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Growth and Development of *Porphyra marcosii* (Bangiales, Rhodophyta) Under Different Temperatures and Photoperiod

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This is the first report on the growth and development of *Porphyra marcosii* Cordero (Bangiales, Rhodophyta) - a tropical *Porphyra* species from the coast of Burgos, Ilocos Norte, Philippines. Blade and conchocelis cultures were incubated at different temperatures (5, 10, 15, 20, 25 and 30º C) and two photoperiod regimes (14L:10D or LD) and (10L:14D or SD) at a photon flux density (PFD) of 60-80 µmol $m^{-2}s^{-1}$. Conchocelis filaments grew at 15-30º C under both photoperiods. Conchosporangial formation and maturation was restricted to 30º C under SD conditions. Induction of conchospore release was at 20-30º C with optimum conchospore production under SD. Thallus blades grew at 15-30º C under both photoperiods. Blade archeospores were copiously released at 20-30º C and developed into new blade germlings. Male and female gametes were produced at 20-25º C and germinated *after fertilization* into free-living conchocelis under both photoperiods. "Phyllospores" from the blades were produced at 30º C and directly developed into conchocelis forming a conchocelis sub-cycle. The conchocelis also produced conchosporangia producing conchospores that grew into (1) normal blade germlings and (2) a *compact* "callus-like free-living conchosporangia". Subsequently, these released conchospores that germinated into another generation of blade germlings. This tropical *Porphyra* species exhibited a typical bi-phasic *P. lacerata*-type life history with considerable variations not commonly observed in most *Porphyra* species. The reproductive variations were considered adaptations to environmental pressures prevailing in its local habitat.

Key Words: archeospore, phyllospores, conchospore, photoperiod, temperature, Bangiales, *Porphyra, P. marcosii*

INTRODUCTION

Porphyra marcosii Cordero is a tropical *Porphyra* species found in northernmost Philippine provinces growing in the upper intertidal zone on rocks and barnacles. It is characterized as light purplish or brownish red monostromatic blades, up to 14 cm in height. The blades are linear-lanceolate, laterally or very rarely basally branched thallus blades, attached by a small disc. It is monoecious with both male and female gametes interspersed with the archeospores and vegetative cells (Cordero, 1974; 1977; 2008). The *Porphyra* vegetation grows well on rocks and barnacles in the upper intertidal zone along with *Padina* sp., *Grateloupia,* sp. *Sargassum* sp., *Enteromorpha* sp. and *Valonia* sp.

The report on the genus *Porphyra* in the Philippines is limited and primarily focused on taxonomy (Sulit 1952,

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Galutira and Velasquez 1963, Cordero 1980, Marcos-Agngarayngay 1983, Silva *et al.* 1987, Masuda *et al*. 1991). To date, there are at least five *Porphyra* species; *P. atropurpurea* (Olivi) De Toni, *P. denticulata* Levring, *P. marcosii* Cordero *P. suborbiculata* Kjellman and *P. crispata* Kjellman reportedly present along the coasts of two northernmost Philippine provinces (Silva *et al.* 1987). Kurogi and Yamada (1986), however, argued that the holotype material of Kjellman's *P. crispata* is not a *Porphyra* but a green alga *Monostroma nitidum (Wittrock)*, and thus requires taxonomic reduction to the genus *Monostronoma*. The abundant growth of these algae is during the cold months of November until January (Sulit, 1952; Galutira & Velasquez, 1963; Cordero, 1974; Marcos-Agngarayngay, 1983; Masuda *et al*., 1991). *Porphyra* spp. or *"gamet"* in local dialect is highly-prized seaweed as food in the region and believed to lower plasma cholesterol (Trono, 1999).

Growth, development and life history studies on *Porphyra* had been mostly limited to specimens found in boreal and temperate regions (Kurogi 1961, Krisnamurthy 1969, Mumford and Cole 1977, Kito *et al.* 1971, Wynne 1972, Hawkes 1978, Notoya *et al.* 1993, Notoya 1997, Notoya and Miyashita 1999, Knight and Nelson 1999). Several reproductive modes on both blade and conchocelis phases have been described (Tseng and Chang 1954, Tseng and Chang 1955, Chen *et al.*1970, Hollenberg 1958, Hawkes 1978, Cole and Conway 1980, Guiry 1990, Magne 1991, Nelson and Knight 1995, Notoya 1997). However, an increasing number of *Porphyra* species are now known to be also present in warm and tropical waters (Tanaka and Ho 1962, Diaz-Piferer 1967, Cordero 1974, Shinmura 1974, Oliviera Filho and Coll 1975, Coll and Cox 1977, Ogawa and Lewmanomont 1978).

To our knowledge, culture studies of the warm and tropical species are limited to *Porphyra spiralis* var. *amplifolia* from Venezuela (Kapraun and Lemus 1987) and *P. vietnamensis* Tanaka *et* Pham-Hoang Ho from Thailand (Ogawa and Lewmanomont 1978, Lewmanomont and Chittpoolkusol 1993, Ruangchuay and Notoya 2003). This study is the first report on the growth, development and life history of *P. marcosii*, a tropical species from northern Philippine waters. The influence of temperature and photoperiod on growth of conchocelis and blade phases, spore production and maturation were investigated. These results provide valuable information for the development of potential farming technology for tropical *Porphyra* species.

MATERIALS AND METHODS

Collection and cleaning of blades

Mature blades of *Porphyra marcosii* were collected at Brgy. Ablan, Burgos, Ilocos Norte, Philippines (18°4'N;120°4'E) on January 15, 2000 (Figs. 1 and 2). Water temperature and salinity at the time of collection was 25ºC and 35.1 parts per thousand (ppt), respectively. Collected samples were preserved in 50 ml. vials containing raw seawater and were gradually replaced with Modified Grund Medium or MGM (McLachlan 1973) until transported to Japan on January 17, 2000.

Blades were segregated and cleaned with a soft artist's brush in sterilized seawater. Samples were washed again in sterilized seawater for 30 minutes to remove epibionts, then soaked in freshwater for 20 minutes to eliminate those that were not removed during the first washing (Avila, et al. 1986, Floreto and Teshima 1998, Monotilla and Notoya 2004) and finally washed and rinsed with sterilized seawater. Excised pieces (1x1 cm) from the upper portion of the mature blades were kept overnight in Petri dishes with sterilized seawater containing 5 ppm of GeO_2 to inhibit diatom growth (Polne-Fuller and Gibor 1987) at room temperature for spore release.

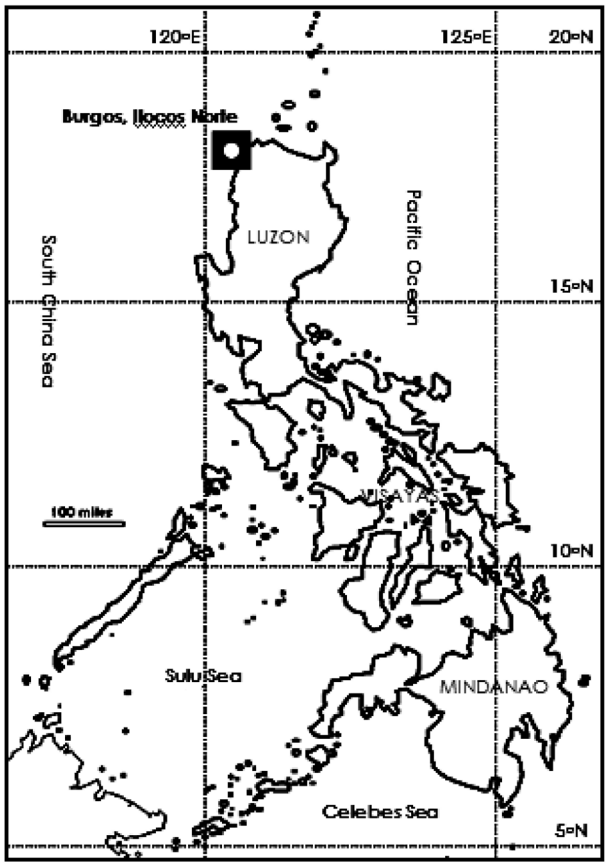

Figure 1. Collection site of *Porphyra marcosii* in Brgy. Ablan, Burgos, Ilocos Norte, Philippines.

Figure 2. Wild thallus blades of *Porphyra marcosii*. Scale bar – 2 cm.

Spore isolation and culture

Initial culture of zygotospores (Guiry 1990, Nelson et al. 1999) released from the wild blade samples were isolated into sterile plastic Petri dishes by glass pipettes and washed three to four times (Notoya and Nagaura 1999, Ruangchuay and Notoya 2003 Monotilla and Notoya 2004). After the zygotospores attached to the glass slides, they were transferred to a new set of sterile plastic Petri dishes (60 x 15 mm) containing sterile MGM and cultured at 25ºC under 14L:10D photoperiod at 60µmol $m^{-2}s^{-1}$ photon flux density (PFD) for 4 weeks, until a uni-algal stock culture of conchocelis was established.

Culture of conchocelis and conchospore release

Free-living conchocelis colonies were cut into lengths of about 150 µm and inoculated into new glass slides placed inside sterile plastic Petri dishes and cultured in a 20ºC growth chamber. After a week, the glass slides with the conchocelis filaments were transferred to 50 ml bottles containing MGM at 5, 10, 15, 20, 25 and 30ºC; 14L:10D (long-day or LD) and 10L:14D (short-day or SD) photoperiods at 60–80 $m^{-2}s^{-1}$ PFD.. Conchocelis filaments were allowed to acclimatize to culture conditions for one week. Weekly monitoring of diameters of 15 conchocelis colonies, conchosporangial formation and conchospore release were undertaken. Measurements during the acclimation period were not included in the statistical analysis. Aliquot samples of 15 conchosporangial colonies were transferred to 10 ml Petri dishes and incubated at 15, 20, 25ºC under both photoperiods to determine optimum conditions for conchospore release. Numbers of conchospores released were counted using an inverted Nikon labophot (Nikon, Tokyo, Japan) microscope.

The relative percentage daily growth rate for four weeks of the conchocelis cultures was calculated as percentage relative growth in diameter using the formula:

$$\% \text{ GR} = ln(d_2\text{-}d_1)/t \text{ x } 100$$

where, d_2 and d_1 represent the mean diameter in every Petri dish culture at the end and beginning of sampling experiments, respectively and t is the number of days.

Isolation and culture of phyllospores and "callus-like" conchosporangia

"Phyllospores" or blade spores with unknown ploidy level (Nelson, et al., 1999) released from the blades and aliquot samples of "callus-like" conchosporangia or short compact conchosporangial branches formed under 30C; SD were isolated. Twenty phyllospores and 10 "callus-like" conchosporangia were separately sub-cultured into 10 new Petri dishes under different temperature, photoperiod and PFD conditions as presented above.

Three trials were conducted and all replicate cultures using cohort zygotospores, and conchocelis filaments isolated from primary cultures were inoculated under similar conditions as discussed above.

Culture of blades

Blade culture was established from isolated conchospores and inoculated into 300 ml flasks containing autoclaved MGM enriched seawater and polyvinyl monofilament strings (6x6 cm) as substrates. Conchospore germlings (approximately 100 germlings) that attached to three monofilament strings were inoculated into each 300 ml aerated flasks and cultured under similar conditions as discussed above. Culture medium was completely renewed once a week.

Blade areas of 15 cultured blades from each monofilament string were measured randomly every week under a microscope for five weeks. Liberation of archeospores, release of sexual reproductive cells and development of marginal cell denticulations were also recorded. A new set of two monofilament strings was placed into each of the culture flasks to monitor spore attachment release. These new monofilament strings were checked and renewed every two days. Larger blades (>1.5 mm) were measured using a calibrated caliper and mounted as herbarium specimens. Blade area and standard deviations of 15 blades from every trial were computed. Archeospore germlings were allowed to acclimatize to respective culture conditions for one week. Measurements at acclimation period were

not included in the statistical analysis. Daily growth rate for four weeks was calculated as percentage relative growth using the formula:

$$\% \text{ GR} = ln(A_2 - A_1)/t \; x100$$

where, A_2 and A_1 represent the mean blade area in every flask culture at the end and beginning of sampling experiments, respectively and t is the number of days.

Statistical Analyses

Statistical analyses were performed using Systat V.8 Software (SPSS, Inc., Chicago, Illinois, USA). Growth rate differences were initially analyzed by two-way Analysis of Variance or ANOVA (growth as a function of temperature and photoperiod, and their interactions). *Post hoc* tests were performed using Tukey HSD Multiple Comparisons test to determine which tested factors were important in the growth of the conchocelis. Level of significance was set at $P < 0.05$.

RESULTS

Growth of conchocelis, conchosporangia formation and conchospore release

The temperature range for conchocelis growth of *Porphyra marcosii* is between 15-30°C. Growth was optimum at 20 and 25ºC under both photoperiods (Fig. 3). Conchocelis filaments at 5-10ºC disintegrated or become bleached and died a week after inoculation. Vegetative conchocelis (Fig. 4-A) developed branching after one week in culture, and grew into a fluffy mass of fine filaments (Fig. 4-B).

Under 30°C;SD, conchocelis colonies become conchosporangial after 30 days of culture. The mature conchosporangia (Fig. 4-C) first released conchospores (*ca*. 12-14 µm) after 35 days in culture at 30º C. Optimum conchospore release at 20 and 25ºC developed into bi-polar blade germlings (Fig. 4-D) in all culture conditions. Some conchosporangia developed secondary conchosporangial branches that were tightly clustered compacted and appearing like a "callus" composed of 2-5 cells (Fig. 4-E) at the outset and as much as 15 bulbous cells as it grows.

Phyllospore and "callus-like" conchosporangia

Isolated archeospores and phyllospores at 30ºC under SD developed into two types: approximately 80% grew into bi-polar archeospore germlings eventually growing into young blades (Fig. 4-F) and 20% of phyllospores (*ca*. 12-18 µm). The latter germinated into unipolar conchocelis filaments (Fig. 4-G) and the conchocelis from these phyllospores produced a normal conchosporangia at 30ºC; SD after 42 days in culture. These conchosporangia released conchospores after 7 days of culture, where approximately (1) 90% germinated into blade germlings under the same culture conditions as above, and (*2*) 10% developed directly into a "callus-like conchosporangial branches" and eventually devoid of vegetative filaments (Fig. 4-H) The former was observed at 15 - 30ºC under both photoperiods after 12 days. The latter type developed restrictively at 30ºC under SD, 14 days after the transfer. When "callus-like conchosporangial branches" were cultured at 15-30ºC under both photoperiods, it subsequently released conchospores at 15-30ºC that germinated into blade germlings within 7 days.

Blade growth and spore production

Archeospore germlings released from the cultured thallus blades grew into blades at 15-30ºC under both photoperiods. Blades were conspicuously observed at 20 and 25ºC under both photoperiods but largest blades were produced at 15ºC under LD at the end of the culture experiment. The average growth rates over the four weeks (after acclimation) showed optimum growth at 20 and

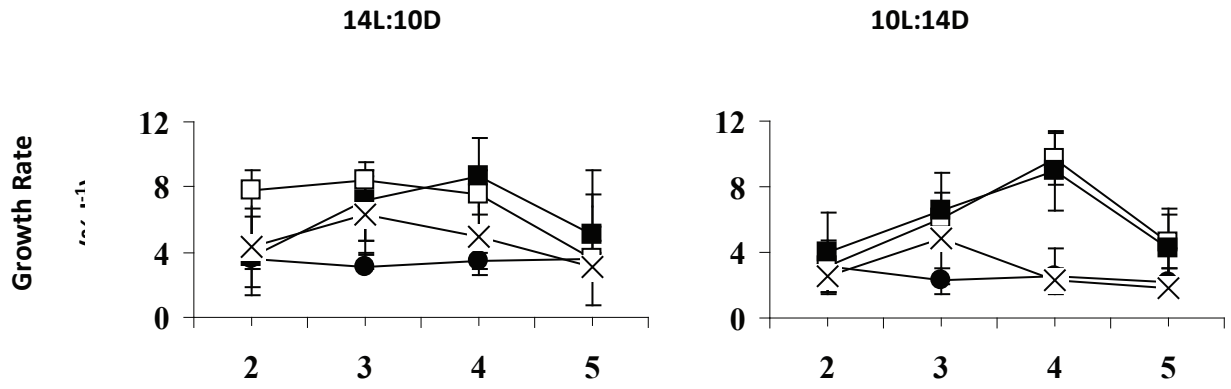

Figure 3. Growth rates of conchocelis of *P. marcosii* during the first four weeks after acclimation period under different temperatures and two photoperiod regimes (X= 30ºC; ■ = 25ºC; □ = 20 ºC; ● = 15 ºC).

25ºC under both photoperiods (Fig. 5). Thallus blades at 20 and 25ºC showed lanceolate shapes, while those at 15ºC grew into linear oblanceolate blades (Fig. 6). Basal shapes of the blades were generally round though umbilicate and cordate shapes were occasionally observed at 15 and 25ºC, respectively. Lateral branching was also intermittently observed at the apical margin of the blades as a result of archeospore release. Some blades at 15ºC under LD were maple leaf-shaped at the first six weeks of culture (Fig. 7-A). This morphology developed some lateral branches at a later stage. Blades at 15ºC, under SD, were smaller than LD cultures. Moreover, 1-3 cell marginal denticulate cells (*ca.* 22-35 µm) were first noticed projecting outwardly and some upwardly after two weeks at 20-30ºC under both photoperiods (Fig. 7-B and C).

The blades at 30ºC under both photoperiods released archeospores (*ca.* 18-25µm) from the apical blade portion after 7 days of culture. The blade phase at this temperature condition was hardly observed because of copious archeospore production that led to eventual blade

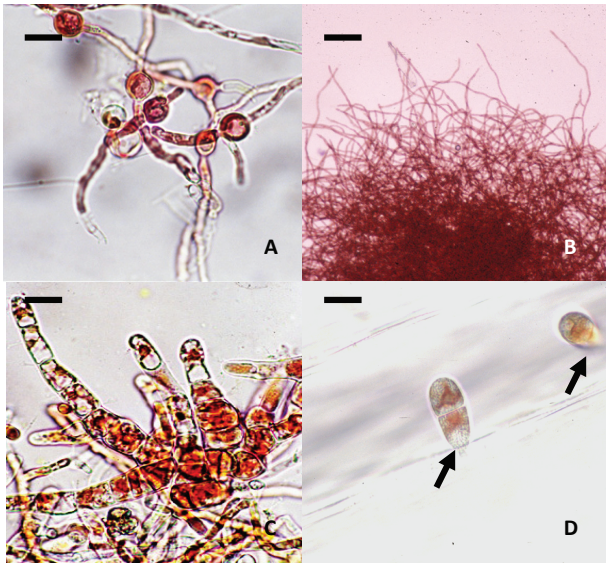

Figure 4. Reproduction of the conchocelis phase of *P. marcosii*: (A) Branching conchocelis filaments originating from zygotospores. (B) Conchocelis filaments at 25ºC under LD. (C) Mature conchosporangia at 30ºC under SD. (D) Blade germlings (arrowhead) from conchospores at 25ºC under LD. (E) Conchosporangia formation of conchocelis from phyllospores at 30ºC:SD. (F) Two-week old archeospore germlings at 20ºC growing on monofilament string. (G) Unipolar conchocelis filaments germinating from phyllospores. (H) "Callus-like conchosporangia" formed at 30ºC:SD from released conchospores. Scale bars: (A) = 50 µm. (B) = 80 µm. (C) = 60 µm. (D) = 30 µm.

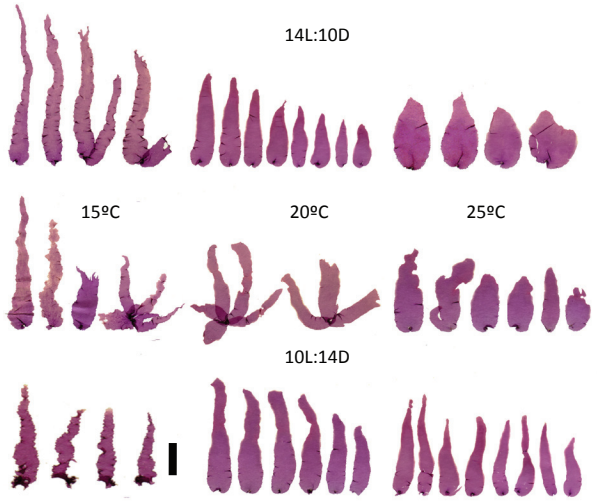

Figure 6. Herbarium samples of cultured blades of *P. marcosii* under different temperature and photoperiod conditions after 5 weeks of culture. Scale bar = 100 mm

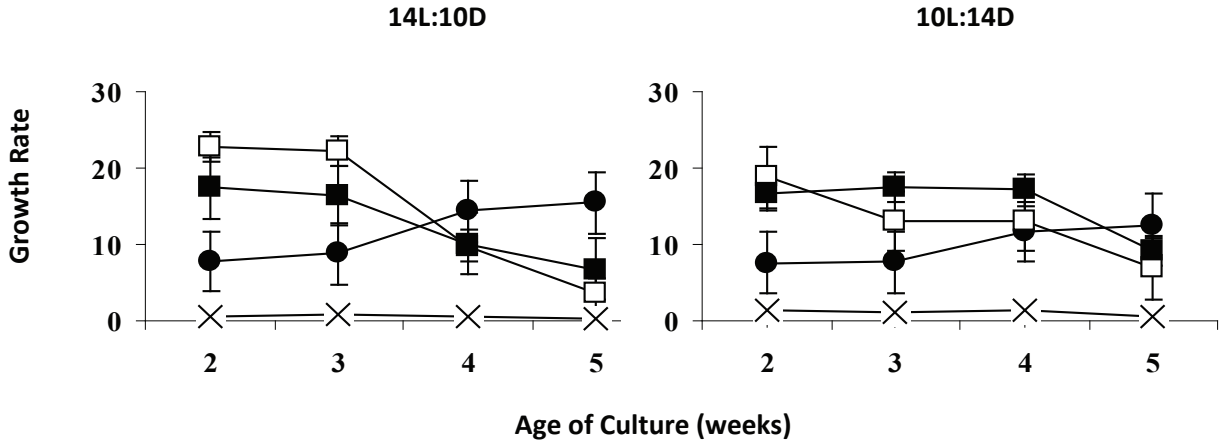

Figure 5. Growth rates of the blades of *P. marcosii* during the first four weeks after acclimation period under different temperatures and two photoperiod regimes (x = 30ºC; ■ = 25ºC; □ = 20 ºC; ● = 15 ºC).

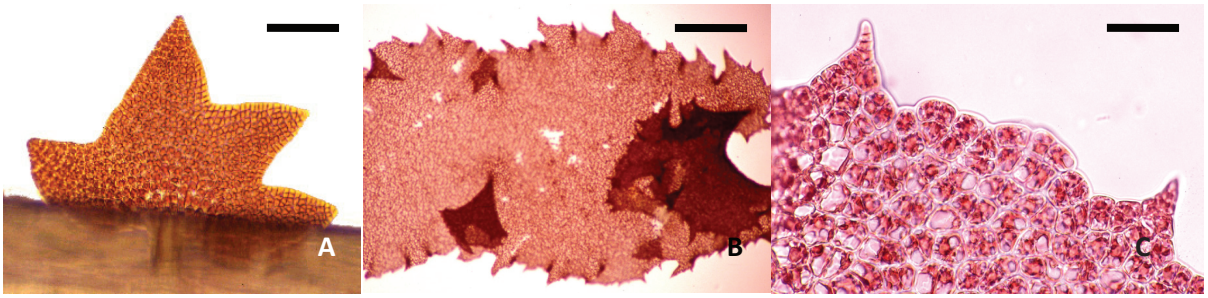

Figure 7. Morphological characters of the blades of *P. marcosii* at early stages of culture. (A) Six week-old maple leaf-shaped blades at 15ºC:LD. (B and C) Denticulate cell margins at 25ºC:LD after 3 weeks of culture. (D) Mixed patches of spermatangia (small arrowhead) and zygotosporangia (bold arrowhead) at 25ºC. Scale bars: (A) = 300 µm (B) = 800 µm (C) = 80 µm (D) = 20 µm..

deterioration and death. The male and female gametes were not observed at 30ºC under both photoperiods.

The blades at 20 and 25ºC under LD released archeospores after 14 days of culture. Spermatangia at these temperature conditions were first observed on the distal apical margin of the blade after 21 days of culture, and released spermatia (*ca*. 6-8 µm) 7 days after formation. The zygotosporangia become distinctly intermixed with spermatangia and vegetative cells as patches within the inner margins of the blade after 27 days (Fig. 7-D). Zygotopores (*ca*. 14-19 µm) at 20 and 25ºC under LD were released after 35 and 28 days, respectively. There were simultaneous and subsequent releases of archeospores, spermatia and zygotospores after 35 days in LD culture. At 25ºC, the SD blades follow similar patterns of release of archeospores, spermatia and zygotospores as with the LD cultures. However, at 20ºC there were simultaneous release of archeospores, spermatia and zygotospores, at the same culture age with the LD **(Fig.8).** Culture blades at 15ºC:LD, released archeospores after 30 days of culture.

Mixed patches of spermatangia and zygotosporangia were observed after 49 days with simultaneous release of sexual spores after 63 days of culture. Blades at SD sporadically produced asexual archeospores and did not mature into sexual reproductive spores until the end of culture. Germlings at 10 and 5ºC died after 7 days of culture.

Life History

The life history of *Porphyra marcosii* alternates between conchocelis and blade phase in culture between 15-30ºC under both photoperiods **(Fig.9).** The conchocelis filaments formed conchosporangia restrictively at 30ºC under SD conditions. The conchosporangia released conchospores 20-30ºC under both photoperiods and developed into blade germlings.

Blade archeospores and phyllospores were produced at the blade phase. Blade archeospores grew into new archeospore germlings while apogamic spores germinated into conchocelis filaments. The conchocelis formed from phyllospores produced mature conchosporangia

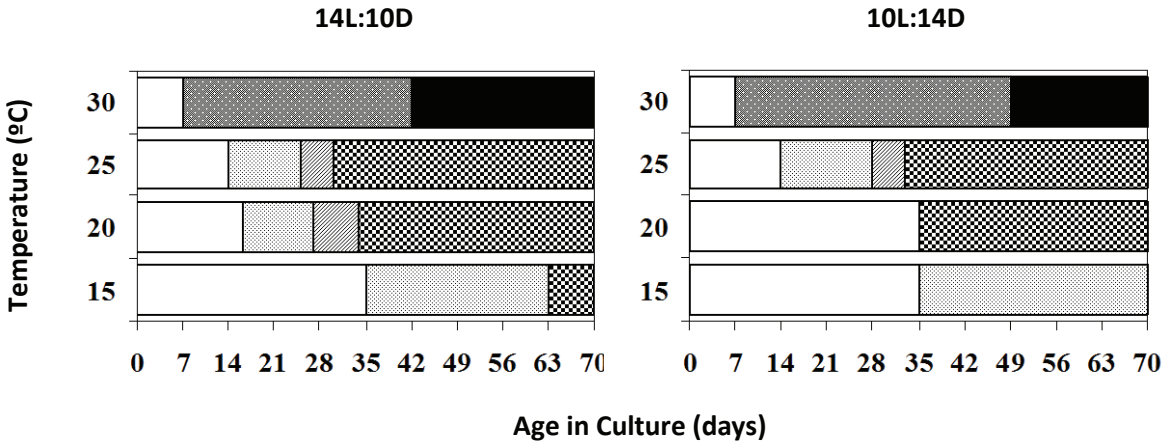

Figure 8. Spore liberation from the blades of *P. marcosii.* (White box = no spore release; black-dotted box = archeospore liberation and some phyllospores; white- dotted box = spermatia release; slanting striped box = zygotospore release; checkered box = spermatia, zygotospore and archeospore releases; black box = blade death.

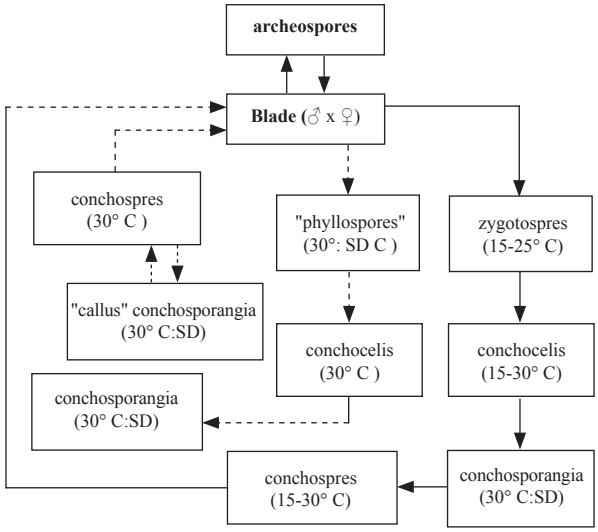

Figure 9 The summarized life history of *P. marcosii* in culture. Bold lines represent the typical *P. lacerata*-type life history pattern at 15 – 25ºC. Broken lines are variations from the typical *P. lacerata*-type life history forming a haploid conchocelis sub-phase at 30ºC. Numbers in parentheses are growth conditions.

restrictively at 30ºC under SD conditions. The conchosporangia released conchospores that germinated into blade germlings and "callus-like conchosporangia". The compact "callus-like conchosporangia" also produced conchospores that grew into blade germlings thus, forming a haploid conchocelis sub-cycle. Production of sexual reproductive cells (male and female gametes) was prevalent at 20 and 25ºC under both photoperiods. Zygotosporangia produced zygotospores germinating into unipolar conchocelis filaments and developed as discussed earlier.

DISCUSSIONS

The temperature range for growth and development of the conchocelis and blade phases of *P. marcosii* clearly suggest tropical affinities. However, photoperiod requirement for the formation of conchosporangia and conchospore release is suggestive of temperate species. It is interesting to note that both temperature and photoperiod, acting either alone or independently, are both significant to blade growth. However, these two environmental parameters influenced growth of the conchocelis independently but not their interactions (Table 1). A clear example of independence is the optimum induction of conchospore release after transfer to lower temperatures and a short photoperiod. This conforms to the studies on some *Porphyra* species from southern China resulting from the manipulation of temperature and photoperiod conditions to achieve

Table 1. Two-way ANOVA on the effect of temperature and photoperiod on growth of the conchocelis and blades of *Porphyra marcosii*. (n = 15; P<0.05)

Parameter	Blade Phase	Conchocelis Phase
Temperature		
df	3	3
F-ratio	65.297	44.096
P	0.000	0.000
Photoperiod		
df	1	1
F-ratio	74.758	7.995
P	0.000	0.000
Temperature x Photoperiod		
df	3	3
F-ratio	34.654	0.994
P	0.000	0.395

growth, development and maturity of the conchocelis phase (Wang, *et al*., 2009).

On the other hand, copious production of blade archeospores was at the highest tolerable temperature conditions. The effect of temperature and photoperiod on production of asexual spores is construed as absolute (qualitative) while sexual maturation appears to be induced by the age of the blades rather than the environmental stimuli. The reproductive variations exhibited by *P. marcosii* are construed as species-specific characters or adaptations to environmental pressures of its tropical habitat.

Porphyra marcosii exhibited a typical bi-phasic life history that alternates with sporophytic conchocelis and gametophytic blades with considerable variation under specific conditions. At 15 – 30ºC growth range, it follows a typical *P. lacerata*-type life cycle (Notoya 1997), except that conchosporangia was formed only at 30ºC:SD. The phyllospores from the blades germinated into sporophytic conchocelis and released conchospores producing a new generation of (1) blade germlings and (2) "callus-like conchosporangia" under a specific temperature (30ºC:SD) condition. The matured "callus-like conchosporangia" produced another set of blade germlings from its conchospores. The conchospore production of *P. marcosii* is almost similar to some reports on *Porphyra* species of temperate origins (Avila and Santelices 1985, Notoya *et al.* 1992, Notoya *et al.* 1993, Frazer and Brown 1995, Notoya and Nagaura 1999) except that it has a sub-cycle of producing conchosporangia from conchospores under specific conditions. The blade phase produced asexual blade archeospores, spermatia and zygotospores, and phyllospores. The zygotospores developed into conchocelis, formed conchosporangia and produced

conchospores similar to conchocelis of most *Porphyra* species. On the other hand, the phyllospores may have undergone mitotic cleavage "without fertilization" and germinated directly into conchocelis filaments. The ploidy level of these spores was not checked but their development suggests apogamic germination. They may have remained haploid because of the absence of male and female gametes at a specific temperature (30ºC, in this case) condition. Nevertheless, it is also highly possible that the sexual gametes at 30ºC may be highly cryptic and remained inconspicuous because of limitations in our methodological design. In this particular case, meiosis may not have occurred at all thus, producing haploid conchocelis as exhibited by *P. okamurae* (Notoya, 1997) and *Bangia atropurpurea* (Notoya and Iijima, 2003; Wang *et al.,* 2006; Shimizu *et al.,* 2008). There were cases when male gametes may have been present yet fertilization was not observed but agamospores *sensu* Kornmann and Sahling (1991), Kornmann (1994) may have been produced. The distinction between female gametangia and agamosporangia (Nelson *et al*. 1999) remains unclear and requires further investigation. Thus, in *P. marcosii*, two types of conchocelis maybe possibly produced – a haploid conchocelis from "apogamic spores" and diploid conchocelis originating from fertilized female sexual gametes. In addition, Kapraun and Lemus (1987) speculated that *Porphyra* species with more southerly distributions likely lack sexual reproduction resulting in both blade and conchocelis being haploid.

Further developmental studies on cytological level is highly recommended to determine the ploidy levels of the putative apogamic spores and the taxonomic placement of *Porphyra marcosii* along with other temperate and tropical *Porphyra* species. Likewise, the taxonomic identity based on molecular approach of *P. marcosii* is also highly desired.

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