeophyceae): bibliographical synthesis and molecular evidence. *Cryptogamie, Algologie* 20: 5–18.

ROUSSEAU F. & DE REVIERS B. 1999b. Phylogenetic relationships within the Fucales (Phaeophyceae) based on combined partial SSU + LSU rDNA sequence data. *European Journal of Phycology* 34: 53–64.

ROUSSEAU F., LECLERC M.C. & dE REVIERS B. 1997. Molecular phylogeny of European Fucales (Phaeophyceae) based on partial large-subunit rDNA sequence comparisons. *Phycologia* 36: 438–446.

ROUSSEAU F., BURROWS R., PETERS A.F., KUHLENKAMP R. & dE REVIERS B. 2001. A comprehensive phylogeny of the Phaeophyceae based on nrDNA sequences resolves the earliest divergences. *Comptes Rendus de l'Académie des Sciences de Paris, Sciences de la Vie* 324: 1–15.

SAUNDERS G.W. & KRAFT G.T. 1995. The phylogenetic affinities of *Notheia anomala* (Fucales, Phaeophyceae) as determined from partial small-subunit rRNA gene sequences. *Phycologia* 34: 383–389.

SERRÃO E.A., ALICE L.A. & BRAWLEY S.H. 1999. Evolution of the Fucaceae (Phaeophyceae) inferred from nrDNA-ITS. *Journal of Phycology* 35: 382–394.

SPARROW A.H. & NAUMAN A.F. 1973. Evolutionary changes in genome and chromosome sizes and in DNA content in the grasses. *Brookhaven Symposium in Biology* 25: 367–389.

STAM W.T., BOT P.V.M., BOELE-BOS S.A., VAN ROOIJ J.M. & VAN DEN HOEK C. 1988. Single-copy DNA-DNA hybridization among five species of *Laminaria* (Phaeophyceae): phylogenetic and biogeographic implications. *Helgoländer Meeresuntersuchungen* 42: 251–267.

STEBBINS G.L. 1971. *Chromosomal evolution in higher plants*. Addison-Wesley, Reading, Massachusetts, 216 pp.

STIGER V., HORIGUCHI T., YOSHIDA T., COLEMAN A. & MASUDA M. 2000. Phylogenetic relationships of *Sargassum* (Sargassaceae, Phaeophyceae) with reference to a taxonomic revision of the section Phyllocystae based on ITS-2 nrDNA sequences. *Phycological Research* 48: 251–260.

STIGER V., HORIGUCHI T., YOSHIDA T., COLEMAN A.W. & MASUDA M. 2003. Phylogenetic relationships within the genus *Sargassum* (Fucales, Phaeophyceae) inferred from ITS-2 nrDNA, with an emphasis on the taxonomic subdivision of the genus. *Phycological Research* 51: 1–10.

SUSINI M.L. 2006. *Statut et biologie de* Cystoseira amentacea *var.* stricta. PhD thesis. University of Nice-Sophia Antipolis. 194 pp.

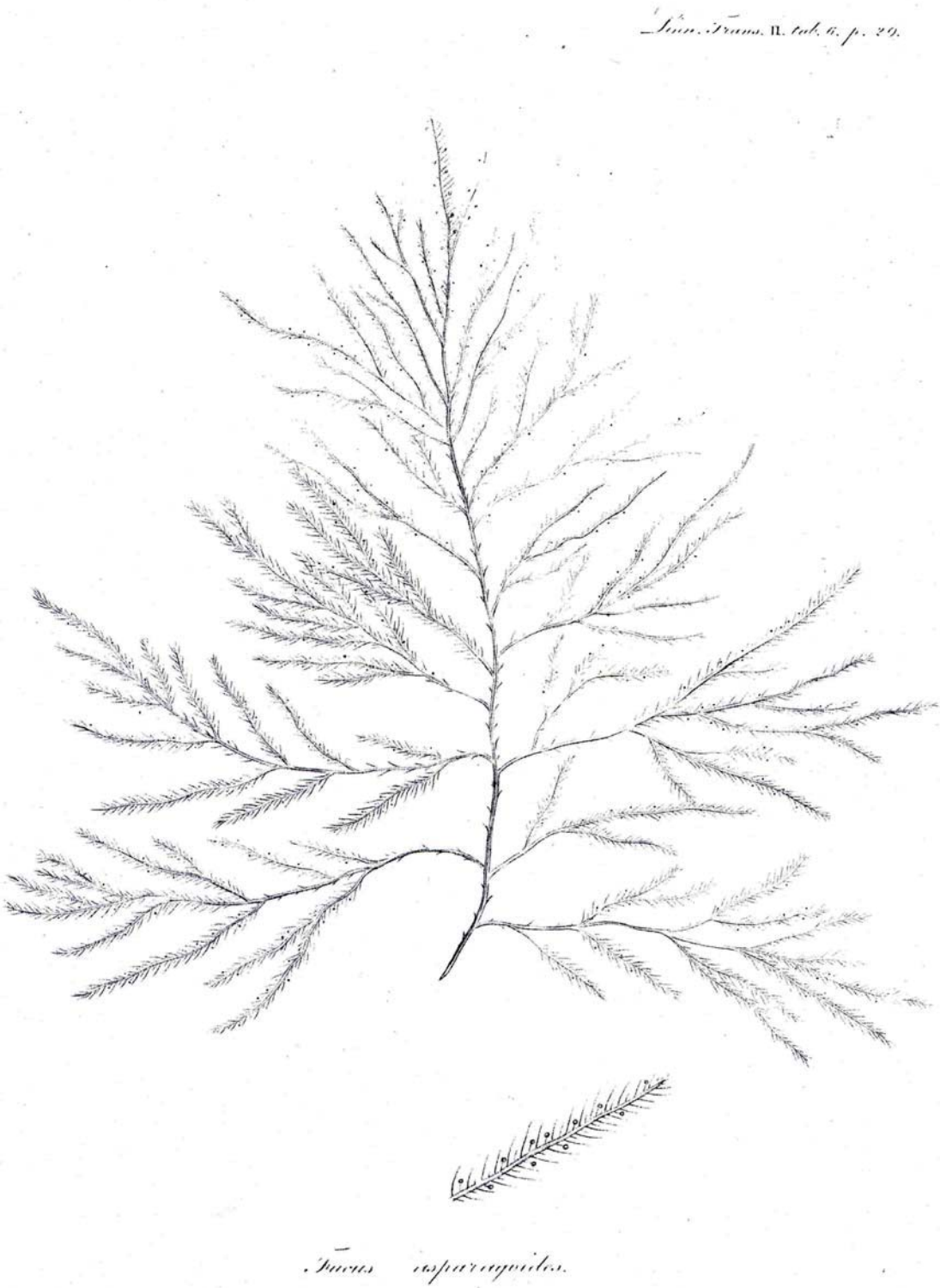
WENZEL W. & HEMLEBEN V. 1982. A comparative study of genomes of angiosperms. *Plant Systematics and Evolution* 139: 209–227.

YABU H. & YASUI H. 1983. Occurrence of a tetraploid in *Sargassum confusum*. *Japanese Journal of Phycology* 31: 86–87.

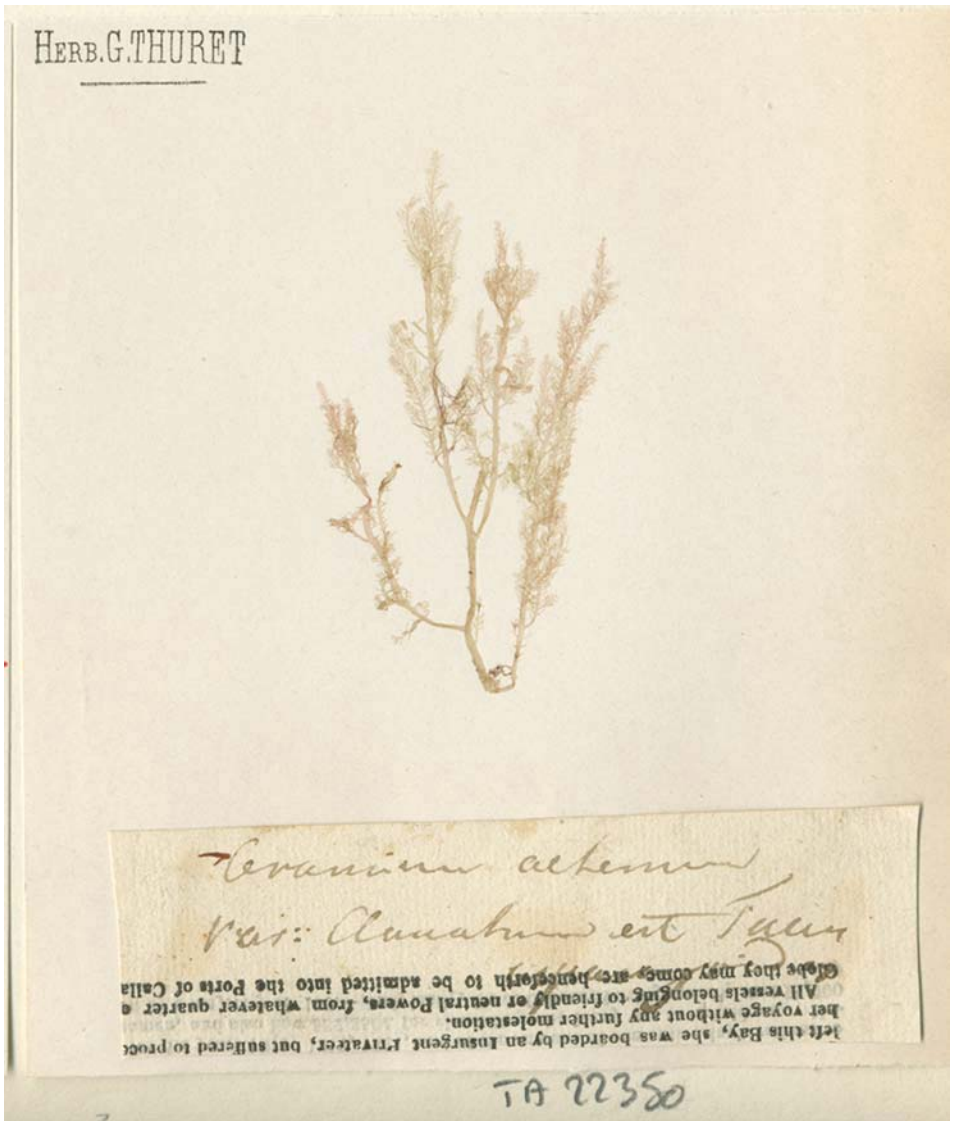
Received 18 February 2009; accepted 6 July 2009
Associate editor:

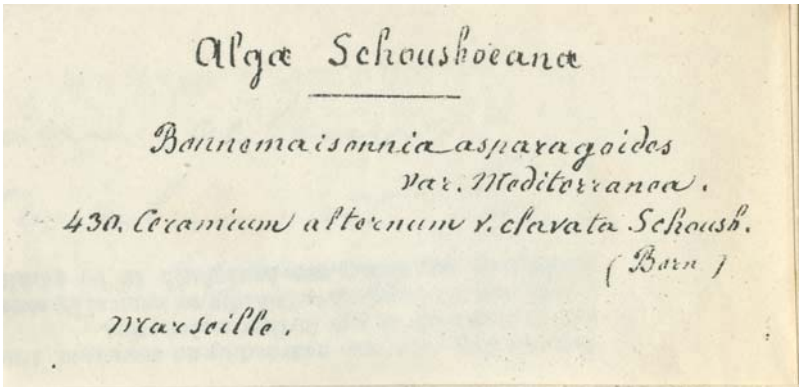
1

2. Tipus nomenclaturals

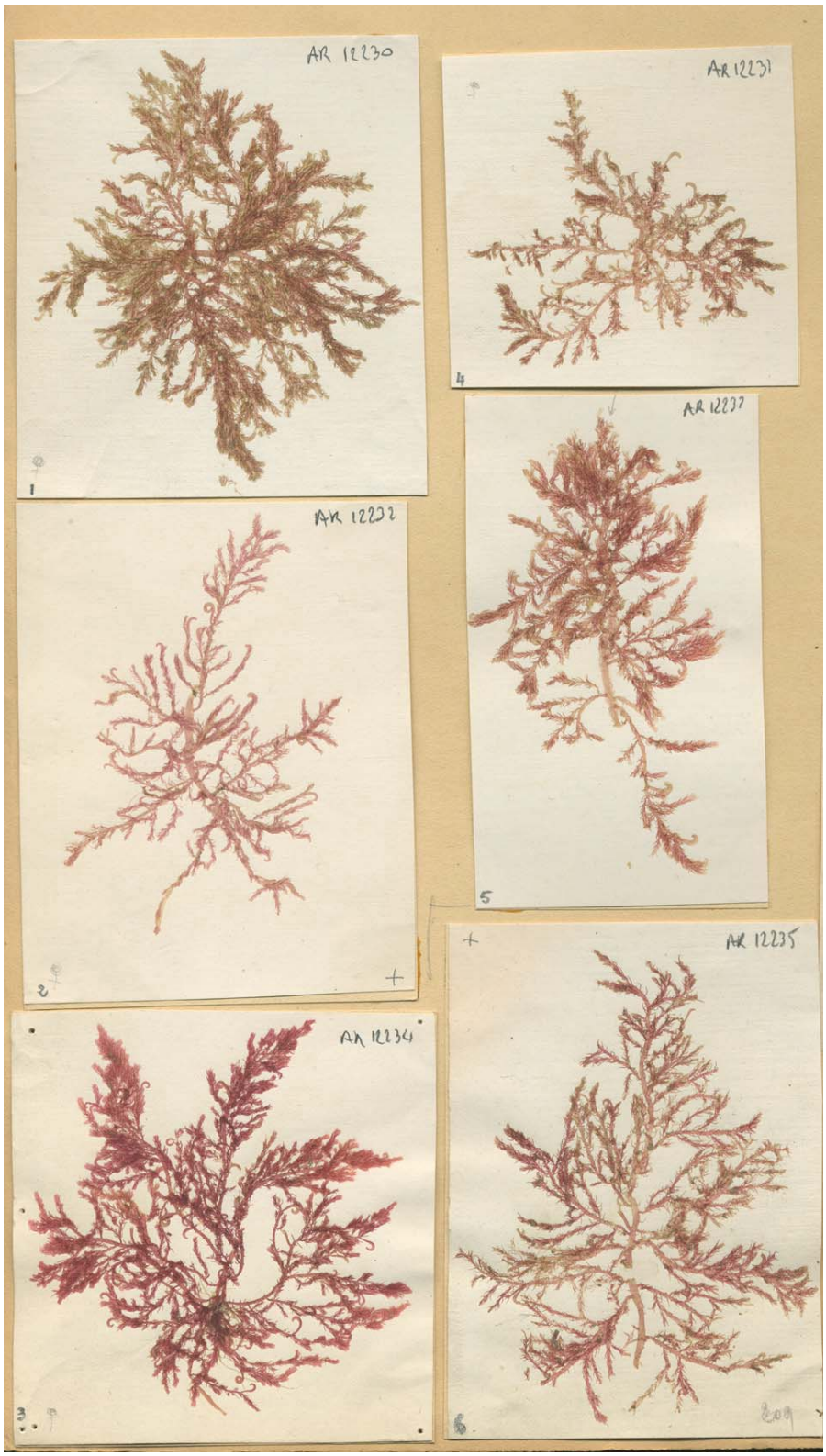


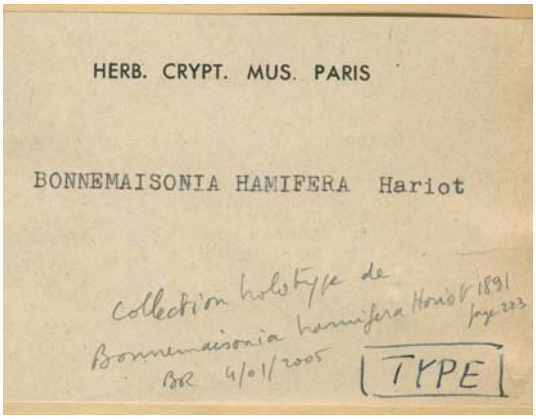
Lectotipus de *Bonnemaisonia asparagoides*





Lectotipus de *Bonnemaisonia clavata*

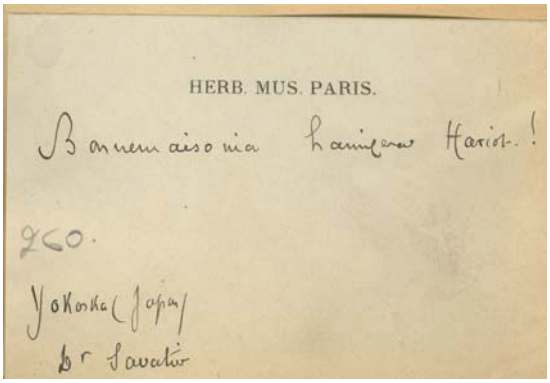



HERB. CRYPT. MUS. PARIS

BONNEMAISONIA HAMIFERA Hariot

collection holotype de
Bonnemaisonia hamifera Hariot 1891 page 223
BR 4/01/2005

TYPE


HERB. MUS. PARIS.

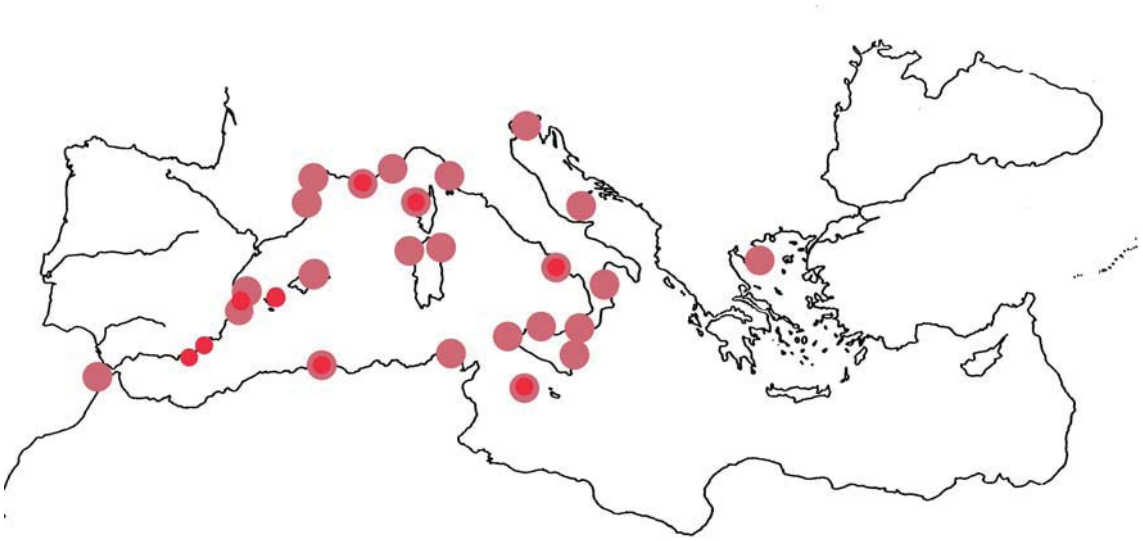
Bonnemaisonia hamifera Hariot. !

200.

Yokoska (Japan)
Dr Savatier

Holotipus de *Bonnemaisonia hamifera*

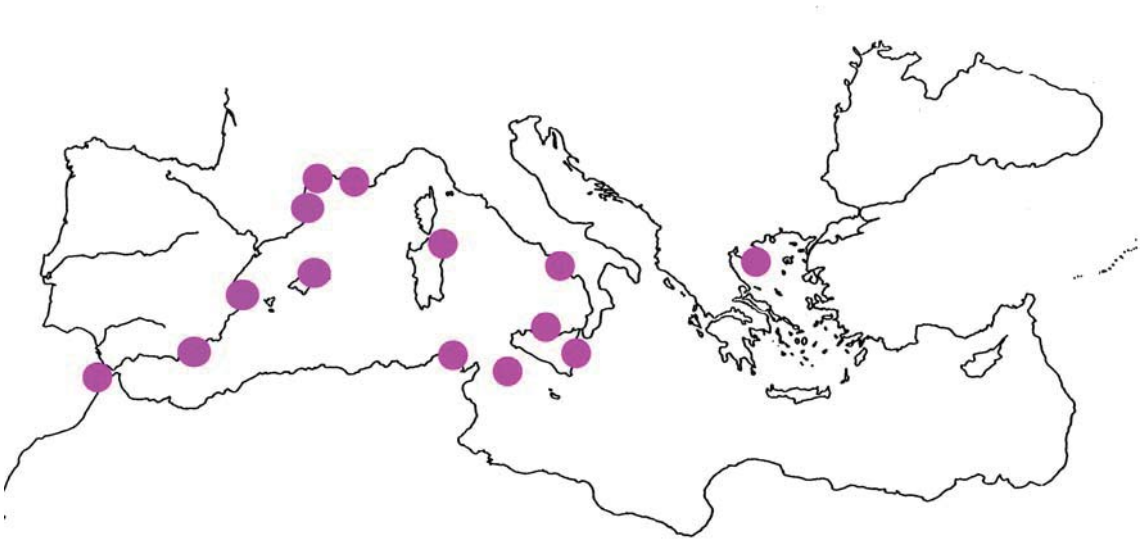
3. Mapes de distribució al Mediterrani



Bonnemaisonia asparagoides

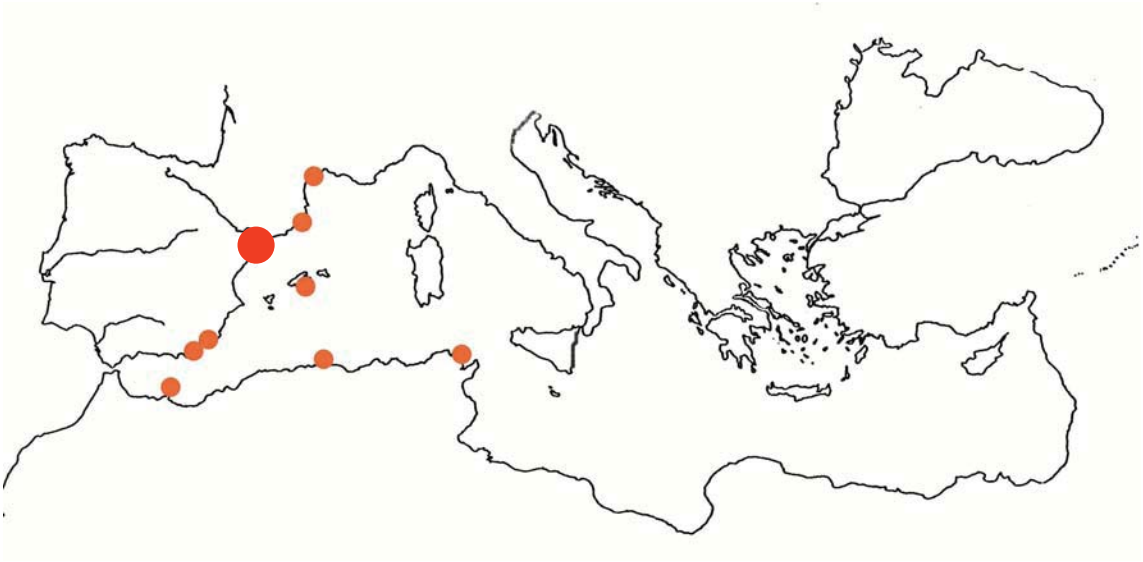
Gametòfit

"Hymenoclonium"



Bonnemaisonia clavata

Gametòfit



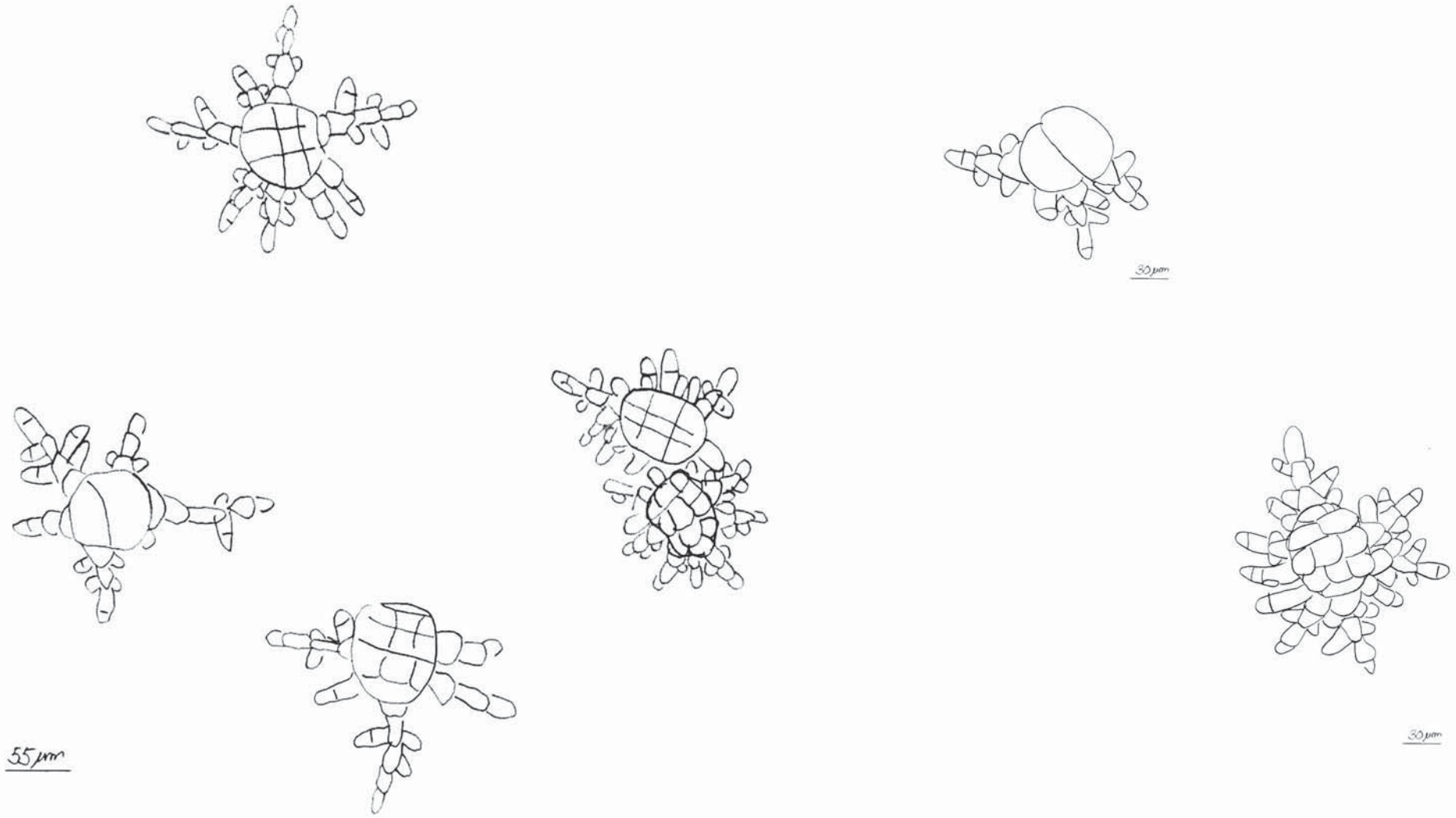
Bonnemaisonia hamifera

Gametòfit

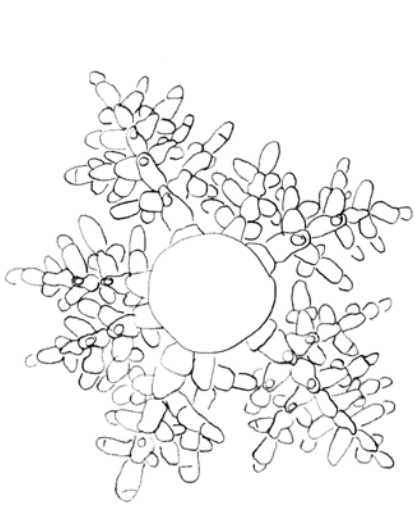
"Trailliella"

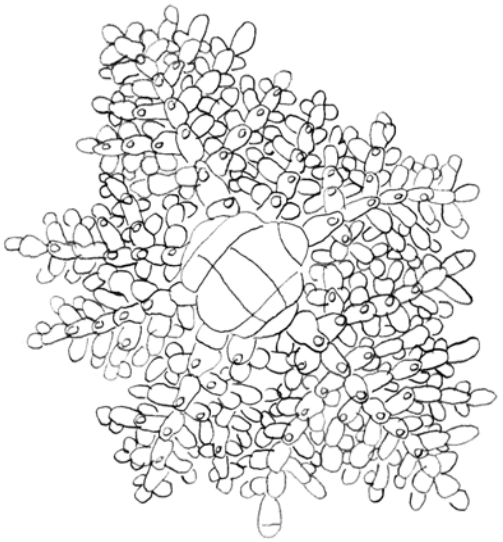
4. Il·lustracions originals

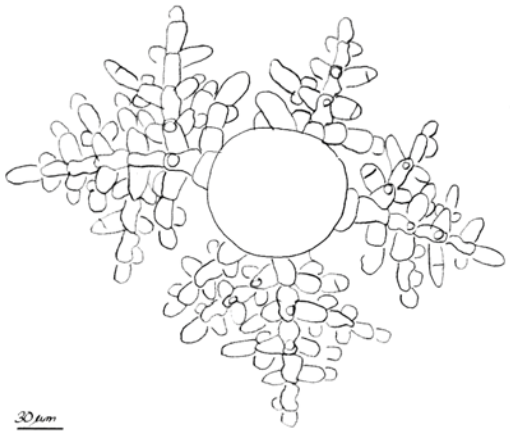
Bonnemaisonia asparagoides

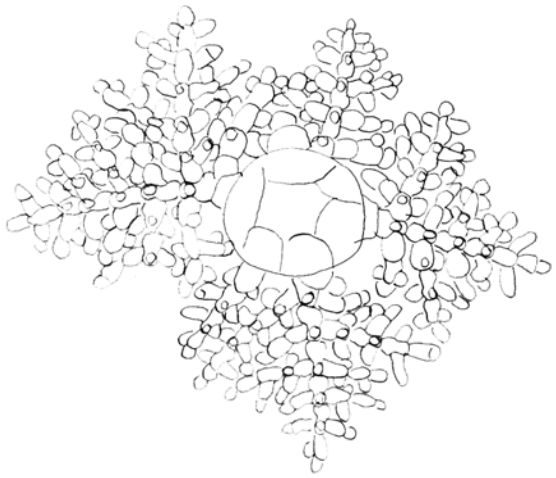


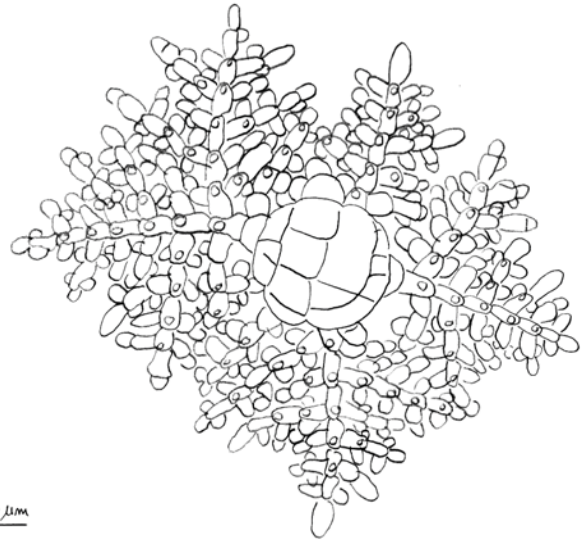
"Hymenoclonium" al 5è dia de cultiu







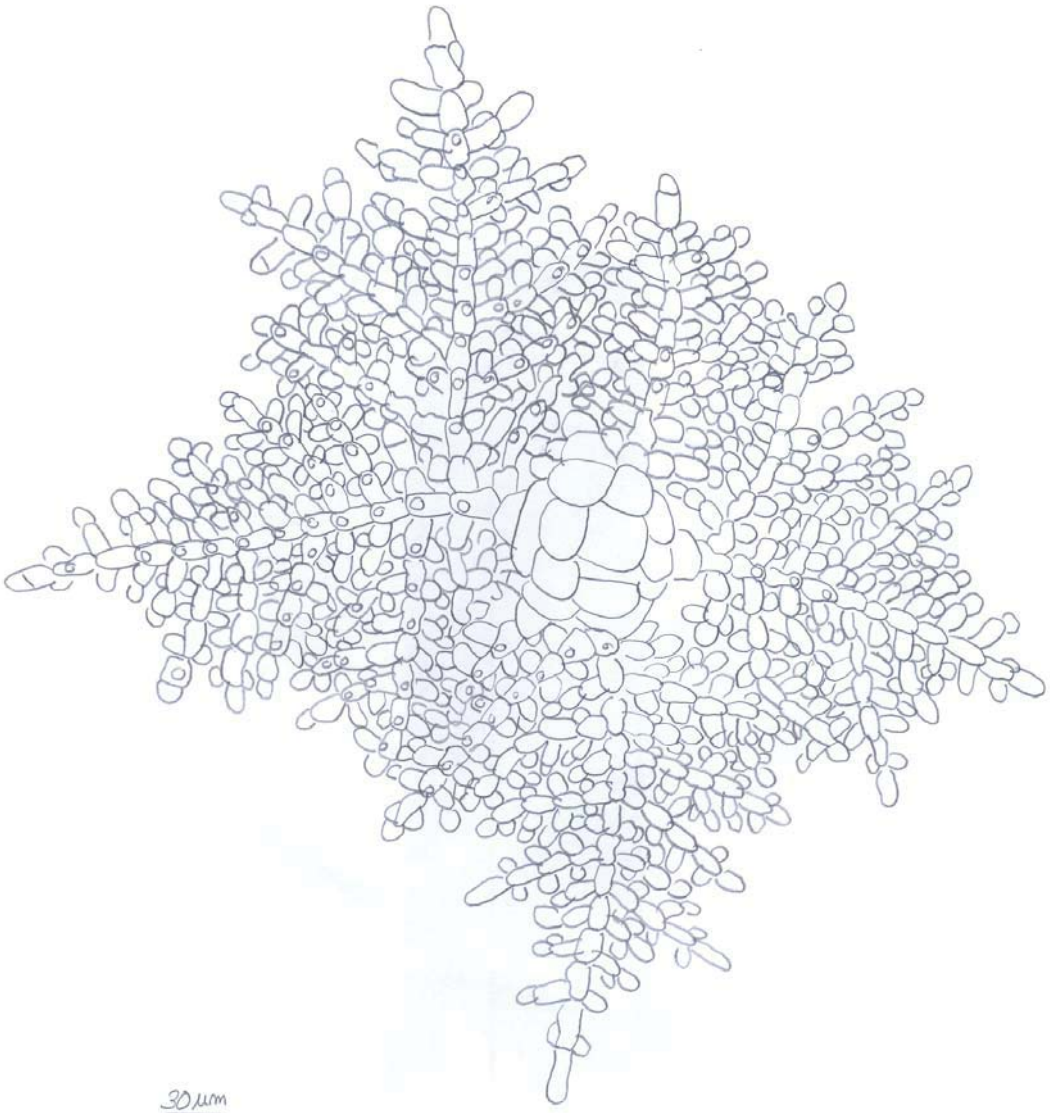




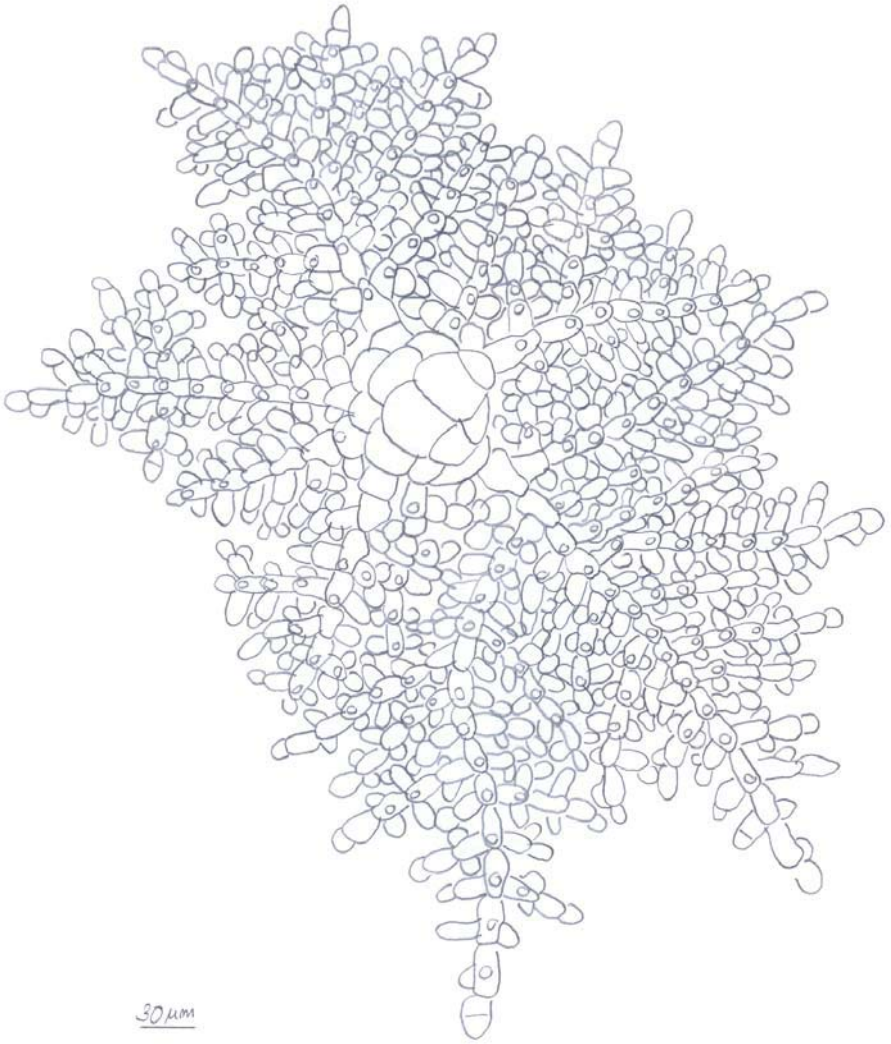
8^{e} dia de cultiu

10^{e} dia de cultiu

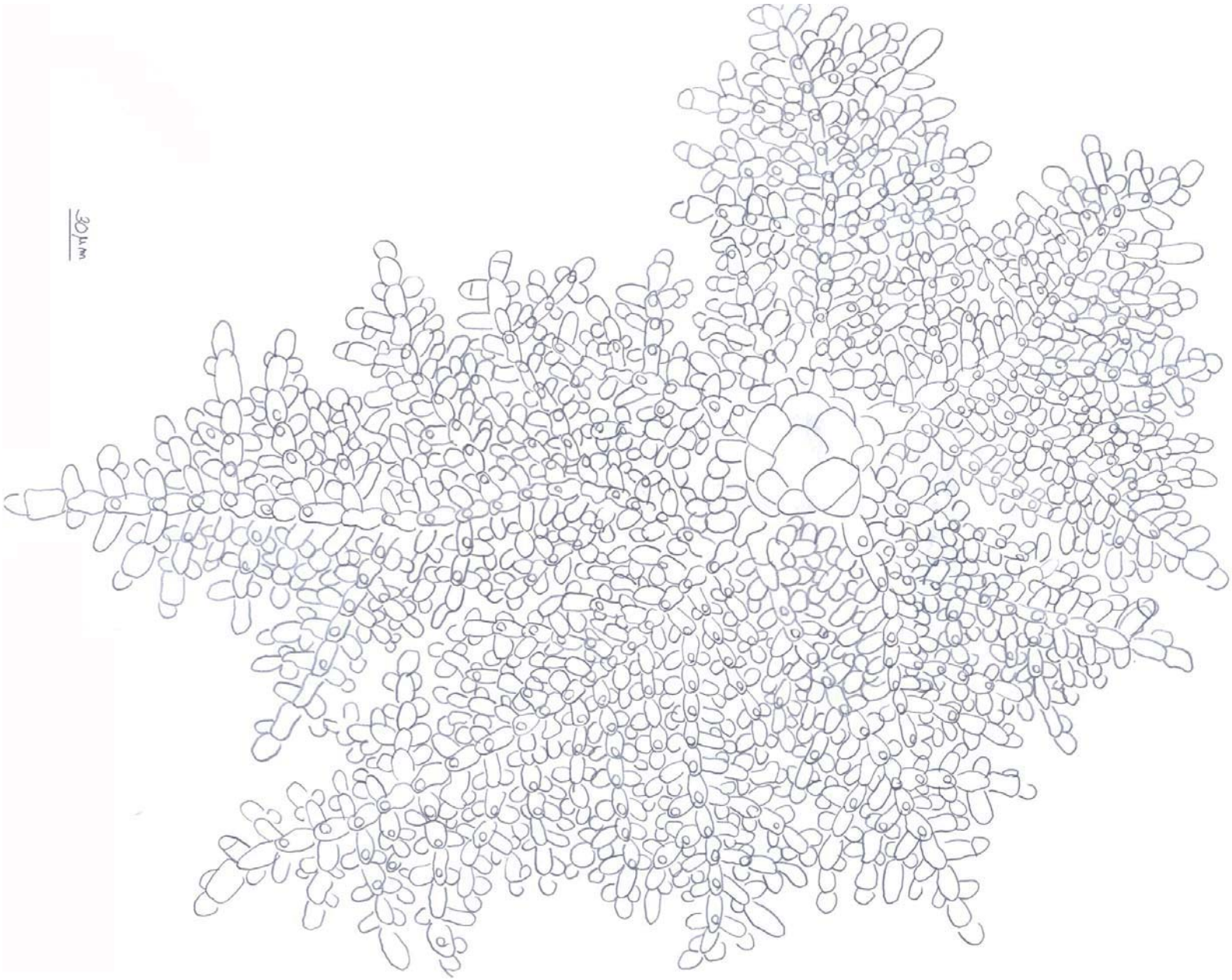
12^{e} dia de cultiu



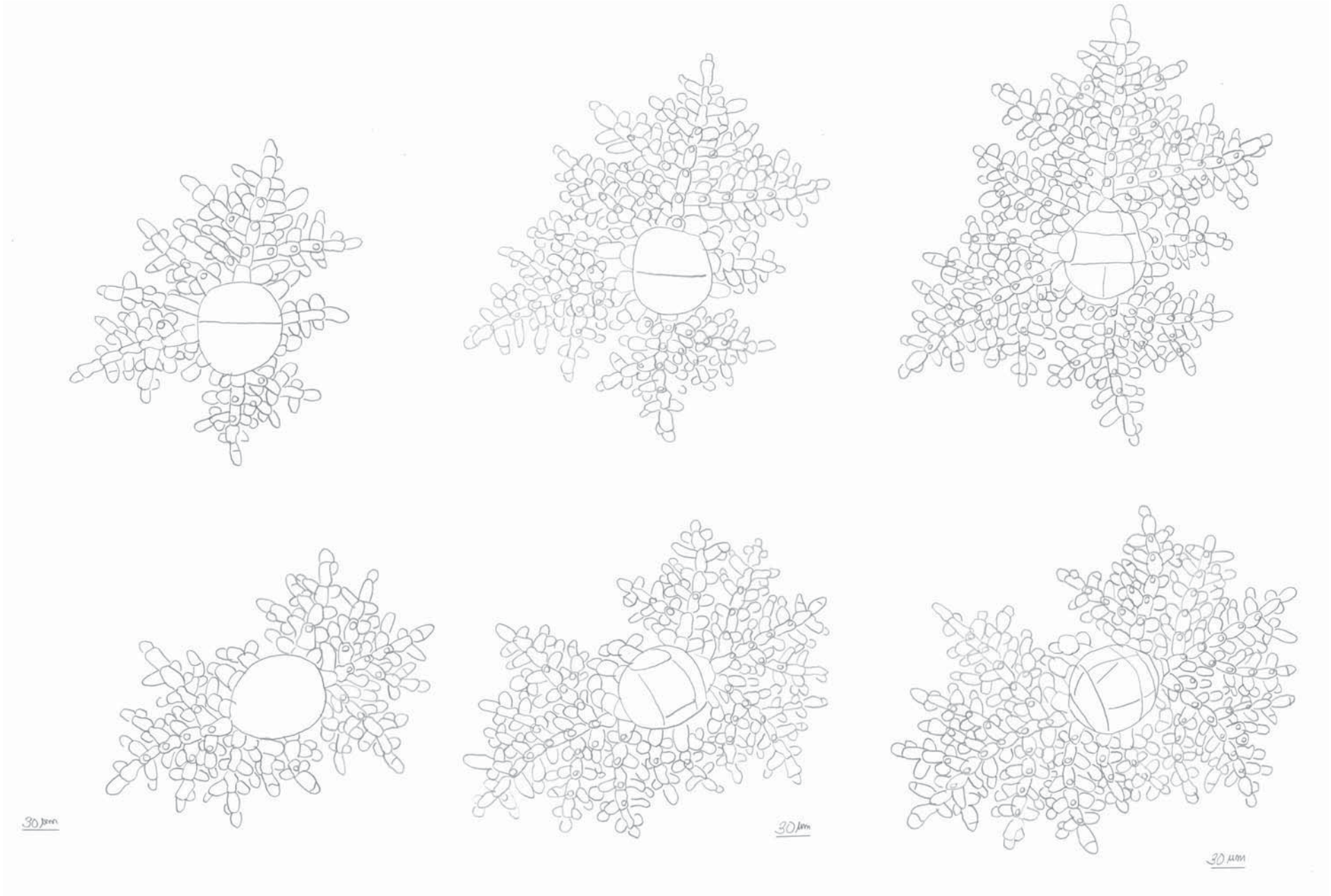
3ª setmana de cultiu



3ª setmana de cultiu



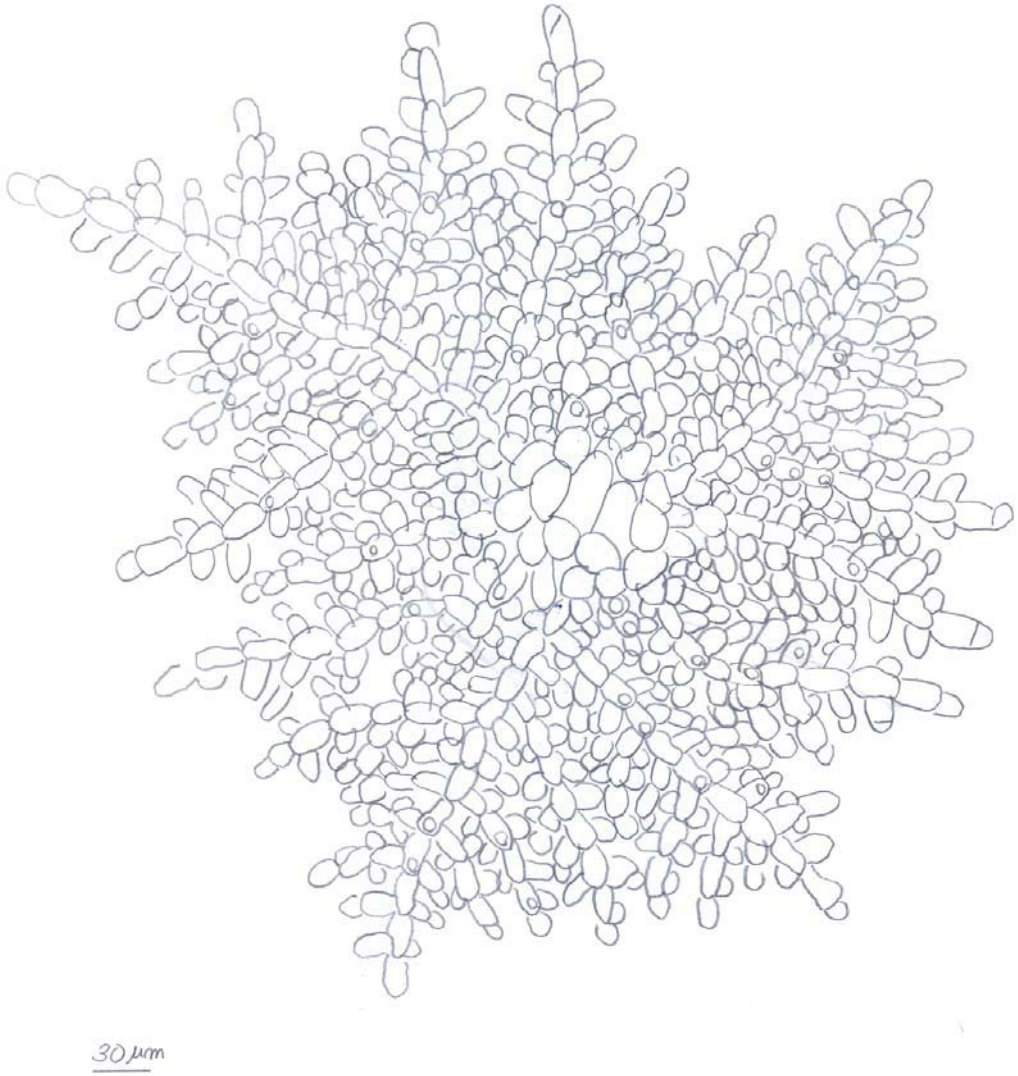
4ª setmana de cultiu



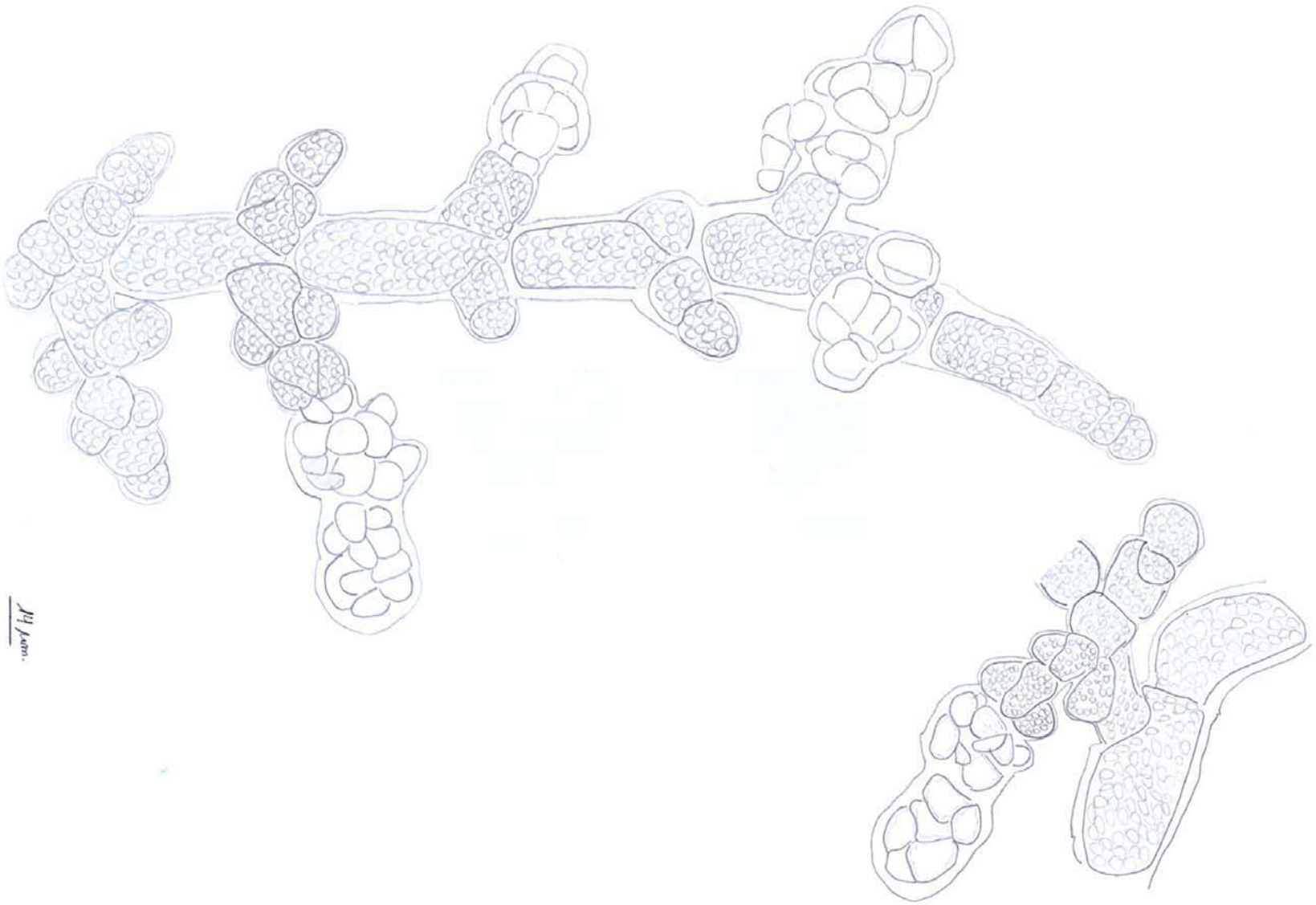
8è dia de cultiu

10è dia de cultiu

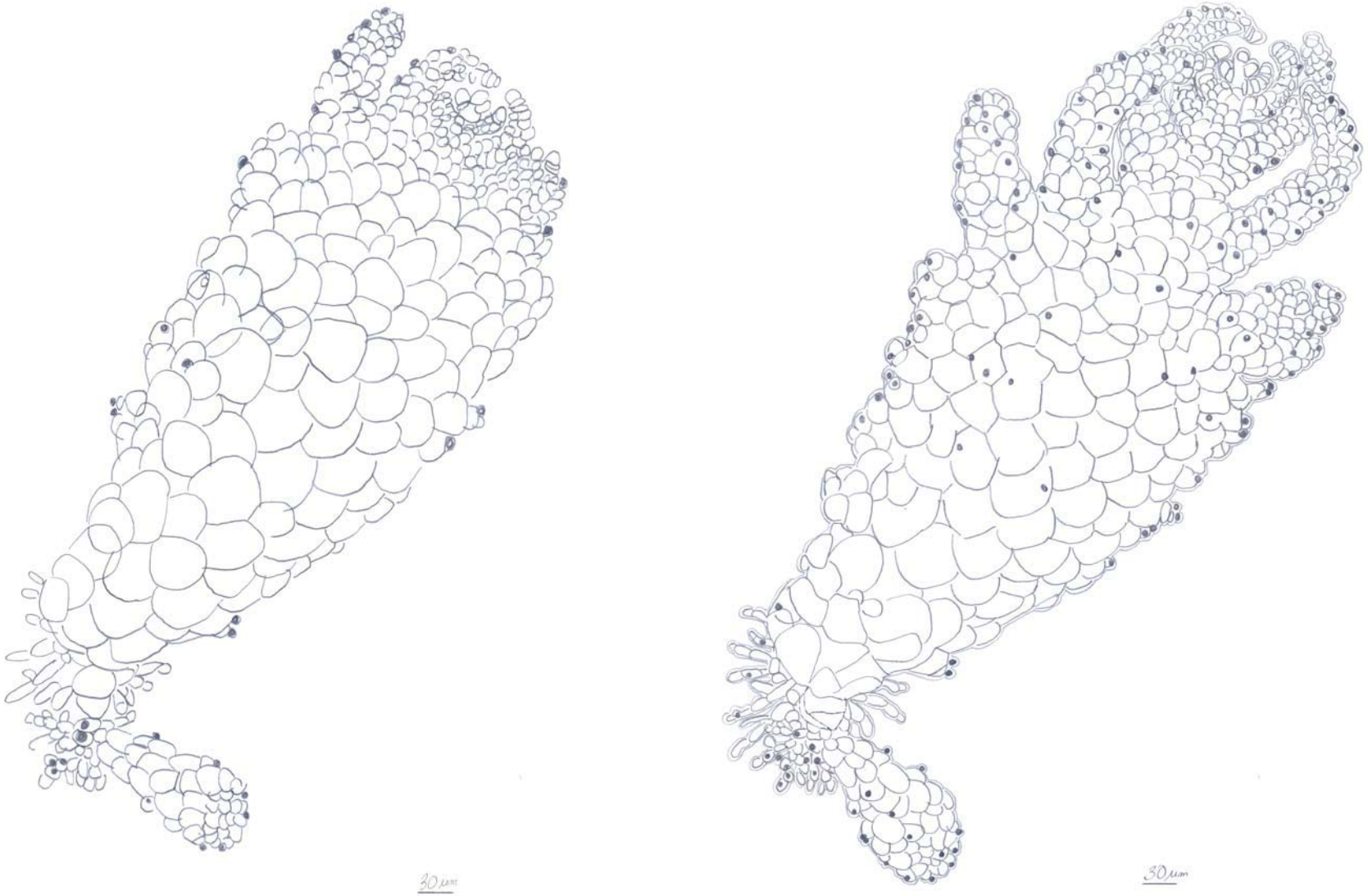
12è dia de cultiu



3ª setmana de cultiu



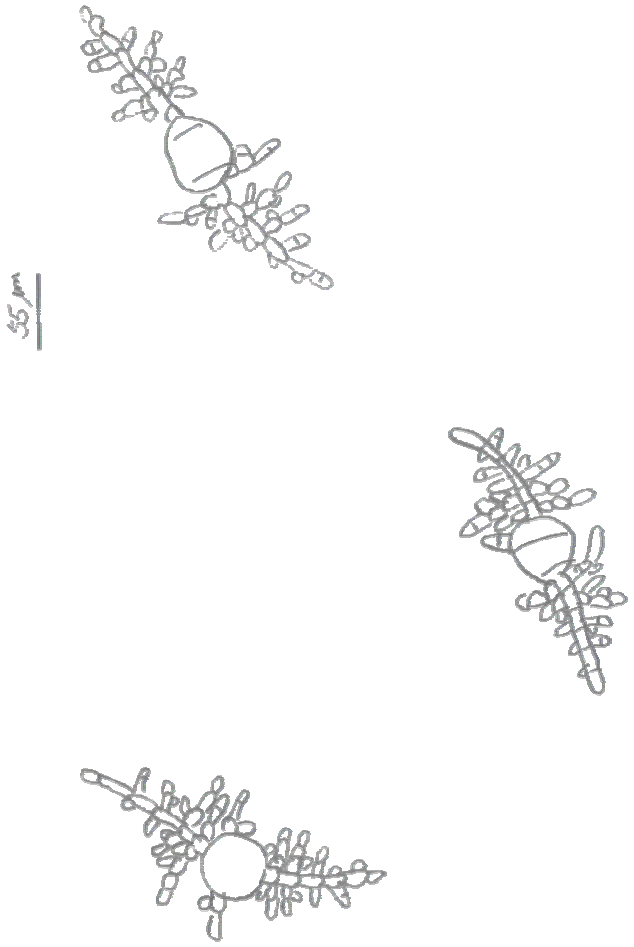
Desenvolupament de diferents gametòfits sobre un filament de la generació “Hymenoclonium”



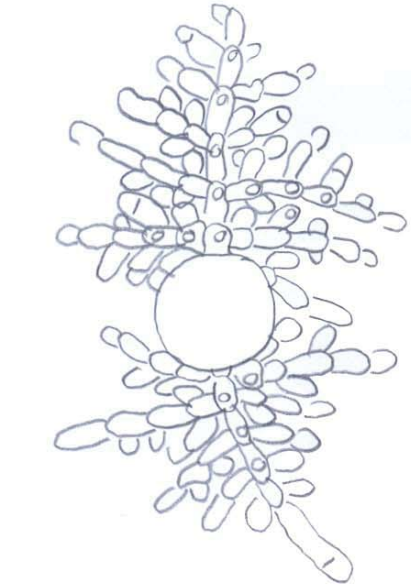
Creixement d'un gametòfit en cultiu

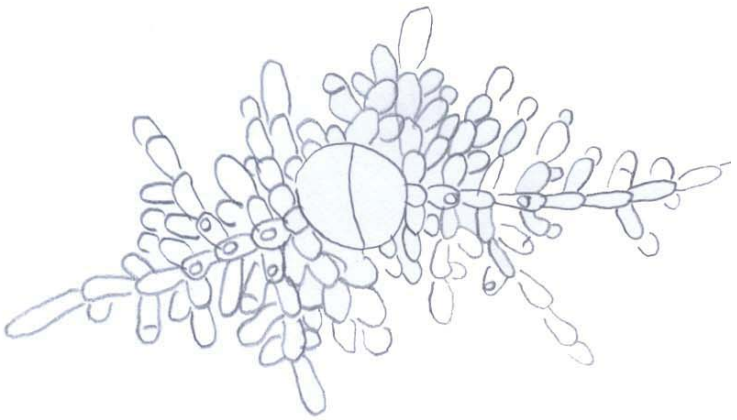
Bonnemaisonia clavata

Carpòspores després de 48h de cultiu

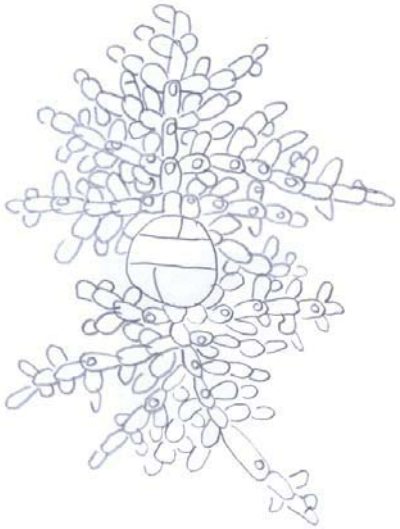


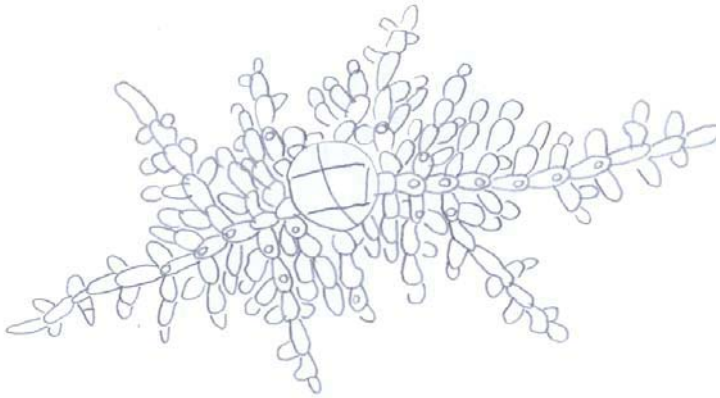
"Hymenoclonium" al 5è dia de cultiu



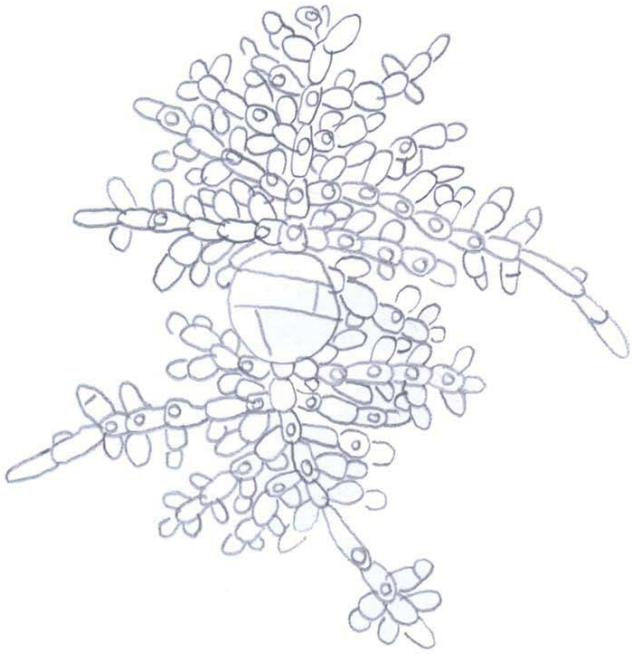


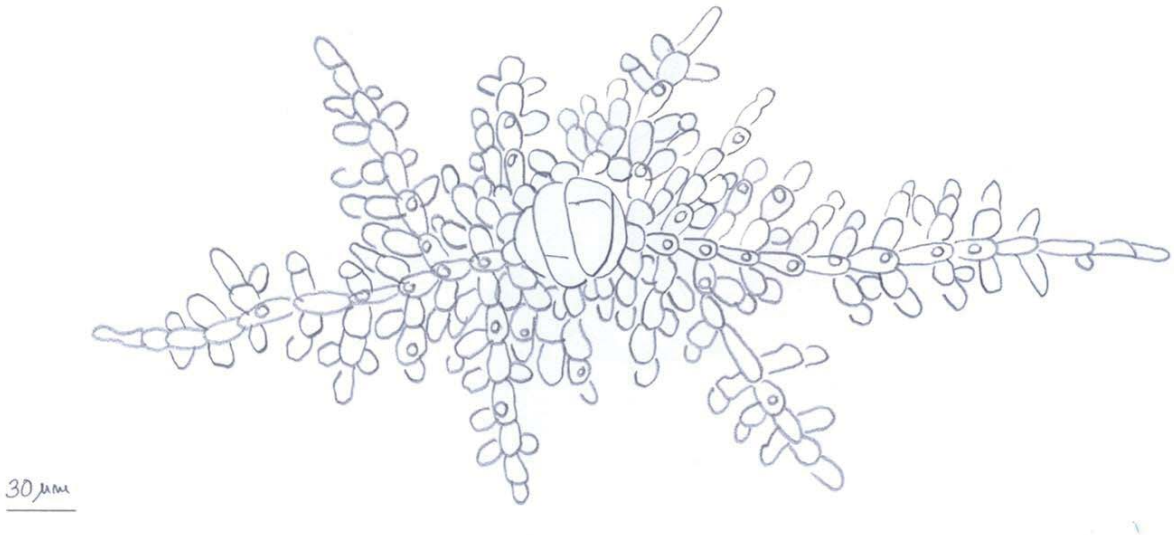
"Hymenoclonium" al 8è dia de cultiu



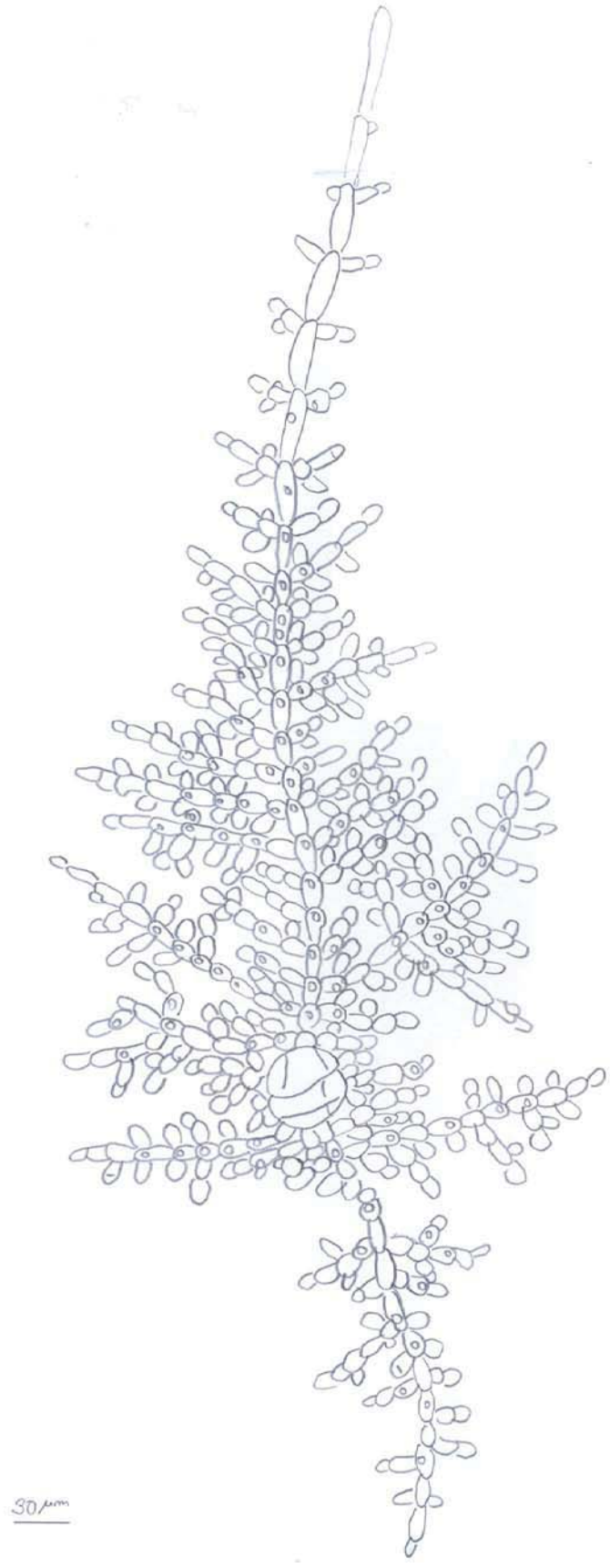


"Hymenoclonium" al 10è dia de cultiu



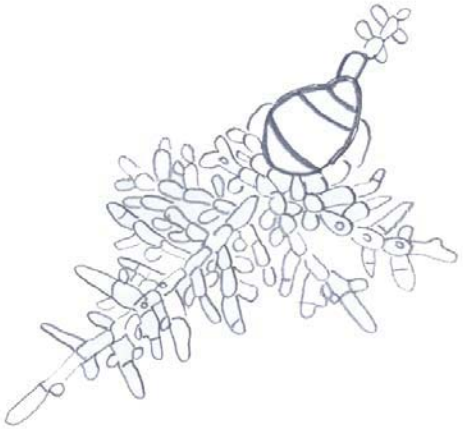


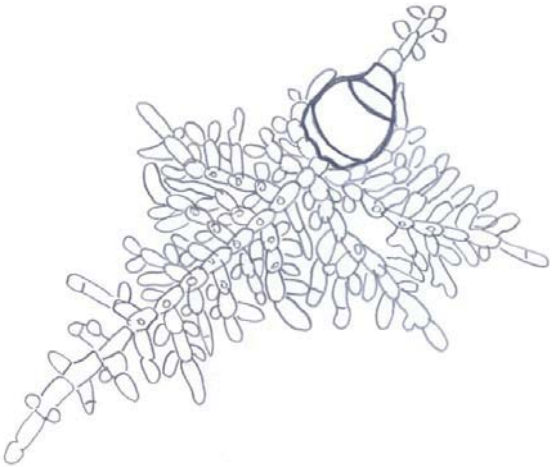
12è dia de cultiu

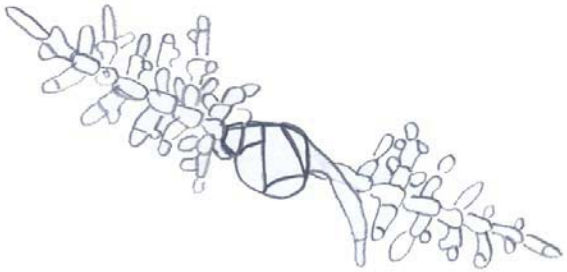


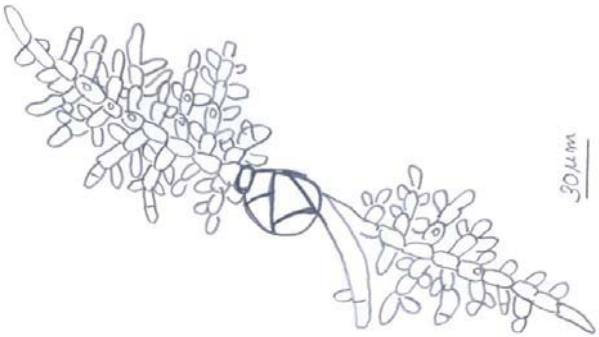
3º setmana de cultiu

"Hymenoclonium" al 26è dia de cultiu



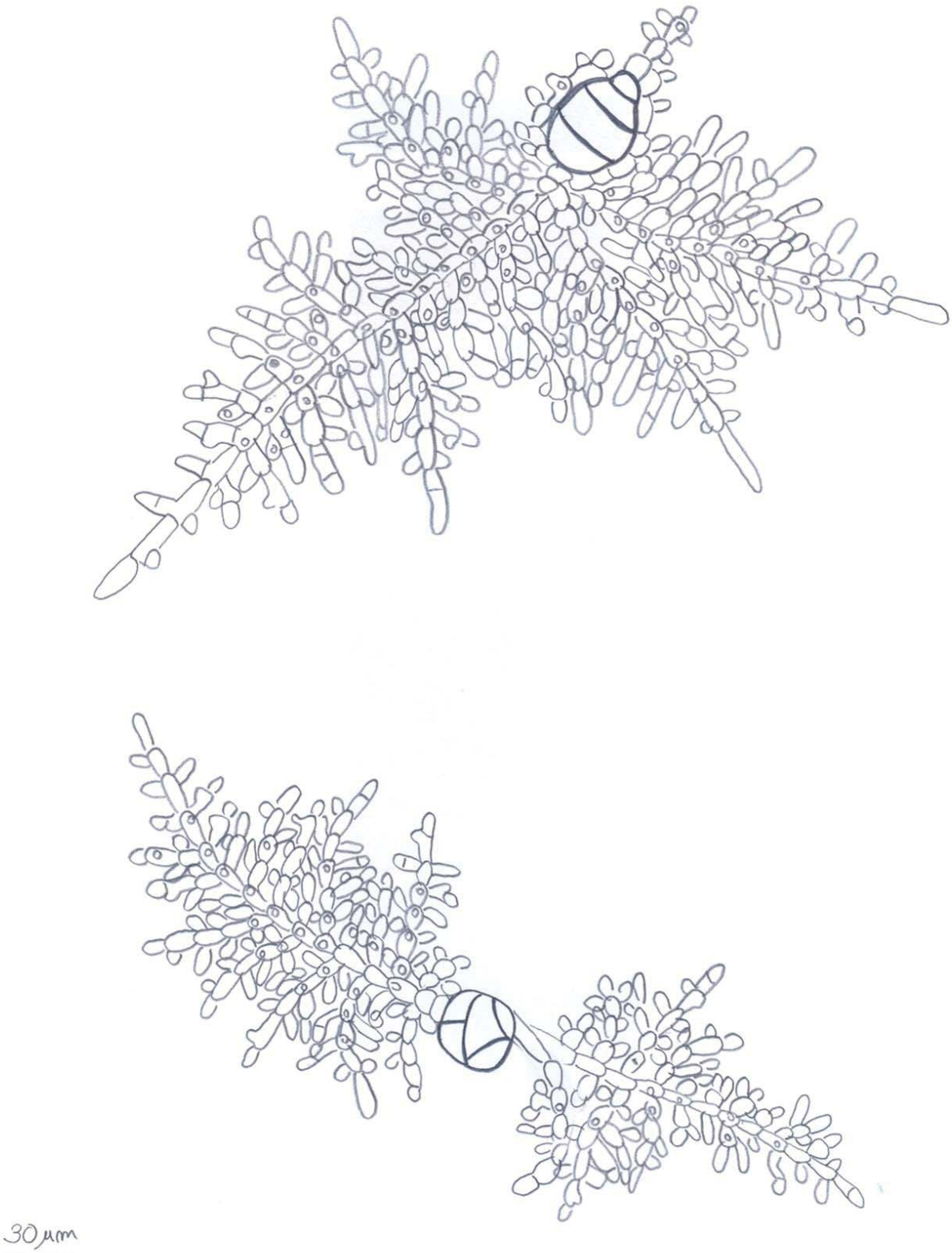




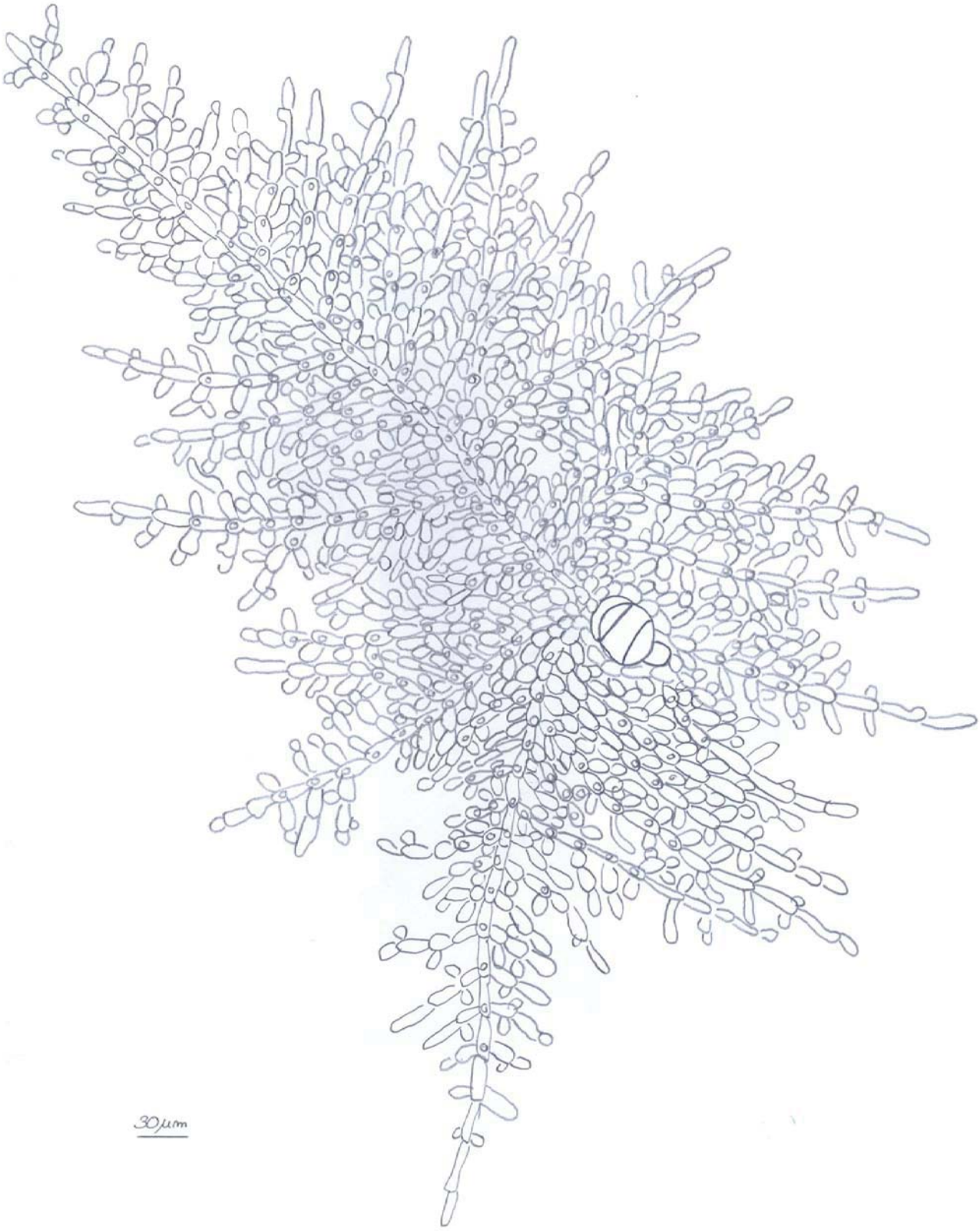


"Hymenoclonium" al 12è dia de cultiu

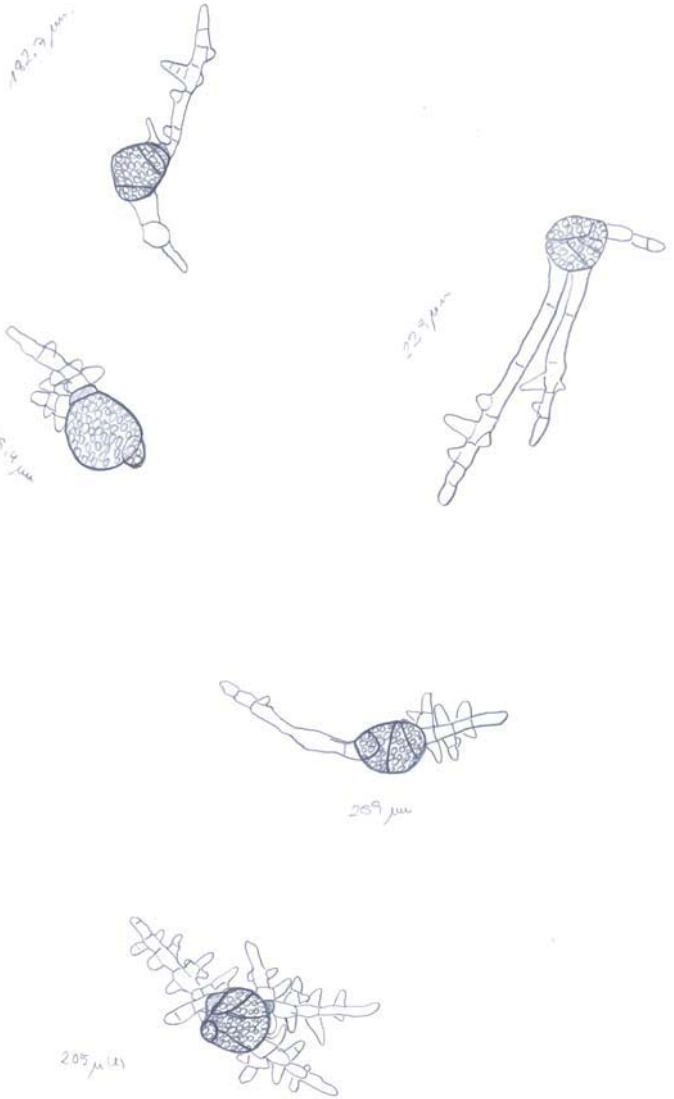
2ª setmana de cultiu



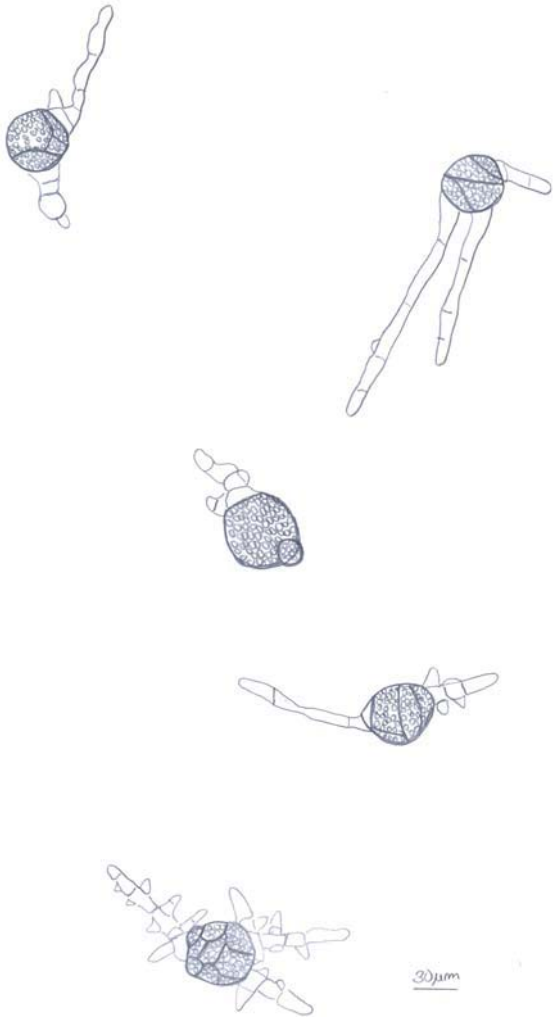
3º setmana de cultiu



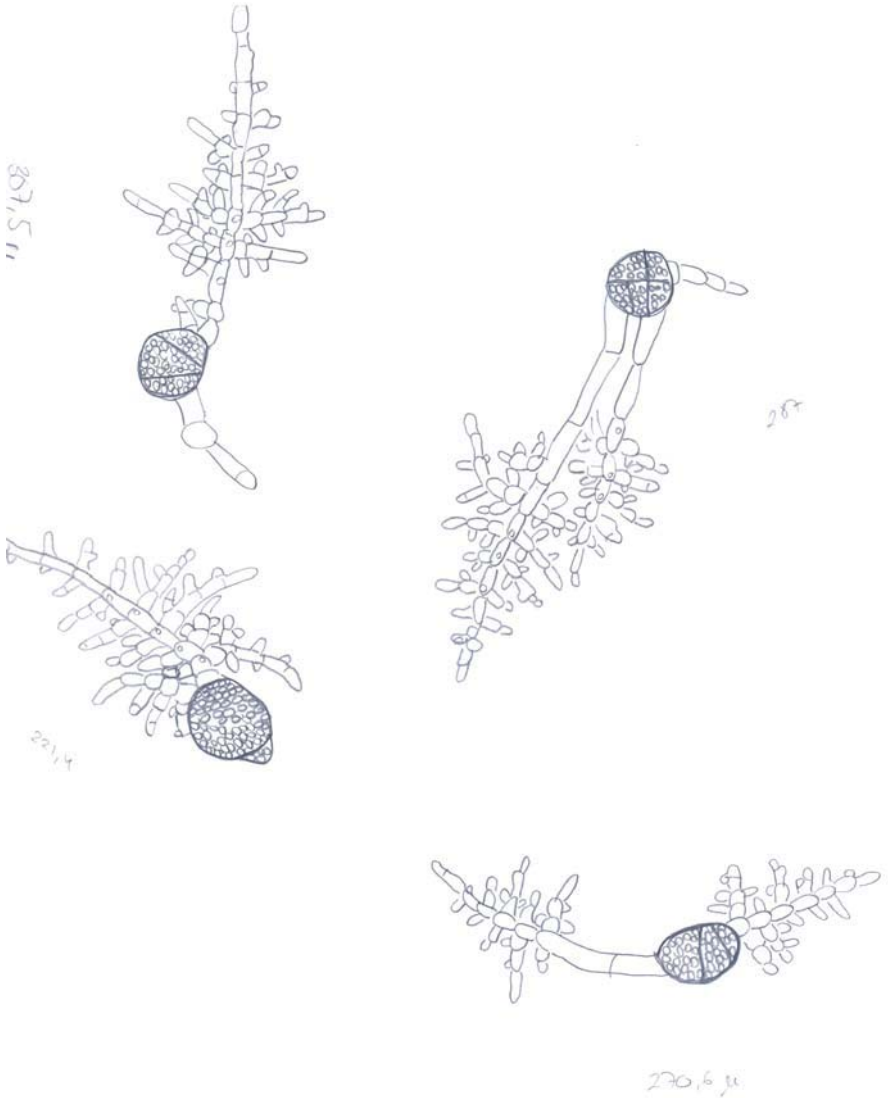
"Hymenoclonium" al 25è dia de cultiu



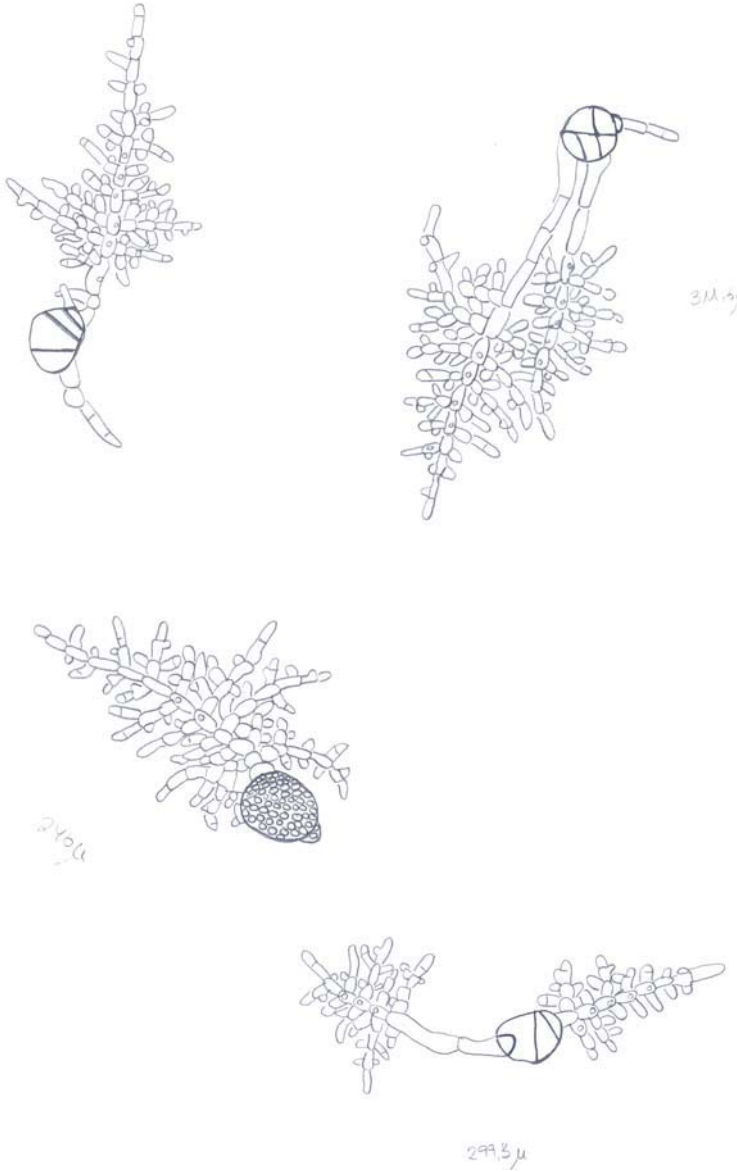
"Hymenoclonium" al 6è dia de cultiu



1ª setmana de cultiu

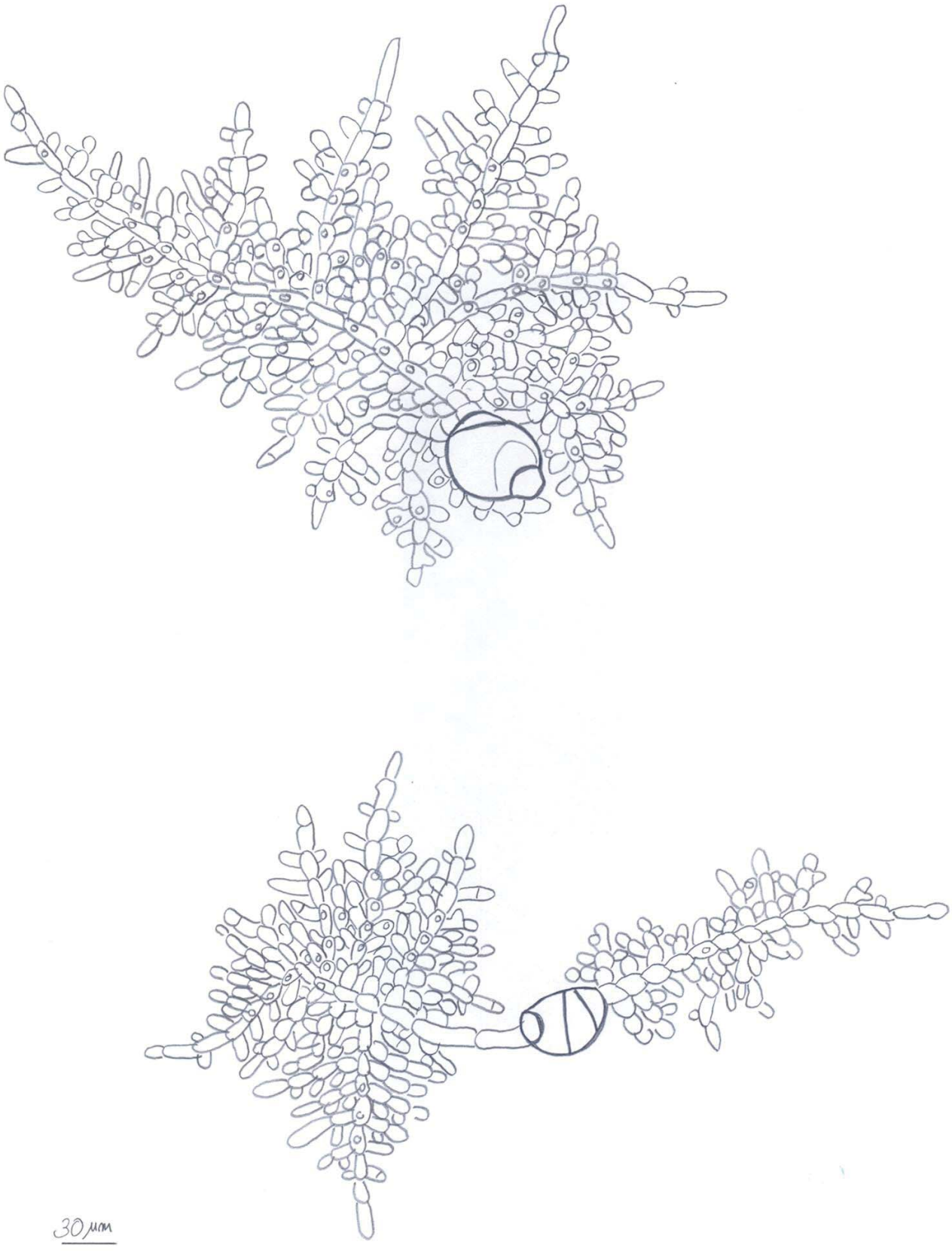


“Hymenoclonium” al 10è dia de cultiu

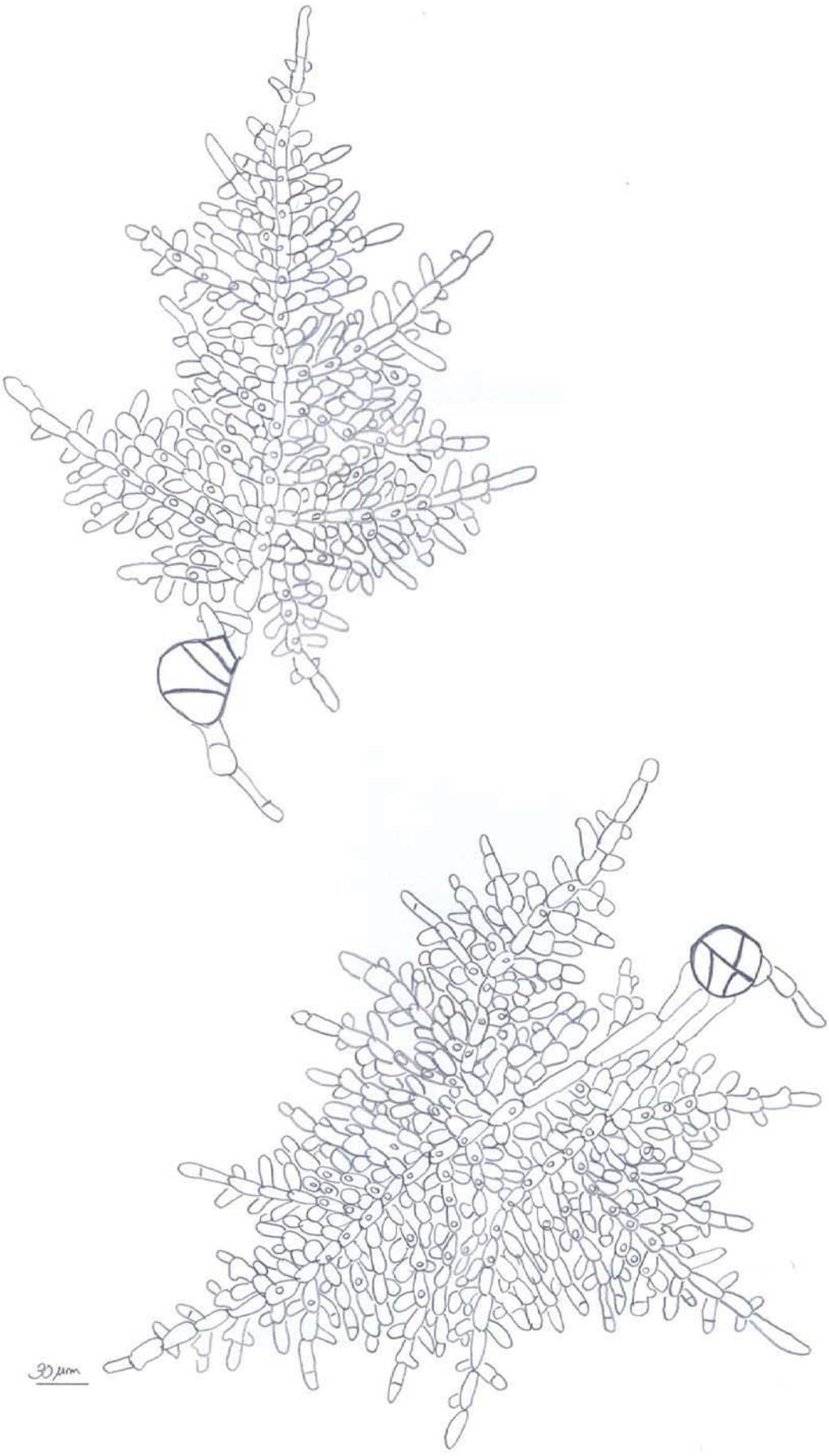


“Hymenoclonium” al 12è dia de cultiu

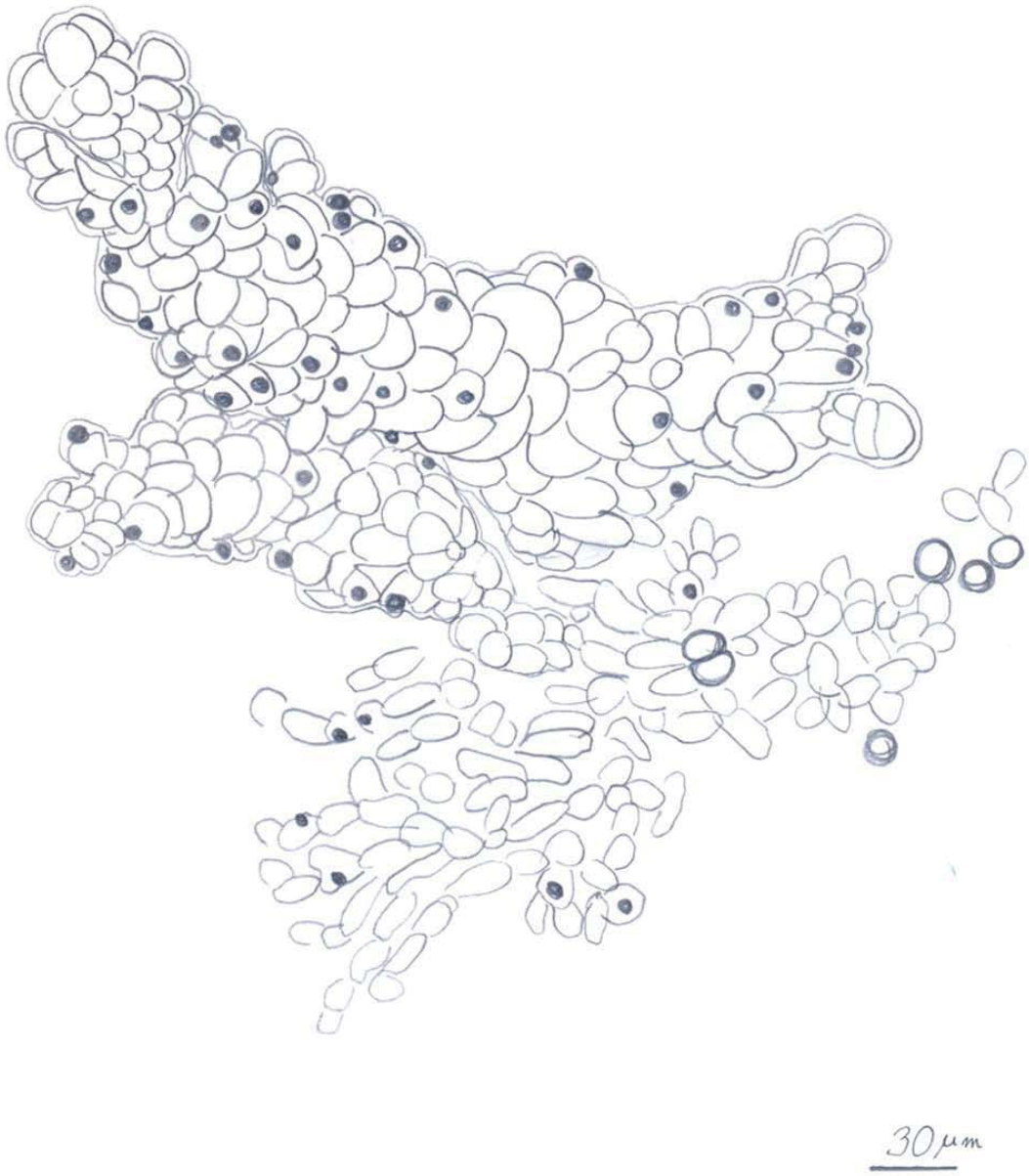
2ª setmana de cultiu



3º setmana de cultiu



3º setmana de cultiu



Gametòfit jove sobre la seva generació “Hymenoclonium”
als dos mesos de cultiu de les carpòspores