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POLYAMINES INFLUENCE MATURATION IN REPRODUCTIVE STRUCTURES OF *GRACILARIA CORNEA* (GRACILARIALES, RHODOPHYTA)[1]

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Naturally occurring levels of putrescine, spermidine, and spermine were analyzed in female gametophyte (nonfertilized) and reproductive tissues (cystocarps) at two different stages of development in the marine red algae *Gracilaria cornea* J. Agardh. Endogenous polyamine levels changed at differential stages of cystocarp maturation. Highest polyamine values were found on tissue from the early post-fertilization stage, decreasing as the cystocarp matured. Incubation experiments revealed that exogenous polyamines induced cystocarp maturation and promoted carpospore liberation, developing cell masses within 4 to 7 days in treatments with spermine. This is the first report on the effect of polyamines on cystocarp maturation in marine algae.

Key index words: **carpospores; cell mass; cystocarp maturation; *Gracilaria cornea*; polyamines; putrescine; spermidine; spermine**

Abbreviations: **PAs, polyamines; PES, Provasoli-enriched seawater; PUT, putrescine; SPD, spermidine; SPM, spermine; TCA, trichloroacetic acid**

Polyamines (PAs), spermidine (SPD), spermine (SPM), and their diamine obligate precursor putrescine (PUT) are small aliphatic amines that are ubiquitous in all plant cells. They have been labeled as a new class of growth substances and a type of plant growth regulator or hormonal second messenger (Galston and Kaur-Sawhney 1990, Lee and Chu 1992, Tiburcio et al. 1993, Gaspar et al. 1997, Scoccianti et al. 2000, Tassoni et al. 2000). The polycationic nature of PAs resulted in covalent bonds with macromolecules and thus affected the synthesis and activity of those molecules. As other plant growth regulators, PAs also react with soluble substances forming conjugates (i.e. phenolic acids). As a result, they can be found in several tissue fractions, such as free acid soluble and bound acid soluble, which must be hydrolyzed to be quantified, and bound acid insoluble, associated tightly to the tissue whose presence are also revealed after hydrolization. The role of each of these fractions remains elusive, but their possible role as a reservoir of PAs that control the endogenous levels of active and free PAs, as occurs with other plant hormone conjugates, has been suggested (Martin-Tanguy 1997, Pandey et al. 2000).

PAs are important for developmental processes such as pollen maturation, germination, and flower development (Smith 1985, Torrigiani et al. 1987, Gerats et al. 1988, Evans and Malmberg 1989). Extensive studies support their role in the modulation of a variety of physiological processes, such as stabilization of cell membranes (Schubert et al. 1983, Roberts et al. 1986, Kaur-Sawhney and Applewhite 1993), stress response (Flores 1990, Aurisiano et al. 1993, Das et al. 1995, Galston et al. 1997, Kakkar et al. 2000), and senescence (Slocum et al. 1984, Evans and Malmberg 1989, Rey et al. 1994, Del Duca et al. 2000). Nevertheless, their precise mode of action is yet to be understood, as is the case for other plant hormones (Walden et al. 1997). In recent years the interest in PA research has increased tremendously and is now being applied to study important agronomic and horticultural crops (Rajam 1997).

In marine algae, PAs have been studied in relation to their occurrence within different algal groups (Hamana and Matsuzaki 1982) and their involvement in cell division (Cohen et al. 1984). Information on endogenous levels, uptake, and transport within the thallus has been reported for *Ulva rigida* (Baldini et al. 1994). Lee (1998) reported accumulation of PUT and

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SPD related to lethal hyposaline stress in several species of intertidal marine macroalgae. Most recently, PAs have been used for studying *in vitro* regulatory events of sporeling morphogenesis for *Grateloupia doryphora* (García-Jiménez et al. 1998, Marián et al. 2000a). To our knowledge, no single study has monitored endogenous levels of PAs during maturation of reproductive structures in marine algae nor the effect of exogenous PAs on its developmental stage, as reported for terrestrial plants (Walden et al. 1997).

Reproduction in red algae occurs by both sexual and asexual processes through the release of sexually or asexually produced spores that are borne in sporangia. The classification of red algal sporangia is best based on morphological attributes (Guiry 1995). Fertilization involves the fusion of a nonflagellated male gamete, the spermatium, with a sessile egg-containing cell, the carpogonium on the female gametangial thalli. After fertilization, the zygote is not released directly but is retained as a parasite on the female gametangial thalli where it develops into a third generation, the carposporophyte. After fertilization, the carposporophyte of the red algae may appear to be surrounded by gametophytic tissue, forming a macroscopically visible reproductive structure, the cystocarp. Cystocarps may appear as small dark spots (1–5 mm diameter) embedded in the thalli as small domoid warts on the surface of branches. The cystocarp may eventually release the carpospores, which serve as the agents of recruitment for the tetrasporophyte generation, a tetraspore producing phase. Tetraspores originate male and female gametangial thalli, completing the life cycle.

In this study we analyzed endogenous levels of PAs at two different stages of cystocarp development, early post-fertilization (immature stage) and mature cystocarp in the carposporophytic phase, of the red macroalgae *Gracilaria cornea* J. Agardh. Furthermore, *in vitro* experiments were conducted to examine the effect of exogenous PAs on cystocarp maturation and carpospore development.

MATERIALS AND METHODS

Plant material. Among the species of *Gracilaria* in the western Atlantic Ocean, the alga known as *G. cornea* is one of the most common and easily recognized. It is widely distributed along tropical and subtropical coasts and is used locally as a source of agar and as a food supplement (Robledo 1998). In *G. cornea* cystocarps are scattered over at least the upper half of the thallus and protrude only moderately, being small relative to the thallus diameter, about 2 mm high and 4 mm wide. From the time of carposporophyte initiation until full maturity, the cystocarp height remains uniform, with the principal expansion in width. The ontogeny of these structures has been reported in detail by Fredericq and Norris (1985).

Female gametophytes without fertilized carpogonia and carposporophytic thallus of *G. cornea* were collected from a natural bed located at Dzilam de Bravo, Yucatán, México. After collection, female gametophytes and carposporophytes were cleaned, brushed, and rinsed several times in sterile seawater to eliminate epiphytes and substrate. The material was maintained 1 week in sterile seawater with Provasoli-enriched seawater (PES) media (Provasoli 1968) at 26° C, 40 μmol photons·m^{-2}·s^{-1}, and an 8:16-h light:dark photoperiod.

In the present study two stages of carposporophyte development were identified and separated based on initial post-fertilization events described by Hommersand and Fredericq (1995) for the order Gracilariales. The first cell derivatives of the fertilized carpogonium give rise directly to gonimoblast filaments and grow over the outermost cortical cells, eventually fusing with them. Additional fusion with neighboring gametophytic cells lead to the formation of a large lobed fusion cell identified by a dark spot on the carposporophytic thallus (immature stage). After the gonimoblast have expanded, filling the cystocarp cavity, they initiate branches of carposporangia and also produce tubular nutritive cells that fuse with pericarp cells or with cells in the floor of the cystocarp, developing a protuberant tissue and leading to cystocarp maturation that is evident by the presence of the ostiole (mature cystocarp). Endogenous levels of PAs were analyzed in female gametophyte without fertilized carpogonia and in the two cystocarp developmental stages described above, easily recognized stages in the *G. cornea* life cycle (Fig. 1).

To evaluate the effect of PAs on cystocarp maturation, 10 mature cystocarps at each stage of development were incubated separately in Petri dishes for 24 h in PES and PES plus common PAs at 10^{-6} M (PES + PUT, PES + SPD, and PES + SPM), which were maintained under 26° C, 40 μmol photons·m^{-2}·s^{-1}, and an 8:16-h light:dark photoperiod, the best conditions for carpospore liberation (Guzmán-Urióstegui and Robledo 1999). Carpospores released from the above experiment were counted in a counting chamber and maintained for 22 days in their original media under 26° C, 20 μmol photons·m^{-2}·s^{-1}, and an 8:16-h light:dark photoperiod to follow carpospore development.

PA analysis. Free (as free cations), bound acid-soluble, and bound acid-insoluble (acid-insoluble forms bound covalently to macromolecules and cell walls) PAs (PUT, SPD, and SPM) were extracted in cold 5% trichloroacetic acid (TCA). PAs were analyzed from 70 to 100 tissue fragments (explants 6 mm length) from nonfertilized female gametophytic and *G. cornea* cystocarps at different stages of maturation in a 1:10 ratio of algae material (g fresh weight):5% TCA (mL). The slurry was centrifuged at 1500*g* for 15 min, and the supernatant was divided in half for the analysis of free TCA soluble and bound TCA soluble, whereas the pellet was used for the analysis of bound TCA-insoluble PAs (Marián et al. 2000a). Bound TCA soluble (200 μL) and bound TCA insoluble (pellet) were hydrolyzed in separated sealed vials with 300 μL of 12 M HCl for 20 h at 100° C to release the bound fraction. After hydrolysis the samples were filtered, dried to evaporate the remaining HCl, and redissolved in 300 μL of 5% TCA for dansylation (24 h, 60° C).

Dansylation of PAs for TLC. The procedure for dansylation and quantification of PAs used a 100-μL sample mixed with 100 μL of a saturated solution of Na_2CO_3 and 200 μL of dansyl chloride (5 mg·mL^{-1} acetone). The dansylation reaction lasted for 10 min at 70° C. Afterward, 50 μL of an aqueous proline solution (100 mg·mL^{-1}) was added to react for 30 min in darkness with the excess dansyl chloride. When the reaction was completed, 500 μL of toluene was mixed with the sample, shaken, and left until the organic and aqueous phases were separated. The organic phase, containing the dansyl-PAs, was dried in a heat-speed vacuum and the residual was dissolved in 600 μL of acetone. The dansyl-PAs were separated with TLC on 7.5 × 2.5-cm plates (F1500/LS, 254, Schleicher & Scuell, Keene, NH, USA). The mobile phase was a chloroform/triethylamine mixture (5:1, v/v). The plates were revealed under UV light (254 nm), and the bands refereed to the dansylated standards of PUT, SPD, and SPM (Sigma, St. Louis, MO, USA). These bands were scraped off the plate and redissolved with 800 μL acetone. The samples were shaken and centrifuged at 6000*g* for 3 min, and 500 μL of this solution was quantified at 365 nm (excitation) and 510 nm (emission) with a high-resolution spectrofluorometer (SFM 25, Kontron Instruments, Zurich, Switzerland). Three replicates were quantified for each sample.

Histology. Mature cystocarps (n = 3) from the incubation experiment were fixed with 5% glutaraldehyde in 10^{-1} M sodium cacodylate buffer containing 0.25 M sucrose (pH 7.4) for 4 h at room temperature (García-Jiménez et al. 1998). This was followed by

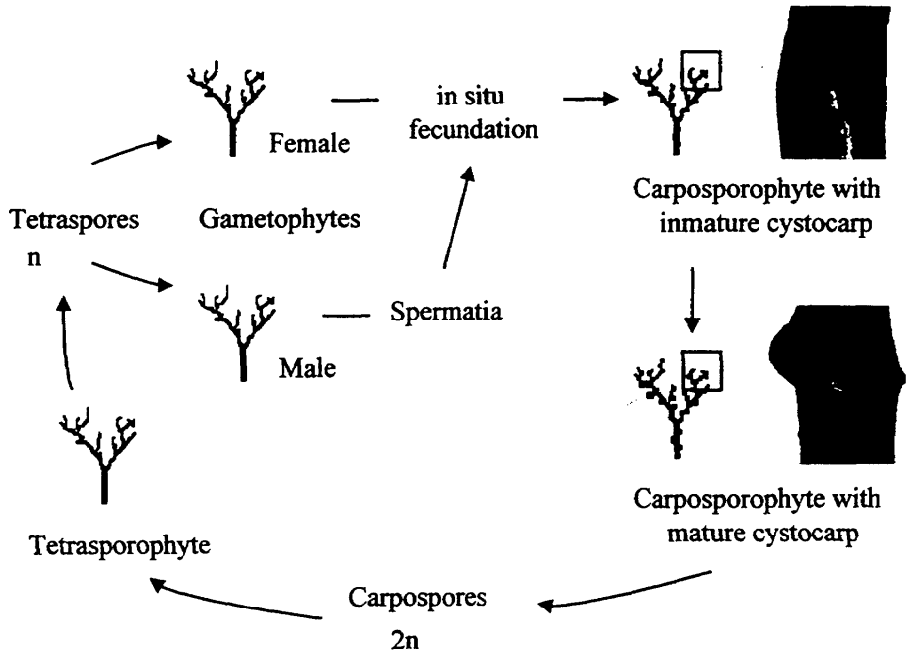

FIG. 1. Triphasic life cycle of *Gracilaria cornea*, showing the external morphology for the two stages of cystocarp maturation studied within the carposporophytic phase.

four washings (one each for 30 min) in the same buffer containing sucrose in descendant concentrations (0.25, 0.12, 0.06, and 0 M) and embedded in glycol methacrylate (Historesin™, Leica, Nussloch, Germany) (Gerrids and Smid 1983). Serial sections 5-mm thick were cut on a Reichert-Jung 2050 microtome and stained for the observation of general morphology with toluidine blue (Tsekos 1983).

Statistical analysis. Statistical analysis was performed with Statgraphics Plus for Windows, version 2.1 (Manugistics, Rockville, MD, USA). The data were analyzed by *t*-tests to find out differences between endogenous PA levels at different developmental stages of the cystocarp and to determine differences between female gametophyte and each stage of maturation of the cystocarp.

TABLE 1. Endogenous PA contents ($\mu g \cdot g^{-1}$ fresh weight) reported in other studies for marine macrophytes and the present study for *Gracilaria cornea*.

	PUT			SPD			SPM		
	Free	BS	BI	Free	BS	BI	Free	BS	BI
Ulva reticulata Forsskål[a]	120	12	1	15	7.5	1.75	3	1.3	0.12
Ulva lactuca Linnaeus[a]	120	45	1	22.5	2	1.75	3	0.4	0.3
Ulva fasciata Delile[a]	22	22	1	5	13	0.5	4	0.22	0.2
Chaetomorpha crassa (C. Agardh) Kützing[a]	42	60	3	6.2	2.5	0.3	6	1.7	0.3
Valoniopsis pachynema (Martens) Børgesen[a]	50	20	2	15	20	1	1	0.75	0.15
Dictyota dichotoma (Hudson) Lamouroux[b]	920	—	19,000	2.3	—	50	1.1	—	20
Gelidium canariensis (Grunow) Seoane-Camba[b]	1500	—	23,000	3	—	28	9	—	29
Grateloupia doryphora (Montagne) Howe[b]	700	—	30,000	5	—	30	3	—	30
Cymodocea nodosa (Ucria) Ascherson[c]	647	10,210	2926	8	15.98	1.42	3.58	24.48	3.32
Gracilaria cornea J. Agardh[d]									
Female gametophyte	390 ± 40	268 ± 28	3140 ± 128	1.9 ± 0.2	1.2 ± 0.4	2.3 ± 1.5	3.8 ± 0.6	1.2 ± 0.04	8.5 ± 1.9
Immature cystocarp	433 ± 13	227 ± 59	4476 ± 6	5.4 ± 0.3	1.9 ± 0.7	7.7 ± 2.9	7.6 ± 2.9	2.6 ± 0.96	15.0 ± 1.2
Mature cystocarp	262 ± 12	240 ± 20	2239 ± 143	3.0 ± 0.5	0.8 ± 0.3	4.2 ± 2.4	5.8 ± 0.1	1.0 ± 0.21	10.8 ± 1.5

[a] Lee 1998.
[b] Marián et al. 2000a.
[c] Marián et al. 2000b.
[d] This study.
—, Not reported; BS, bound acid soluble; BI, bound acid insoluble.

RESULTS

Endogenous levels of PAs. PA analysis revealed that PUT was the predominant amine, followed by SPM and SPD. The bound acid-insoluble forms of PAs were more abundant, followed by the free and bound acid-soluble forms. This was more evident for PUT, where a 10-fold difference was observed. PA contents were in range with those obtained for other algae (Table 1).

The different forms of PAs significantly increased ($P < 0.05$) from female gametophyte (nonfertilized carpogonia) to immature cystocarp (early post-fertilization), except for PUT free and bound acid soluble and SPD bound acid soluble (Fig. 2). Mature cystocarps had lower levels of PUT (free, bound acid soluble, and bound acid insoluble), SPD (bound acid soluble), and SPM (bound acid soluble) than female gametophytes.

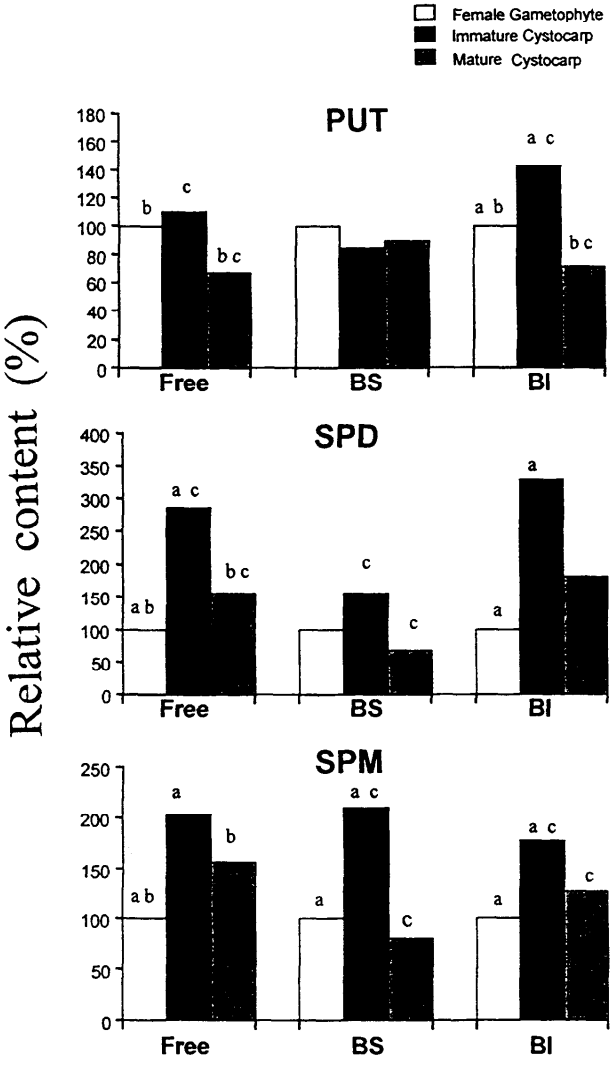

FIG. 2. PA contents at various stages of cystocarp development in the female gametophyte of *Gracilaria cornea*. PA contents in nonfertilized female gametophyte are 100%. BS, bound acid soluble; BI, bound acid insoluble. Same letter indicates significant differences ($P < 0.05$) between female gametophyte, immature cystocarp, and mature cystocarp.

The concentrations of all three PAs were higher in immature cystocarps (early post-fertilization stage) than in mature cystocarps. PA analysis for immature cystocarps revealed that the PUT content (bound acid insoluble) was highest, decreasing for mature cystocarps; this was also observed for all forms of SPD and SPM.

PA incubation experiments. Histological observations on mature cystocarps after PA incubation showed the effect of exogenous PAs on maturation of cystocarp. Exogenous addition of PAs promoted high disorganization of carposporangial branches inside cystocarps. This disorganization was more evident for SPM (Fig. 3, A and B).

Incubation of cystocarps in PAs promoted carpospore release; the number of carpospores liberated increased at least 40% when compared with liberation in PES (Table 2). A higher number of carpospores were liberated in SPM, with a lower output in PUT and SPD. During cultivation in media with PAs, the growth and development of carposporelings was enhanced. In PES, successive cellular divisions were evident until the formation of one main axis after 21 days of release, whereas PAs increased cell division during carpospore development, produced cell masses, and induced morphogenesis. In PUT, cell masses were formed between 12 and 15 days after release. A most evident effect on cell division was observed in SPM, where cell masses were obtained after 4–7 days. Differences in axes number and size were also evident for cell masses obtained in treatments with PAs. In PUT axes were larger, whereas in SPD and SPM more than one axis developed (Fig. 3, C–F).

DISCUSSION

PA patterns differ greatly among algae not only in the amount of PUT, SPD, and SPM, but also in the amount of free, bound acid-soluble, and bound acid-insoluble fractions. Endogenous levels of PAs in *G. cornea* revealed a higher amount of PUT. The presence of PAs has been reported for other algae, with PUT being the most abundant in green algae (Baldini et al. 1994, Lee 1998). SPM in *G. cornea* was found in higher amounts than SPD, contrary to trace levels of SPM found in green algae (Hamana and Matsuzaki 1982). Nevertheless, PA levels in the nonreproductive (female gametophyte) and reproductive tissue (cystocarps) of *G. cornea* are within the range described earlier for other algae.

A natural increase in endogenous levels of PAs during cystocarp development at early post-fertilization events was evident when compared with PAs found in female thalli without fertilized carpogonia. Our results suggest that endogenous PAs levels in reproductive tissue of *G. cornea* are associated with differential stages of cystocarp maturation. Carposporophyte evolution has involved the progressive modification of gametophytic tissues and the structural and functional compartmentalization of the cystocarp. Hommersand and Fredericq (1995) recognized three compartments of the cystocarp: the outer photosynthetic tissues, the modified inner ga-

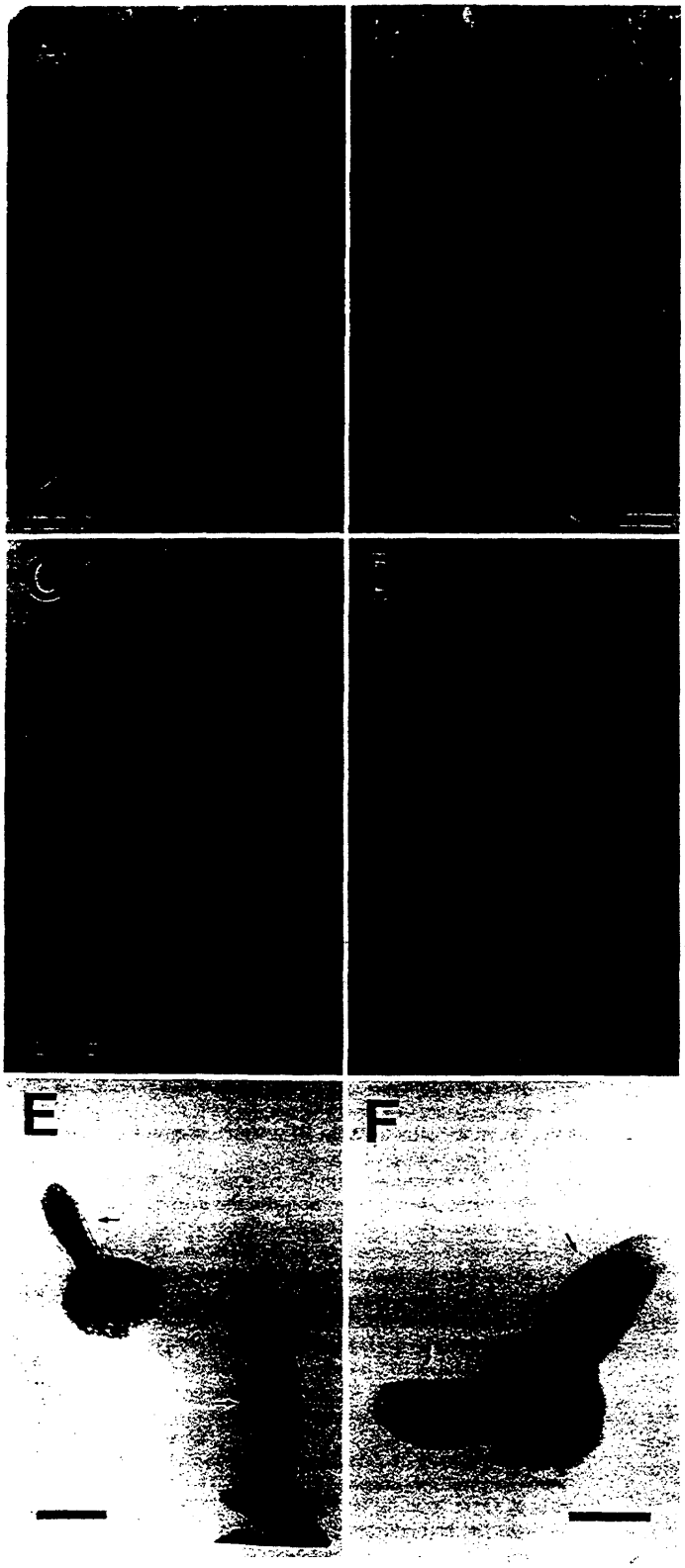

FIG. 3. Cystocarp and carpospore development in *Gracilaria cornea*. (A) Longitudinal section of mature cystocarp incubated in PES and (B) in PES plus SPM 10^{-6} M: Note differences in the arrangement of carpogonial branches (arrows). (C) Cell masses originated from carpospores in PES plus SPM 10^{-6} M, 7 days old and (D) carpospore development in PES, 22 days old. Axes development after 22 days in (E) PUT 10^{-6} M and (F) SPM 10^{-6} M. Scale bar, 0.2 mm.

TABLE 2. Carpospore release of *Gracilaria cornea* mature cystocarps after incubation for 24 h in PES and PES plus common PAs at 10^{-6} M.

Incubation media	Carpospores·cystocarp^{-1}
PES	1223 ± 231
PES + PUT	1870 ± 209[a]
PES + SPD	1922 ± 380[a]
PES + SPM	2418 ± 496[a]

[a]Significant differences ($P < 0.05$) in carpospore release between PES media and PES plus PAs.

metophytic tissues that process and store the metabolites of photosynthesis, and the developing carposporophyte.

Increased nutritional dependency on the gametophyte is evident in the fact that carposporophyte growth is not a continuous process; it proceeds in stages, with periods of rapid growth followed by intervals of slow growth or apparent inactivity during which the pattern of development may change. These patterns of development may be attributed to endogenous PA levels, as is shown in this study; PAs were up- and down-regulated during cystocarp development. Each stage is preceded by the transformation of existing gametophytic cells into special protein-rich tissues, or new secondary gametophytic tissues are formed that are rich in protein content. Fusion cell formation and organelle autolysis is a process of senescence designed to cannibalize compounds, particularly nitrogen containing substances, for use during the final stages of carposporophyte development (Hommersand and Fredericq 1995). This may explain lower endogenous levels of PAs found in mature cystocarps of *G. cornea*. Endogenous levels of PAs have not been related to physiological events in algae, except for those described by García-Jiménez et al. (1998) and Marián et al. (2000a) on morphogenesis during *in vitro* cultivation of the red algae *Grateloupia doryphora* and *Gelidium canariensis*.

It is also evident from the incubation experiment that short exposure to PAs in cystocarps at different development stages enhanced cell division and growth of carpospores, most probably due to the increased cellular PAs levels. High levels of free PAs (PUT) have been reported in growing and dividing tissues of higher plants (Kaur-Sawhney et al. 1989). Moreover, when cystocarps were incubated in PAs, they induced cystocarp maturation, seen as the number of carposporangial lines and mature carpospores, particularly with SPM, in contrast to maturation process without PAs, as observed in the histological sections. In the green algae *Acetabularia* the inhibition of ornithine descarboxylase, a key enzyme of PA metabolism, profoundly affects the development of nucleate and anucleate fragments (Brachet et al. 1978). Besides regulation of cystocarp maturation by endogenous PAs levels, our results also suggest that PAs may promote carpospore release and further growth and development.

In conclusion, PA levels during development of the reproductive structures (cystocarp) in the red algae *G. cornea* increased from nonfertilized tissue (female gametophyte without fertilized carpogonia) to early post-fertilization event (immature cystocarp), most probably due to increased cell division. Addition of exogenous PAs during the final stages of carposporophyte development promoted carpospore release, cell division, and morphogenesis. Further studies are needed to understand the mode of action of PAs and other plant hormones.

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