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INFLUENCE OF POLYAMINES ON THE SPORULATION OF *GRATELOUPIA* (HALYMENIACEAE, RHODOPHYTA)[1]

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Polyamines (PAs) such as putrescine (PUT), spermidine (SPD), and spermine (SPM) are ubiquitous aliphatic amines involved in widely varying physiological behavior, but particularly they are actively involved in cell growth, division, and differentiation during reproductive events in plants. The contents of PUT, SPD, and SPM in infertile and fertile thalli of the red macroalga *Grateloupia* sp. were compared, and the results revealed a significant decrease in quantity from infertile to fertile status. At the enzymatic level, L-ornithine decarboxylase (ODC) was mainly detected, and L-arginine decarboxylase activity was not diminished by the inhibition of ODC. The maximum enzymatic activities, within the range of activities observed, correlated with the lower levels of polyamines in fertile thalli. In culture, SPM promoted the maturation of cystocarps to the eventual liberation of spores from aseptic fertile explants. PAs accumulated in cultivated explants as compared with noncultivated, but exogenous SPM addition further increased the endogenous SPM. The addition of berenyl, cordycepin, cyclohexylamine, dicyclohexylamine, and aurintricarboxylic acid blocked the synthesis in culture at the level of PUT, and partially at SPD and SPM synthesis, but the addition of SPM restored the levels of SPD and SPM as SPM accumulated, and they appeared to interconvert each other. The results obtained suggest that the culture in presence of SPM restored a deficient SPM situation in fertile explants, thus promoting sporulation.

Key index words: **arginine decarboxylase; *Grateloupia*; ornithine decarboxylase; polyamines; spermine; sporulation**

Abbreviations: **ADC, arginine decarboxylase; DFMO, DL-α-difluoromethylornithine; DTT, dithiothreitol; ODC, ornithine decarboxylase; PAs, polyamines; PMSF, phenylmethylsulfonyl fluoride; PUT, putrescine; SPD, spermidine; SPM, spermine**

The polyamines (PAs) putrescine (PUT), spermidine (SPD), and spermine (SPM) are small, ubiquitous, aliphatic amines usually found in all organisms studied so far and play multiple physiological roles. They have been classified as a new group 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). Extensive studies support the fact that they modulate 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). Their important role at the cellular level has justified extensive studies to clarify, at the molecular level, their function as modulators of cell viability or stimuli for cell proliferation (Igarashi and Kashiwagi 2000). In higher plants, PAs are actively involved in reproductive processes such as pollen maturation, germination, and flower development (Smith 1985, Torrigiani et al. 1987, Gerats et al. 1988, Evans and Malmberg 1989, Walden et al. 1997).

The commonly occurring PUT is predominantly synthesized by the key enzyme ornithine decarboxylase (ODC, EC.4.1.1.17) from the nonproteic amino acid L-ornithine. In higher plants, arginine decarboxylase (ADC, EC.4.1.1.19) is also involved in the production of the diamine PUT, via decarboxylation of the arginine and deamination of the intermediate agmatine. One or two aminopropyl groups, resulting from the decarboxylated S-adenosylmethionine, are

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then added to convert PUT into SPD or SPM, respectively. SPM may be degraded to SPD and PUT by the cooperation of SPM/SPD n(1)-acetyltransferase and polyamine oxidase (Fig. 1). It is possible to inhibit ADC and ODC using the irreversible suicide inhibitors DL-α-difluoromethylarginine and DL-α-difluoromethylornithine (DFMO), respectively. Berenyl, cordycepin, cyclohexylamine, dicyclohexylamine, and aurintricarboxyilic acid (Fig. 1) have also been reported as less specific inhibitors (Marton and Pegg 1995, Bouchereau et al. 1999).

In marine algae, PAs have been studied in respect to their occurrence in various algal groups (Hamana and Matsuzaki 1982) and their involvement in cell division (Cohen et al. 1984, García-Jiménez et al. 1998). In marine macrophytes, data concerning the endogenous level and uptake by and /or transport within the thallus have been given for *Ulva rigida* (Badini et al. 1994) and several red and brown algae and also for the marine spermatophyte, *Cymodocea nodosa* (Marián et al. 2000a). In the green alga *Acetabularia*, the inhibition of ODC affects the development of nucleate and anucleate fragments (Brachet et al. 1978). Lee (1998) reported the accumulation of PUT and SPD in response to lethal hyposaline stress in several species of intertidal marine macroalgae. Marián et al. (2000b) showed that addition of DFMO caused a decrease in growth rates and morphogenesis of sporelings of *Grateloupia* in glycerol-containing media.

Recently, Guzmán-Urióstegui et al. (2002) provided evidence for the implication of PAs in reproductive events in algae by reporting variations in the endogenous levels of PAs in different stages of cystocarp maturation in *Gracilaria cornea*. In Cryptonemiales algae such as *Grateloupia*, the cystocarps may appear in the female gametophyte as small dark spots (less than 1 mm in diameter) completely embedded in the thalli, which can be distinguished under the stereomicroscope in natural collected samples, though the extent of their maturation is not as evident as in *Gracilaria cornea*. Explants from gametophyte thalli bearing cystocarps may release spores if cultivated under axenic culture conditions (García-Jiménez et al. 1996, Rodrigo and Robaina 1997).

The aim of this work was to compare the endogenous levels of the PAs of gametophyte thalli with and without cystocarps (henceforth fertile and infertile axes, respectively) from samples collected in the field. Understanding the role of PAs in alga reproduction may require more than a mere description of the characteristics of changes in the endogenous contents. Therefore, in this work we checked for the enzymatic

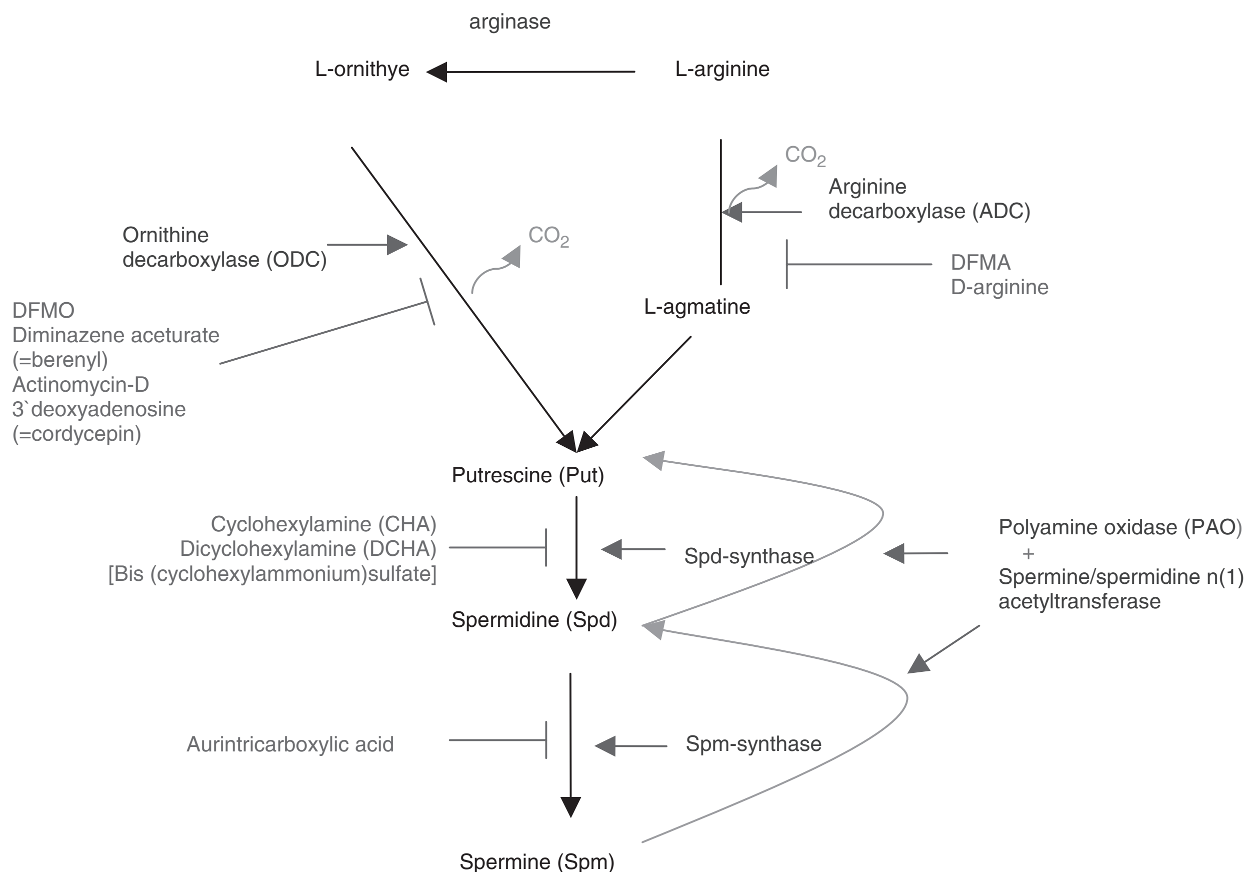

FIG. 1. Routes of the metabolic synthesis of PAs from L-arginine and L-ornithine with the participating enzymes and their inhibitors (data were compiled from various bibliographic sources).

activities of the enzymes responsible for the synthesis of PAs and worked upon the hypothesis that enzymatic activity must correlate with changes in endogenous content during the development of the cystocarp. We focused on whether the starting points of the biosynthetic route, the ODC and/or ADC, were present in the thallus. We also tested the effect of the addition of exogenous concentrations of PUT, SPD, and SPM on axenic samples cultured *in vitro* to determine their endogenous levels and whether sporulation was affected.

MATERIALS AND METHODS

Plant material. Grateloupia (Halymeniaceae, Rhodophyta) is an intertidal red alga found along the northeast coast of Gran Canaria (Canary Islands, Spain). Formerly defined as *G. doryphora* in the taxonomic checklist for Canarias (Haroun et al. 2002), its taxonomic position is now being revised since the work of Gavio and Fredericq (2002). A voucher specimen of the species used in this and previous works was deposited as sheet 6100 in the BCM herbarium (Biology Department, University of Las Palmas de Gran Canaria, Canary Islands). Thalli were collected and processed within 2 h after collection. Infertile and fertile axes were identified, separated, and used for analysis and/or culture.

Extraction and dansylation of PAs for HPLC. PAs are polycationic substances that can be found in three different cell fractions: free acid soluble, conjugated acid soluble (i.e. bound to phenolic compounds), and conjugated acid insoluble (i.e. conjugated to macromolecules). All fractions were extracted with cold 5% perchloric acid in a 1:10 ratio of material (g fresh weight:mL) from infertile and fertile samples ($n = 3$). The supernatant containing the acid-soluble fractions (free and conjugated) and the pellet with the insoluble PAs were separated. The pellet and 300 μL of the supernatant were hydrolyzed with 300 μL of 12 M HCl overnight. After hydrolysis, the samples were filtered and dried and resuspended in 300 μL of perchloric acid for derivatization. Dansylation and HPLC quantification of PAs were carried out according to the method described by Marcé et al. (1995).

ODC and ADC enzyme activity analysis. Samples of fertile and infertile axes ($n = 30$) were processed within 2 h of collection. They were ground in a mortar with liquid N_2 and proteins extracted in phosphate buffer (100 mM, pH 7.5), 10 mM dithiothreitol (DTT), 0.3 mM pyridoxal phosphate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The slurry was centrifuged (5000 g, 4 min, 4° C), the pellet was discarded, and 250 μL (0.3–0.6 mg proteins $\cdot$ mL^{-1}) of the supernatant was used for each enzyme activity quantification.

ODC activity was assayed in Warburg respirometer vessels containing 0.3 μCi of L-[1-^{14}C]ornithine (52 mCi $\cdot$ $mmol^{-1}$, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and L-ornithine (0.7 mM total L-ornithine in the reaction mixture) in 250 μΛ crude extract in 100 mM K-phosphate buffer, pH 7.5, 10 mM DTT, 1 mM PMSF, 0.3 mM pyridoxal phosphate. ADC was assayed in incubations containing 0.21 μCi of L-[U-^{14}C]arginine (300 mCi $\cdot$ $mmol^{-1}$, Amersham Biosciences, UK) and L-arginine (0.8 mM total L-arginine) in 250 μL crude extract in 100 mM K-phosphate buffer, pH 7.5, 10 mM DTT, 1 mM PMSF, 0.3 mM pyridoxal phosphate. The $^{14}CO_2$ released in 60 or 30 min for ODC and ADC, respectively, was trapped in 100 μL Solvable trapping solution (NEN, Northampton, UK) and counted using Formula 989 (NEN) as scintillant. Protein concentration was determined using detergent and reductant compatible system for protein quantification and BSA as standard (RCDC BioRad, Hercules, CA, USA). To check for the conversion of L-arginine into L-ornithine by arginase and its further decarboxylation by ODC, instead an actual ADC activity, 3 mM of DFMO was added to the enzyme reaction mixture in some infertile samples ($n = 4$).

Culture conditions. The culture conditions used to test the combined effects of PUT, SPD, and SPM were defined using the simplified block statistical design Box-Behnken (Box and Behnken 1969) for three factors (PUT, SPD, and SPM) and at three levels (low, 10^{-9} M; null, 0; and high concentrations, 10^{-6} M) as generated by the statistical software (see below). Response surfaces were obtained for the variable "mature cystocarps" (i.e. number of cystocarps that mature and release spores after 15 days of culture). Three replicates (three petri dishes with three explants in each) were used for each treatment. This design was run in a single block that was repeated twice with the same results.

Provasoli enriched seawater medium (Provasoli 1968) was used as a base medium. All cultures were maintained aseptically, using the previously described methods (Robaina et al. 1990a,b) at 20 ± 2° C and 30 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ in petri dishes in a growth chamber.

Effect of exogenous SPM on endogenous PAs. Based on the results obtained in previous experiments, we focused on the effect of the addition of SPM (10^{-6} M) on endogenous content of free PUT, SPD, and SPM in the presence or absence of inhibitors (10^{-6} M berenyl, cordycepin, cyclohexylamine, dicyclohexylamine, or aurintricarboxyilic acid, Sigma Inc., St. Louis, MO, USA) to check whether SPM was being accumulated and/or transformed into other PAs. Extraction and dansylation of the free fraction of PAs were as described above.

Statistical analysis. Statistical design and calculations were carried out using Statgraphics Plus 3.1 for Windows (Statistical Graphics Co., Rockville, MA, USA).

RESULTS

Content of PAs and enzyme activities of ADC and ODC in natural collected samples. As shown in Figure 2, the contents of PUT, SPD, and SPM in samples from fertile axes were significantly lower than those found in infertile samples, particularly in the acid free fractions. Table 1 shows the range of specific activities of ADC and ODC in crude extracts from fertile and infertile samples. Maximum activities were observed for both ODC and ADC in infertile thalli, with ODC always the highest record. The addition of DFMO inhibited the decarboxylation of L-orthinine but not that of L-arginine.

Effect of exogenous PAs and sporulation in vitro. The culture of the axenic explants in three concentrations of the PAs produced the results shown in Figure 3, which represents the response surface for the variable called mature cystocarps. The analyses of the block design clearly detected SPM as the main factor affecting sporulation, because it increased significantly the amount of cystocarps that matured and released carpospores. In the response surface, the presence of SPM at 10^{-9} M (Fig. 3, −1) and 10^{-6} M (Fig. 3, 1) enhanced the value of this variable, whereas a decrease was witnessed when this PA was absent (value 0) (Fig. 3 A and B). The presence of PUT or SPD clearly decreased the number of maturing cystocarps. Also, it seems that spores were mostly liberated when totally absent (Fig. 3C).

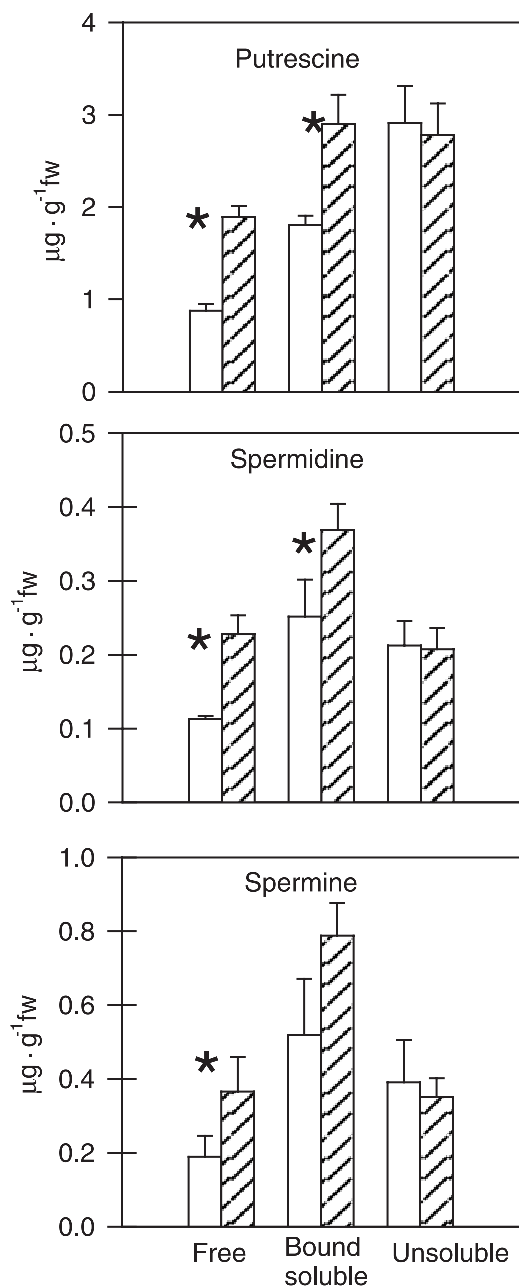

FIG. 2. Contents of PAs in the free, bound, soluble and insoluble fractions in infertile samples (i.e. axes from female gametophyte without evident carpogonia [solid bars]) and fertile female gametophyte with cystocarps (open bars). $n = 3$; vertical bars are SE. $^{*}P < 0.05$.

The results in Figure 4 show that as compared with freshly collected material, the endogenous levels of PAs increased significantly in cultivated explants. The addition of inhibitors totally suppressed the production of PUT and partially that of SPD and SPM. The addition of SPM raised the levels of SPM in all treatments except in berenyl and SPD except in cordycepin, but PUT was not affected. The explants cultivated in SPM without inhibitor showed the highest endogenous levels of SPM.

TABLE 1. ADC and ODC enzymatic activities (nmol $^{14}CO_2$ liberated · mg^{-1} protein · h^{-1}) in samples from fertile and infertile axes of the thalli ($n = 30$).

	Substrate	
Sample	L-Arginine	L-Ornithine
Fertile	n.d. to 0.73	0.10–2.63
Infertile	0.08–5.15	0.02–16.6
Effect of the inhibitor DFMO on infertile		
– DFMO	0.85 ± 0.12	1.56 ± 0.82
+ DFMO	0.73 ± 0.08	0.15 ± 0.07[a]

Data are the range of activities observed. n.d., not detected. Data in DFMO experiments came from crude extracts of infertile thalli, divided into subsamples with or without inhibitor. Data are means ± SE from $n = 4$ samples.

[a]$P < 0.05$.

DISCUSSION

In Guzmán-Urióstegui et al. (2002), a sharp increase in endogenous levels of PAs during cystocarp development was detected at early postfertilization events as opposed to the PAs found in female thalli without fertilized carpogonia and mature cystocarp. These results suggested that endogenous PAs levels in reproductive tissue of *G. cornea* may be associated with differential stages of cystocarp maturity (Hommersand and Fredericq 1995). New evidence with respect to the PA implication during cystocarp maturity is provided in this work in which infertile axes from gametophyte thalli clearly accumulated more PAs than those axes with cystocarps (Fig. 2) at the same time that they reached maximum enzymatic activities (Table 1). As opposed to what occurs in the case of *G. cornea*, it is difficult to distinguish more stages of cystocarp development in *Grateloupia* than those which were assumed on the basis of conspicuous absence or presence of cystocarp spots, and probably multiple stages of maturation actually occurred within samples that were assumed to belong to only two, infertile and fertile, classes. Regardless, the results in the latter species, as in the case of the former, indicate a decrease (perhaps deficiency) in the amount of PAs and enzymatic activity as the cystocarp matures.

The results in Table 1 would appear to indicate the presence of the two enzymatic activities, ADC and ODC, as responsible for PAs synthesis in *Grateloupia*, though ODC seems to be more active. The cooperation of arginase and ODC in the dercaboxylation of L-arginine (Primikirius and Roubelakis-Angelakis 1999) should be discarded because DFMO, an inhibitor of ODC, did not inhibited arginine decarboxylation. In higher plants, it is assumed that PA biosynthetic pathways proceed via the ADC and ODC enzymes; the ADC is an alternative route mainly reported in the chloroplasts, and it is linked to stress responses, whereas the ODC is an ubiquitous enzyme occurring in the cytosolic and nuclear fractions, and it is possibly related to cell division (Slocum et al. 1984, Evans and Malmberg 1989, Tiburcio et al. 1993). Although this simplistic

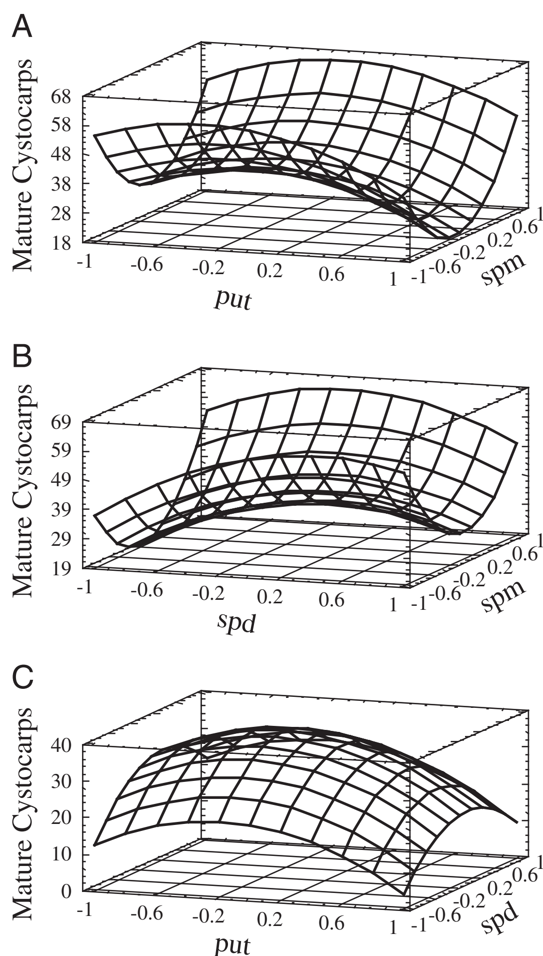

FIG. 3. Response surfaces resulting from simplified block design in which explants were cultivated axenically in media containing 10^{-9} M (−1), 0 M (0), or 10^{-6} M (1) of PUT, SPD, and SPM. The response variable (mature cystocarps) represents the number of cystocarps that mature up to time of liberation of spores after 15 days in culture. (A) Combined effects of SPM and PUT, (B) effects of SPM and SPD, and (C) effects of SPD and PUT.

picture of the roles and distribution of ADC and ODC is now being revised (Bouchereau et al. 1999, Hanfrey et al. 2001, Dudkowska et al. 2003), the results in this work made PA synthesis in *Grateloupia* closer to higher plants than to organisms, like bacteria, in which ODC is the unique activity reported. Interestingly, the results in Figure 4 (discussed below) point out that the synthesis of PUT was totally suppressed in explants cultivated in presence of all the inhibitors tested, despite that only berenyl and cordycepin were expected to preferentially inhibit PUT synthesis (Fig. 1). New enzymatic techniques and approaches, including compartmentalization and enzyme purification to avoid crude extract variability, should be tested to check for ADC and ODC enzyme kinetics in macroalgae, together with genetic probes, which may definitively help to throw light on these questions.

It is evident from the experiments *in vitro* that exposure to PAs affected cystocarp maturity with SPM as the main factor increasing the number of cystocarps that liberated spores (Fig. 3). This and other examples studied reveal so far the important role played by SPM in the sporulation and further growth of the liberated spores in macroalgae: In G*rateloupia*, SPM induced growth in sporelings cultivated axenically (García-Jiménez et al. 1998), whereas in *G. cornea*, the largest number of spores liberated and the greatest further growth were also obtained using this PA (Guzmán-Urióstegui et al. 2002). It is also evident from the results in Figure 4 that whatever the mechanism is, the effect of SPM on sporulation may come from inside, because SPM levels increased in axenic explants cultivated in media with SPM. One may suspect the same effect of SPM on sporulation in *Grateloupia* as in the *Chlamydomonas* cell cycle (Theiss et al. 2002): PUT and SPD are inhibitory and the addition of SPM may (retro)inhibit their accumulation, thus the latter promotes sporulation. The endogenous PUT and SPD changes, not the SPM, were reported as the actual modulators during onset of sporulation in Azolla, the heterosporous mosquito fern (Marsh et al. 1998). However, in *Grateloupia*, the results obtained with the contents of PUT and SPD (Fig. 4, A and B) clearly show that the three PAs increased in cultivated explants and that the addition of SPM did not decrease them. In fact, a fair increase in SPD was observed in explants cultivated with SPM, which became significantly different in most treatments in which the explants were cultivated in media supplemented with inhibitors and SPM. Because the route of PA synthesis in cultivated explants was stopped by inhibitors at PUT synthesis (Fig. 4A), the data in Figure 4 would support PA interconversion in a range that could be estimated as denoted by the numbers in Figure 4, B and C: The range denoted by (1) would be the maximum conversion of endogenous PUT into SPD, comparing the maximum endogenous levels of SPD in explants cultivated with an inhibitor (I4 or dicyclohexylamine, in this case) and SPD levels in collected samples; (2) would be the minimum SPM conversion into SPD, comparing SPD levels in explants cultivated in dicyclohexylamine with that in explants cultivated in the same inhibitor plus SPM; and (3) would be endogenous SPD conversion into SPM, comparing the maximum endogenous levels in explants cultivated with an inhibitor (I1 or berenyl, in this case) and SPM levels in collected samples. Taken together, the data in Figures 3 and 4 appear to point out that SPM accumulation further promoted the sporulation in fertile cultivated explants. The contribution of PUT and SPD remains obscure, because they were seen to increase in cultivated explants as well, and even SPM could be transformed into SPD, but their exogenous addition seems to be inhibitory (Fig. 3C).

In conclusion, PA endogenous levels decreased during development of the reproductive structures (cystocarp) in the red algae *Grateloupia* from infertile to fertile tissue. ADC and ODC enzymatic activities were detected in the alga, which correlated with the higher contents of PAs in infertile samples. The cultivation of

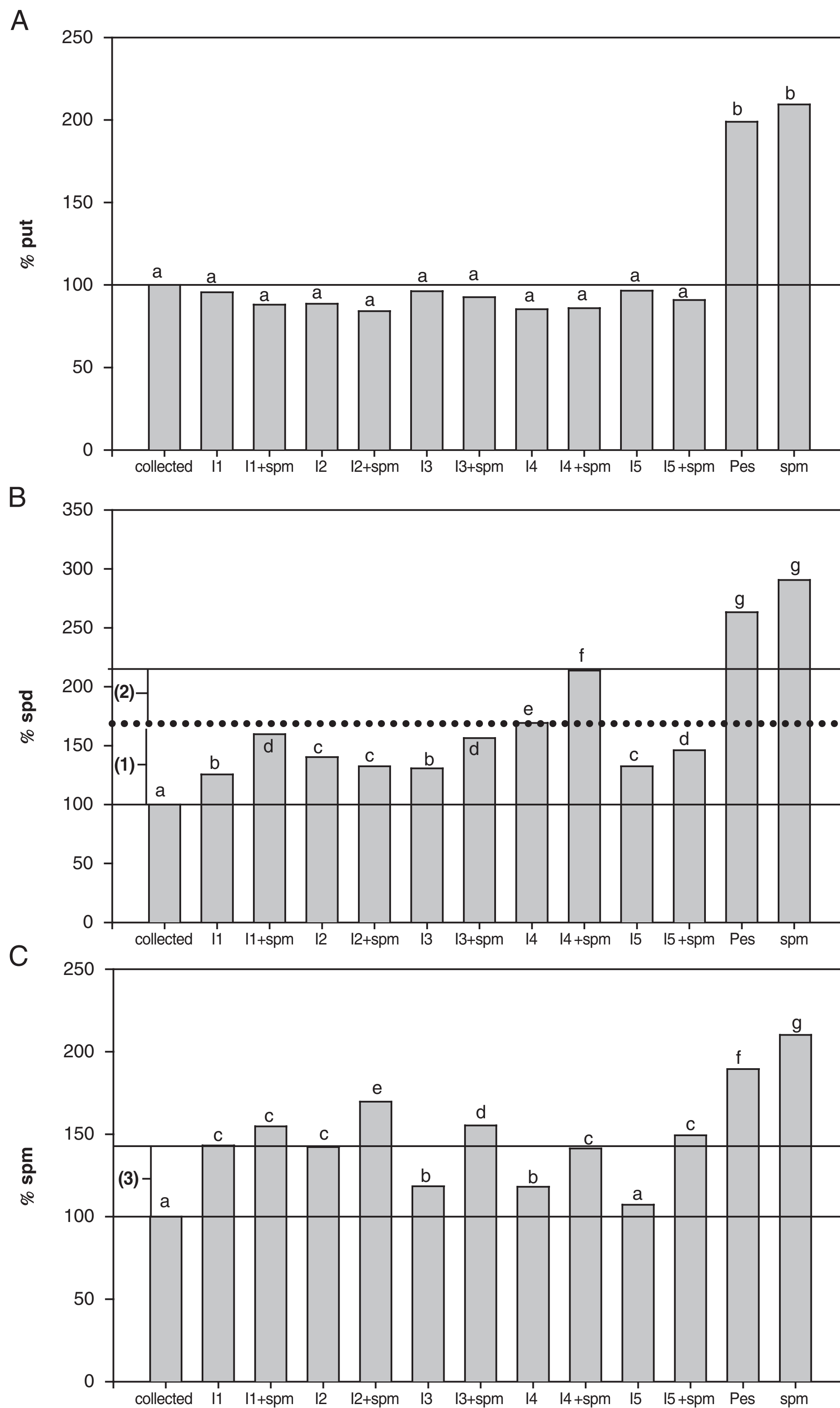

FIG. 4. Endogenous accumulation of PUT (A), SPD (B), and SPM (C) in fertile explants cultivated under axenic conditions in media with (10^{-6}M, SPM) or without spermine (PES) and inhibitors (10^{-6} M, I1, berenyl, I2, cordycepin, I3, cyclohexylamine, I4, dicyclohexylamine, I5, aurintricarboxyilic acid). $n = 3$; vertical bars are percentage over the endogenous levels in the collected samples before cultivation. 100% = 1.45 ± 0.06, 0.117 ± 0.01, and 0.245 ± 0.01 $\mu g \cdot g^{-1}$ fresh weight of PUT, SPD, and SPM, respectively. Same letter denotes statistical homogenous group. (1), (2), and (3) in B and C estimate the range of conversion between endogenous PUT into SPD, SPM into SPD, and SPD into SPM, respectively.

fertile explants revealed that SPM promoted sporulation and that it occurred an immediate increase of SPM endogenous levels as compared with explants cultivated without SPM and natural collected material. This SPM increase may affect SPD, because they can be metabolically interconverted. The results points toward an SPM-deficient status in fertile explants that may be restored by their *in vitro* cultivation but that is accelerated in media with SPM, thus promoting further the sporulation.

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