Bot. Bull. Acad. Sin. (1994) 35: 189-193

Regeneration of protoplasts from the brown alga, *Endarachne binghamiae* (Phaeophyta; Punctariales, Scytosiphonaceae)

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(Received November 11, 1993; Accepted April 19, 1994)

Abstract. The regeneration of protoplasts isolated from erect and crust phases of the brown alga *Endarachne binghamiae* was studied. The protoplasts were prepared by enzymatic degradation of cell wall, using cellulase (Yakult Co.), macerozyme (Yakult Co.), and limpet acetone powder (Sigma Co.) in a ratio of 3:4:2 or 2:1:0 (w/w), depending on the material. Three developmental patterns were displayed by the protoplasts from algal blades: **1**) develop into crusts only, **2**) proceed further to form erect thalli directly on the crusts, or **3**) (seldom) divide diffusely to form cell clumps. The first pattern was followed by protoplasts from the crust phase and by zooids released from mature blades. The life history of the studied species is discussed in relation to the regeneration patterns.

Keywords: Brown alga; Protoplast; Regeneration.

Introduction

Regeneration is a prerequisite for using protoplasts as a tool for studying plant genetics and breeding. The inability to regenerate protoplasts has been a significant problem encountered with many species of seaweed. Species that have been successfully raised from protoplasts are limited to those with relatively simple forms. Among the brown algae, the filamentous form, including *Sphacelaria* spp. and *Pilayella littoralis*, was the first found to have this potential (Ducreux and Kloareg, 1988; Mejjid et al., 1992). More recently, the tubular form of *Cladosiphon okamuranus* has been found to regenerate (Uchida and Arima, 1992). Our previous work (Chen and Shyu, 1993) on the isolation of protoplasts from four brown algae showed that the two blade-forms, i.e. *Petalonia fascia* and *Endarachne binghamiae*, were capable of regeneration. Protoplasts isolated from *E. binghamiae* were observed further, and we now report on the regeneration patterns of protoplasts prepared from its erect and crustose phases. The developmental patterns of the protoplasts are also compared with those of the zooids.

Materials and Methods

Algal blades of *E. binghamiae* were collected from an intertidal area near Keelung in 1992 and 1993, during the period of their active growth. Young blades, emerging directly from the crustose phase that developed from protoplasts, were also used. Algal blades were cleaned of epiphytes, and then surface sterilized for 5 min in 1% betadine solution. Filamentous materials were raised from zooids released from mature blades. The zooids were collected, using their phototactic behavior. The collected zooids were dispersed into fresh sterilized seawater. This was repeated three times to clean the zooids. Protoplasts were isolated using an enzyme mixture of cellulase Onozuka RS (Yakult Co.), macerozyme (Yakult Co.), and limpet acetone powder (Sigma Co.) (OML) in a ratio of 3:4:2 (w/w) for blades, and using OM in a ratio of 2:1 for both young blades (sporeling) and filaments. Algal material was mixed with the enzyme mixture at a rate of 0.4% (w/v). Bovine serum albumin (BSA) was added to the enzyme mixture (0.4%) to protect algal blades from being over degraded. A hypo-osmotic condition of 1200 milliosmols kg^{-1} was maintained in the digestion mixture by the addition of sorbitol to a molarity of 1.2. The digestion was conducted under darkness on a rotatory shaker plate at 60 RPM in a 20 °C incubator. Removal of the cell wall took approximately two hours. The completeness of cell wall removal was determined under a fluorescent microscope (Nikon Diaphot-TMD with TMD-EF) using Calcofluor white (Sigma) staining.

The isolated protoplasts were further cultured in Provasoli's enriched seawater (PES) medium (Provasoli, 1968) supplemented with sorbitol to a molarity of 0.8, with an osmotic pressure of 1791.5 milliosmols kg^{-1}.

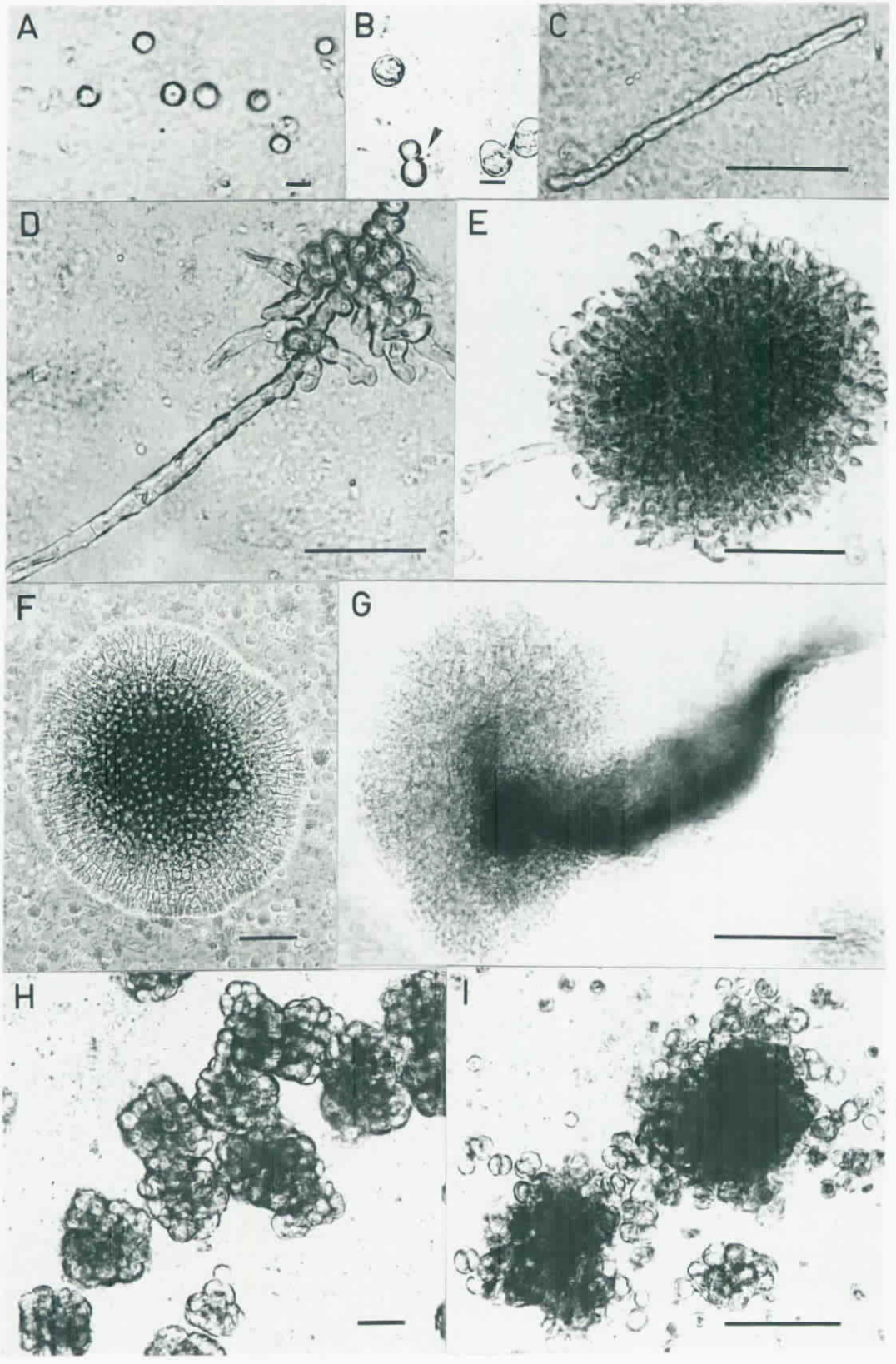

Figure 1. **A**) Protoplasts isolated from thallus of *Endarachne binghamiae*; **B**) two-cell stage; **C**) earlier filamentous stage; **D**) filamentous stage with branches formed from the distal half end; **E**) earlier ralfsioid (crustose) stage with filamentous tail; **F**) ralfsioid stage; **G**) young sporeling raised directly from the ralfsioid stage; **H**) earlier stage of cell clumps; **I**) later stage of cell clumps. Bar = 50 μm

Culturing took place at 20 °C, illuminated by cool white fluorescent lamps at 42 μE m^{-2} s^{-1} in an incubator with a 12h:12h photoperiod. Over a one-week period, the osmotic pressure of the culture medium was progressively reduced to that of seawater at each renewal of the medium (three times per week). During the first week, the culture was supplemented with 10 ppm of 2,4-D to improve the protoplast survival rate. The development of protoplasts and zooids was monitored on an inverted microscope (Nikon Diaphot-TMD). The zooids were used as a reference in the developmental study.

Results

On the second day after transfer to enriched full-strength seawater, most *Endarachne* cells (post protoplast) entered the two-celled stage (Figure 1B), and on about the fifth day entered the 3-celled stage; some cells remained quiescent. The subsequent development of dividing cells followed three patterns. A large proportion (95%) of protoplasts developed an initial filament by continuous transverse divisions (Figure 1C). Having reached a length of 50–150 μm, primary branches were formed bilaterally from the distal half of the filament (Figure 1D). Secondary and tertiary branches were formed subsequently. The branches contacted and interweaved to form a crust, roughly spherical in shape, with the proximal end of the filament retained as a tail (Figure 1D). The tail was eventually incorporated into the crust as the latter grew larger (Figure 1F). Two pathways were taken during further development of the crust. The first pathway was characterized by the formation of unpigmented filaments on the crust. No further development was observed. This pathway occurred mainly in protoplasts isolated from mature blades collected in late spring. A similar developmental pathway was observed among zooids released from mature blades. The second pathway involved the direct formation of blade primordia on the crust (Figure 1G); this occurred 40–50 days after initiation of the culture. They were found only in protoplasts from immature blades and young plantlets that regenerated directly from the protoplasts. A very low proportion of cells were found to divide diffusely, forming loose cell clumps (Figure 1H). At a later stage, the outer cells of the clumps sloughed off to form new clumps (Figure 1I). Development of protoplasts isolated from the crust phase was also monitored. Except for the non-dividing cells, the cells showed only one developmental pathway; each stage in the pathway resembled its corresponding first regeneration pattern of protoplasts prepared from blades.

Discussion

The life cycle of *E. binghamiae* has been reported to vary according to geography. Plants collected from northeastern Pacific water formed bladed macrothalli directly on discoid holdfasts that developed from their motile cells (Wynne, 1969). A similar direct differentiation type of life cycle has been observed among plants of northern South Wales (Clayton, 1982) and southern California (Thomas and Steven, 1989). In contrast, a heteromorphic life cycle has been observed in plants from western Pacific waters; their life cycle includes sexual fusion of motile cells as well as a parthenogenetic development of the non-fused cells (Nakamura and Tatewaki, 1975). The development of zooids from northern Taiwan remains undetermined. As shown in this study, all *E. binghamiae* zooids developed to the discoid crust stage with no further development. Presumably, their development includes sexual fusion of motile cells that are formed on the crusts, to complete a heteromorphic life history, since no direct blade formation was observed. The direct regeneration of blades from discoid crust that developed from protoplasts prepared from immature blades implies parthenogenesis which is not observed in nature. Both aspects can be related to the formation of either unilocular or plurilocular sporangia under different combinations of temperature and photoperiod, as in some species of *Scytosiphonales*. (Nakamura and Tatewaki, 1975; Wynne and Loiseaux, 1976). Further study of these aspects is in progress.

The regenerative potential of protoplasts has been reported to vary among algae. Thus far, it has not been possible to induce protoplasts isolated from species characterized by advanced organization to undergo complete regeneration, e.g. those of *Sargassum muticum* would not divide (Fisher and Gibor, 1989); those of *Laminaria* thalli could only resynthesize cell wall (Butler et al., 1989); those from *Undaria pinnatifida* formed callus (Tokuda and Kawashima, 1988); and those from *Macrocystis* regenerated only to the microthallus stage (Kloareg et al., 1989). Incomplete regeneration in plants may be related to differentiation. The higher the level of differentiation in a plant's tissues, the less likely it is to cycle through de-differentiation and re-differentiation. The callus from *Sargassum muticum* is capable of regenerating complete plants (Polne-Fuller and Gibor, 1987), suggesting the regenerative potential of protoplasts from callus. The few examples of complete regeneration from protoplasts are associated with algae with a relatively low degree of organization, such as the filamentous *Sphacelaria* spp. and *Pilayella littoralis* (Ducreux and Kloareg, 1988; Mejjid et al., 1992), tubular *Cladosiphon okamuranus* (Uchida and Arima, 1992), and another filamentous form (crustose phase) and blade form of *Endarachne binghamiae* (the present study), which possesses a medulla consisting of densely interwoven hyphae, and may represent the most advanced form with a high potential for regeneration.

Regenerative potential might also vary among protoplasts prepared from the same plant. Filamentous algae, such as *Sphacelaria* spp. exhibit complete and spontaneous regeneration of protoplasts from apical cells (Ducreux and Kloareg, 1988), but protoplasts from thallus cells require hormone treatment before they will

regenerate (Ducreux et al., 1992); The simple leafy thallus of *Porphyra perforata* can be divided into four zones according to the regenerative potential of protoplasts derived from each zone (Polne-Fuller and Gibor, 1984). Therefore it is reasonable to find that more than one developmental pathway may be followed by protoplasts from complex thalli. For example, protoplasts from *Cladosiphon okamuranus* follow one of four regeneration pathways (Uchida and Arima, 1992), and those of *E. binghamiae* follow three pathways. The cell clumps that occurred in cultures of *E. Binghamiae* represent protoplast-derived cells that have lost their regenerative potential but retain their ability to divide. This has also been encountered in *Porphyra linearis*, and has been used to establish cell suspension cultures (Chen, 1989). Experiments have shown that these cells can retain their regenerative potential under certain diurnal photoperiod and temperature regimes. A probable cause of heterogeneity in protoplast regeneration is the diversity of differentiation stages within a single thallus. Coexisting bacteria has also been reported to affect the morphogenesis of some algae (Provasoli and Pinter, 1980; Tatewaki and Provasoli, 1983).

Acknowledgement. The authors express their gratitude to the National Science Council, ROC, for financial support (NSC-81-0418-B-019-513-BG).

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小海帶原生質體之再生

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本文主要探討褐藻小海帶 *Endarachne binghamiae* 直立相及匍匐相材料所製備之原生質體之再生過程原生質體之製備係採用酶解法，即以纖維素軟化酶及笠螺丙酮萃取粉末依 3:4:2 或 2:1:0 之比例混合（依材料而異）酶解細胞壁。由葉體製備之原生質體之發育型式有三種：(1) 僅發育至盤狀體 ；(2) 進一步由盤狀體上直接長出直立之葉體；(3) 形成細胞團（頻率低）。由絲狀體製備之原生質體及由成熟葉體釋出之游孢子之發育與前述第一型相同。此外亦由再生之型式進一步討論本種之生活史。

關鍵詞：褐藻；原生質體；再生。