Early development of carpospores of *Gracilaria verrucosa* (Rhodophyta, Gracilariaceae) under laboratory conditions

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Abstract

The aim of this work was to study the effect of environmental factors, irradiance and photoperiod on carpospores development of *Gracilaria verrucosa* under laboratory conditions. Relationships of germination rate, diameter and survival rate of carpospores with grads of photoperiod and irradiance were determined, respectively. Carpospores spent approximately six weeks to develop into young gametophytes. It was shown that irradiance variation (15, 40, 75 and 100 µmol m^{-2} s^{-1}) has significant effects on the three growth parameters: germination rate, growth (diameter) and survival rate. The optimal germination rate and growth were detected at irradiance of 75 µmol m^{-2} s^{-1}. The photon flux density which exceeds 40 µmol m^{-2} s^{-1} has apparently positive effect on survival rate. The three photoperiods tested show that long day condition (16 h) has a negative effect on three growth parameters. The optimal germination rate was achived at the photoperiod of 12 h:12 h, but it was shown that there is no apparent effect on growth between photoperiods 8:16 and 12:12.

Keywords: *Gracilaria verrucosa*, Carposores, Photon flux density, Photoperiods, Survival, Growth

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Introduction

Gracilaria verrucosa (Gracilariaceae, Rhodophyta) is an important economic alga used for agar extraction (Freile-Pelegrín and Murano, 2005), being the main source of agar (Polifrone *et al.*, 2006), the most valuable phycocolloid in the world market. The total harvest is slightly in excess of 37000 dry tons (Mantri *et al.*, 2009). Their cultivation for commercial

purpose has been developed in several countries on a very large (Tseng 2001; Lüning and Pang, 2003) and medium scale (Alveal *et al.,* 1997) since the increasing demand of agar caused a rapid depletion of natural populations, several cultivation have been used (Alveal *et al.,* 1997; Buschmann *et al.,* 2001; Tseng, 2001) to increase the production of agar.

In the past, most of the *Gracilaria* harvest come from wild stocks as the marine aquaculture only accounts from a little part of the total biomass (Ye *et al.,* 2006). From 1991 approximately one-third of the harvest was from cultured sources of *Gracilaria* (Glenn *et al.,* 1996). Although in the last few years, great deals of data have been obtained from basic research, there is still little information concerning their reproductive strategy in the field, particularly about Mediterranean populations (Polifrone *et al.*, 2006).

Recently, most of the culture methods rely on vegetative fragments, rather than tetraspores or carpospores of mature thalli, as the propagating units to provide plants (Ye *et al.,* 2006, Mantri *et al.,* 2009). Being one of mini-phases of life history of *Gracilaria,* tetraspores connect phases of sporophytes and gametophytes. However, differing from tetraspores, carpospores are rarely used as seeds to propagate the important economic marine algae and the information about the development of the tetraspores is still limited. In this study, we used sporophytes thalli of *Gracilaria verrucosa* as a matrix to study the morphologic changes of carpospores and the effect of extra environmental factors, irradiance and photoperiods, on the survival of carpospores during their early developing period under laboratory conditions. The cultivation from spores (via sexual reproduction) is better than cultivation from fragments of thalli (vegetative propagation). Indeed, vegetative propagation is inefficient because it requires large amounts of propagating material to start or revitalize mass plantings**.**

Materials and methods

Collection site

The Bizerta lagoon, located in the north of Tunisia (37°8’-37°14’N, 9°46’-9°56’E), has a surface area of 150 Km², with mainly muddy bottom and a maximum depth of 8 m (Commissariat régional au développement agricole de Bizerte CRDA, 2002), a maximum width 11 km, and a maximum length of 13 km. This lagoon is connected to the sea via a canal of 6 km long, as well as to Ichkeul Lake to the east via the 5 km long river of Tinja. The Bizerte lagoon has been exploited in conchyliculture since 1964. At the same time this area represented a receptor of several industrial wastes, pesticides and chemical fertilizers through soil erosion and runoff. Saline and temperature variations induced by meteorological and

climatic conditions are relevant. In summer salinity increases up to 40 ‰ (Ksouri *et al.*, 1999).

Treatment of thalli used for carpospores' release

Forty reproductive mature cystocarpic and tetrasporic thalli *G. verrucosa* were collected in April 2008 from the lagoon of Bizetre. The main period of reproduction was in spring when annual maximum density coincided with a high percentage of fertile thalli. The active growth periods of *Gracilaria verrucosa* are characterized by slightly important temperatures and light intensity. Two hundred fertile carposoprophytic fronds were selected and rinsed in distilled water twice. Then 100 g of fertile fronds were kept in Crystallizing vessel each containing 1000 mL of sterile seawater under 20°C and with a constant day length (12:12).

To inhibit the growth of bacteria and Cyanophyceae, 1 mL/L Sodium Penicillin G (Orduna-Rojas and Robledo, 1999) was added to the medium because bacteria can inhibit algal spore germination (Egan *et al.*, 2001). Five mL/L of germanium dioxide (stock solution of 1:1 mg GeO_2 ml of distilled water) were added to suppress growth of diatoms (Lewin, 1966). The determination of carpospores distribution were observed directly under light microscope (Nikon, type 104).

Effect of photon flux density upon carpospores

After 48 h, segments of 10 cm were eliminated and twenty carpospores were isolated using a capillary Pasteur pipette and placed in Petri dishes with enriched seawater with 34 g/L (McLachlan, 1979). The Petri dishes containing spores were cultured in cultivation chambers. Photons flux density treatments were performed by culturing sets of spores at 4 photons flux densities (15, 40, 75, 100 µmol m^{-2} s^{-1}) under temperature of laboratory.

The culture medium was changed fully every 3 days. During the developing period some of carpospores became colourless and died, specific growth rates of spore lings were calculated when development of the multicellular basal disks and development of young thalli (Polifrone *et al.*, 2006). Parameters of germination rate, growth (diameter) and survival rate of carpospores were determined after six weeks. We used 20 samples per treatment and these experiments were repeated 3 times. Diameters were measured directly using a microscope with a ruler.

Effect of photoperiod

Three photoperiods were studied by culturing sets of spores: 8 h light : 16 h dark (winter conditions), 12 h light : 12 h dark (intermediate conditions) and 16 h light : 8 h dark (summer conditions). Under temperature of 22°C, light of 40 µmol.m^{-2}.s^{-1} provided by cool-white fluorescent tube of 40 W.

Results

Development of carpospores under different photon flux densities

The effect of different photon flux densities on the development of carpospores were showed in figure 1. Analysis indicated that variance of photon flux densities had significant effect on three parameters.

It is shown that optimal germination rates were obtained at 75 µmol m^{-2} s^{-1} photon flux density, whereas the minimum value appeared at 15 µmol m^{-2} s^{-1}.

The values of diameter in the range of 15 to 75 µmol m^{-2} s^{-1} increased with the enhancing of photon flux density, nevertheless the value significantly declined with higher photon flux density (100 µmol m^{-2} s^{-1}). Survival rates of carpospores slightly increased with the increase of the photon flux densities, but the minimum value appeared at 100 µmol m^{-2} s^{-1}. Statistics is missing.

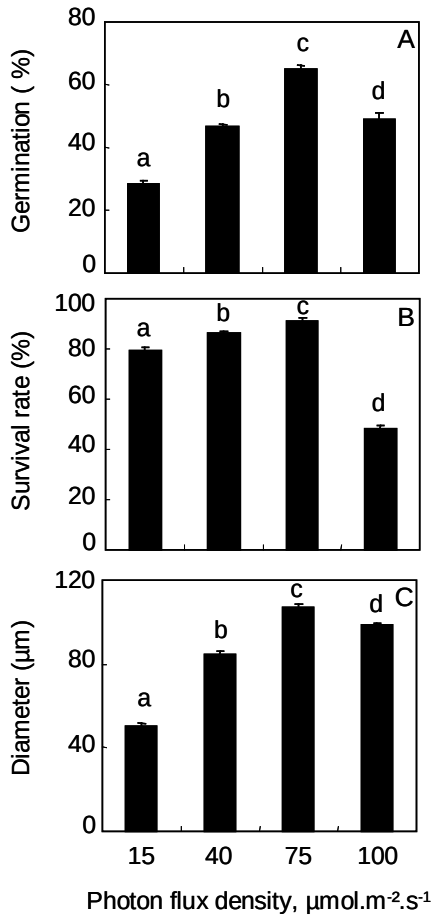

Fig. 1. The effect of light intensity on the germination rate (A), survival rate (B) and diameter of carpospores (C) cultured under room temperature. Means of 20 plants and confidence intervals for p = 0.05. Means sharing a same letter are not significantly different at *p* = 0.01 (ANOVA, and post hoc mean comparison with Newman-Keuls test).

Development of carpospores under different photoperiods

The effect of photoperiods evaluated by the same three parameters and same times as temperature on the development of carpospores were in remarkable differences (Fig. 2). The analysis indicated that except germination rate, the photoperiod variations significantly affected the survival rate and growth (diameter). At the photoperiod of 16 h light / 8 h dark the survival rate is detected to be around 56%, which was significantly lower than for other photoperiods. Concerning the growth (diameter), 12 h light / 12 h dark is the optimal photoperiod.

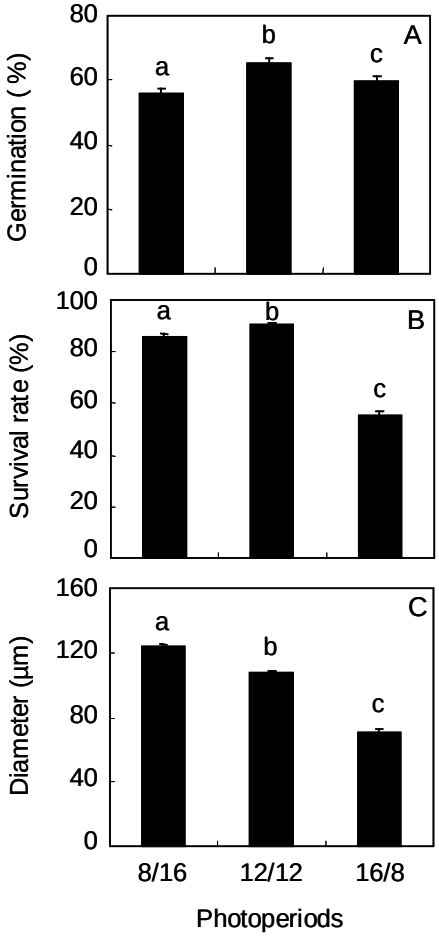

Fig. 2. The effect of Photoperiods on the germination rate (A), survival rate (B) and diameter of carpospores (C) cultured under room temperature. Means of 20 plants and confidence intervals for p = 0.05. Means sharing a same letter are not significantly different at $p = 0.01$ (ANOVA, and post hoc mean comparison with Newman-Keuls test).

Morphological change of carpospores under different photon flux densities

G. verrucosa released a large amount of spores able to settle down, then to attach and develop to new individuals. Carpospores appeared on slides as dark red, spherical cells approximately 25 µm in diameter with some undergoing cell division after 72 h (Fig. 3).

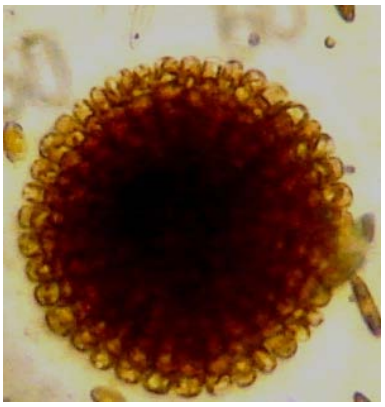

Fig. 3. Multicellular disk originated from carpospores (80 µm).

Carpospores adhered to the substratum within 24 h after they were released. The germination and development of initial thalli from carpospores at 15 µmol m^{-2} s^{-1} photon flux density were slower than from carpospores at 75 µmol m^{-2} s^{-1} (105 µm of diameter, Fig. 4).

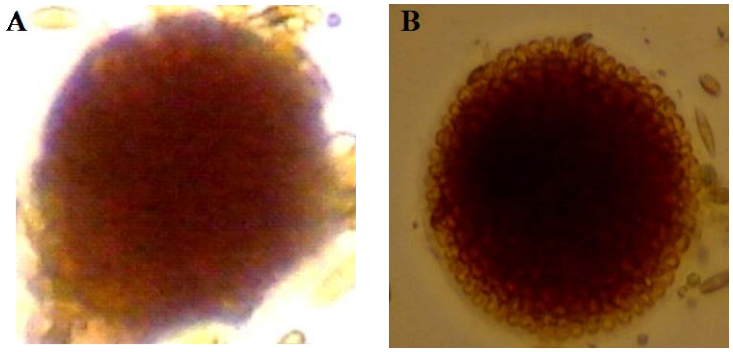

Fig. 4. The first division of carpospores, two weeks after the release. (A) carpospores at 15 µmol $m^{-2}s^{-1}$ (52 µm). (B) carpospores at 75 µmol $m^{-2}s^{-1}$ (105 µm of diameter).

The first division is early for carpospores under high intensities than those provided by low intensities (Fig. 5A). Multicellular basal disks originating from carpospores produced spherical agglomerates of cells (135±5 µm) that were released and able to proliferate to develop to new thalli (Fig .5B).

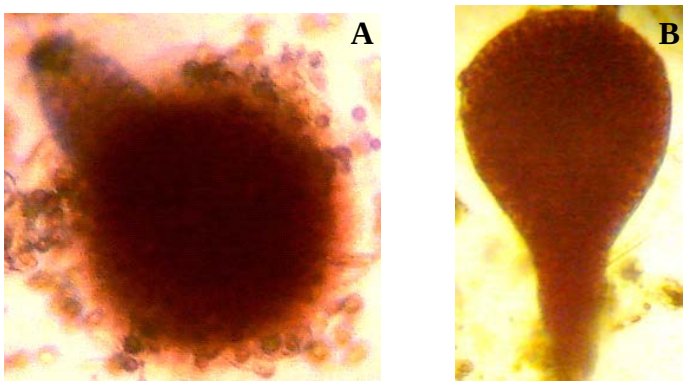

Fig. 5. Gametes with the bourgeon, three weeks after the release (150 µm) (A). Proliferation in to new Thallus (200 µm) (B).

Morphological change of carpospores under different photoperiods

The evolution of development of carpospores at photoperiods of 8 h light : 16 h dark (winter conditions), 12 h light : 12 h dark (intermediate conditions) are more important than for 16 h light : 8 h dark (summer conditions). Further, cell divisions formed a multicellular disk, which divided into more planes due to the rapid multiplication of cells, forming an erect cylindrical thallus (Fig. 6A). After one month, the young carposporophytes from photoperiods 12 : 12 showed an important higher growth rate than carposporophytes from photoperiods 16 : 8. Release carposopres bourgeoned with one or two erect axis (Fig. 6B).

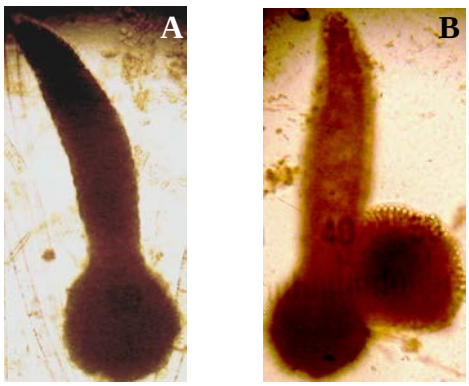

Fig. 6. Young Thallus of *G. verrucosa* (0.6 cm) (A). Carposopres bourgeoned with two erect axis (B).

Discussion

The increasing demand of agar in several countries caused a decrease of natural population of *Gracilaria*, give prominence to the necessity of developing a method to recover the productivity of this species.

In practice, therefore, efforts to develop cultivation of this species through control of its reproductive cycle should focus on the potential use of tetraspores and carposopores as seed stocks. Culture of tetraspores in a season can result in pure gametophytes according on the purpose (Ye *et al.,* 2006). At present the dominant method for cultivation is the use of vegetative fragments as propagating units to provide new plants but the development of more suitable methodologies is urgently needed (Ye *et al.,* 2006). Small-scale culture studies have demonstrated the feasibility of growing *Gracilaria* from either carpospores or tetraspores (Destombe *et al.,* 1993, Alveal *et al.,* 1997; Glenn *et al.,* 1996; Mantri *et al.,*, 2009).

The results of this study demonstrate the technical possibility of culturing *Gracilaria* from isolated carpospores under laboratory conditions. Our objective is to scale up a combination of different environmental factors and their influence on the attachment of carpospores. This technique is a potential source of material for the edible seaweed market. It might be possible to transfer this technique to open seawater using alternative substrata.

Based on the result of the experiments, the optimal conditions for culture of *Gracilaria verrucosa* in the laboratory are: a temperature around 20°C, a light/dark photoperiod of 12/12 and under a light intensity of 40 to 75 µmol m^{-2} s^{-1}). That's why in the lagoon of Bizerte, fertile gametophytes reached the maximum in spring and winter, while they decreased considerably in summer. The disappearance of the alga during the warm season is due to local environmental conditions, including the extreme photoperiodicity, temperature and salinity and the proliferation of *Chaetomorpha linum* which covered the surface of the lagoon. Similar finding have been reported for the population of *G. gracilis* from the Sicilian Lake (Polifrone *et al.,* 2006), but a different behaviour was observed in populations of *G. gracilis* from the Strait of Dover (Destombe *et al.,* 1993).

Conclusion

Carpospores seems to be the best option owing to their rates of settlement and germination, although in tetraspores should also be included. Comparative study of two methods will provide further information about the maintenance of *Gracilaria* populations.

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