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ORIGINAL ARTICLE

Use of plant growth regulators in micropropagation of *Kappaphycus alvarezii* (Doty) in airlift bioreactors

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Abstract The effects of plant growth regulators on callus induction rate and regeneration of *K. alvarezii* explants was evaluated. *K. alvarezii* calluses were induced *in vitro* with kinetin (K), 6-benzylaminopurine (B), 1-naphtalene acetic acid (N) and spermine (S). After 30 days, *K. alvarezii* explants produced filamentous calluses and isolated crystalline filaments growing from the medullar region and from cortical cells at the cut edge. The plant growth regulators 1-naphtalene acetic acid (1 mg L^{-1}) and 6-benzylaminopurine (1 mg L^{-1}) and the 1-naphtalene acetic acid + kinetin + spermine (1, 1, 0.018 mg L^{-1} respectively) combination produced 85 to 129% more calluses, with significant differences versus the control ($p < 0.05$). Spermine at 0.018 mg L^{-1} produced calluses in the apical, intercalary and basal regions of explants. Spermine also reduced callus induction time to 7 days, which is faster than previously reported induction times with other plant growth regulators. An airlift bioreactor was designed and characterized to micropropagate *K. alvarezii* calluses. The bioreactor had mixing times ranging from 4.6–10.3 s at T_{90} and T_{95}, which is shorter than those for the Fernbach (5.2–13.4 s) and balloon flasks (6.3–17.3 s). Mixing time standard deviations were smaller for the bioreactor (1.1–4.6) than for the Fernbach (9.3–13.6) and balloon flasks (5.5–15.8), suggesting an adequate flow regime within the bioreactor. The results are useful for improving callus induction in *K. alvarezii* and propagating microplantlets in an airlift bioreactor, and provide baseline data for macroalgal bioreactor culture.

Keywords Airlift bioreactor · Callus · Plant growth regulators · Spermine

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Introduction

Kappaphycus alvarezii (Doty) is the largest worldwide source of k-carrageenan in the phycocolloid industry. Annual commercial production has increased from less than 1000 t dry weight when first began to approximately 100,000 t worldwide in recent years (Ask & Azanza, 2002). The market for carrageenan has been growing exponentially at a rate of 5% annually for the last 25 years (Bixler, 1996).

Farming of *K. alvarezii* is currently based on vegetative propagation of sterile strains, although this involves problems such as growth rate decay, disease, reduced carrageenan yields and lack of continuous seed material supply (Ask & Azanza, 2002). In an effort to overcome seed material shortages and thus expand *K. alvarezii* farms, micropropagation techniques have been developed at different stages, from callus

cultures for maintenance and propagation of select seed stocks (Dawes & Koch, 1991; Dawes et al., 1993) to embryogenesis and regeneration of somatic embryos (Reddy et al., 2003). Plant growth regulators such as auxins (1-naphthalene acetic acid) and cytokinins (6-benzylaminopurine, kinetin) are known to accelerate callus induction and regeneration (Bradley, 1991). Other growth regulators, like polyamine spermine, have been poorly studied, though this regulator has been shown to enhance cell division (Cohen et al., 1984) and morphogenesis (García-Jiménez et al., 1998), and to promote spore development and growth (Guzmán-Urióstegui et al., 2002; Sacramento et al., 2004).

Algae cultivation has been performed in different systems. Bioreactors are used extensively to propagate microalgae because of the major advantages (control, scalability and repeatability of bioprocesses) they offer over conventional culture systems (Sánchez-Miron et al., 2004). Only three bioreactor configurations have been experimentally tested to date for red macroalgal suspension cultures: bubble column (Rorrer et al., 2001; Huang & Rorrer, 2002); stirred tank (Barahona & Rorrer, 2003); and airlift internal draft tube (Polzin & Rorrer, 2003). Rorrer and Cheney (2004) stated that airlift bioreactors are the most suitable system for macroalgal cell culture because they improve gas exchange, light transfer and reduce shear damage. Mixing in airlift bioreactors is normally done by sparging, mechanical agitation, or a combination of both. These maintain a uniform concentration of chemical species (e.g. pH, gases and nutrients) in the bulk phase and increase the mass transfer rate (Sajc et al., 2000). Mixing is apparently the key parameter for equal distribution of cells and nutrients in the liquid phase, and understanding liquid-phase mixing times is crucial to airlift bioreactor design, scale up and operation.

Establishing effective suspension cultures with seaweed bioprocessing technology requires adequate photobioreactor design and performance, as well as determining tissue culture development. The present study was aimed at evaluating the effects of plant growth regulators (auxins, cytokinins and polyamines) on *K. alvarezii* explant callus induction rate and regeneration. An airlift bioreactor was designed, built and characterized to micropropagate *K. alvarezii* calluses and provide baseline data for further research.

Materials and methods

Sterile vegetative thalli from a *K. alvarezii* brown strain were used as explants. The *K. alvarezii* brown strain used here was provided by Edison José de Paula (University of São Paulo, Brazil) in December 1999, and has been under cultivation at an experimental farm at Ubatuba, Brazil, since 1995 (Paula et al., 2002). Plants of this strain were originally introduced from Northern Bohol, Philippines, and selected from vigorous and sterile plants propagated in Uranochi Inlet in Tosa Bay, Japan (Ohno et al., 1994). Since being brought to Mexico, the strain has been kept for 5 years growing vegetatively at $25 \pm 1^{\circ}$C and 100 μmol photon m^{-1} s^{-1} at Cinvestav, 30 km from the coast. Quarantine protocol considering the Mexican Official Code NOM-011-PESC-1993 was used (Muñoz et al., 2004).

Explant size determination and disinfection protocol

Selection of *K. alvarezii* explants of adequate size for micropropagation was done by culturing three sizes of apical and intercalary explants: (a) <2 mm; (b) 3–4 mm and (c) 4–5 mm. Explants were cultured in 20 mL Petri dishes under 10 μmol photon m^{-2} s^{-1}, a 12L:12D photoperiod and $26 \pm 1^{\circ}$C for 14 days. Each Petri dish contained five explants and each treatment consisted of four Petri dishes. All culture procedures were done under a sterile laminar flow hood. Provasoli Enriched Seawater (PES, Provasoli, 1968) was used as culture medium. Solid media were used at an agar concentration of 8% (w/v) and prepared with 0.2 μm filtered, sterilized seawater (33 psu). A disinfection protocol was defined after testing four disinfection treatments: chlorine 1% 15 s + iodine 1% 15 s + antibiotics 24 h; chlorine 1% 25 s + antibiotics 24 h; iodine 1% 25 s + antibiotics 24 h; osmotic shock 10 s + iodine 1% 10 s + antibiotics 24 h. The stock antibiotic solution was prepared with penicillin (100 mg), ampicillin (25 mg), nystatin (25 mg) and germanium dioxide (10 mg), and dissolved in 100 mL distilled water. Antibiotic treatments used 1 mL of this stock antibiotic solution per 9 mL sterile seawater. Osmotic shock was done by soaking explants for 10 s in distilled water. Survival rate in both experiments was determined by counting unviable explants; an explant was considered unviable when more than 50% of total explant surface was bleached.

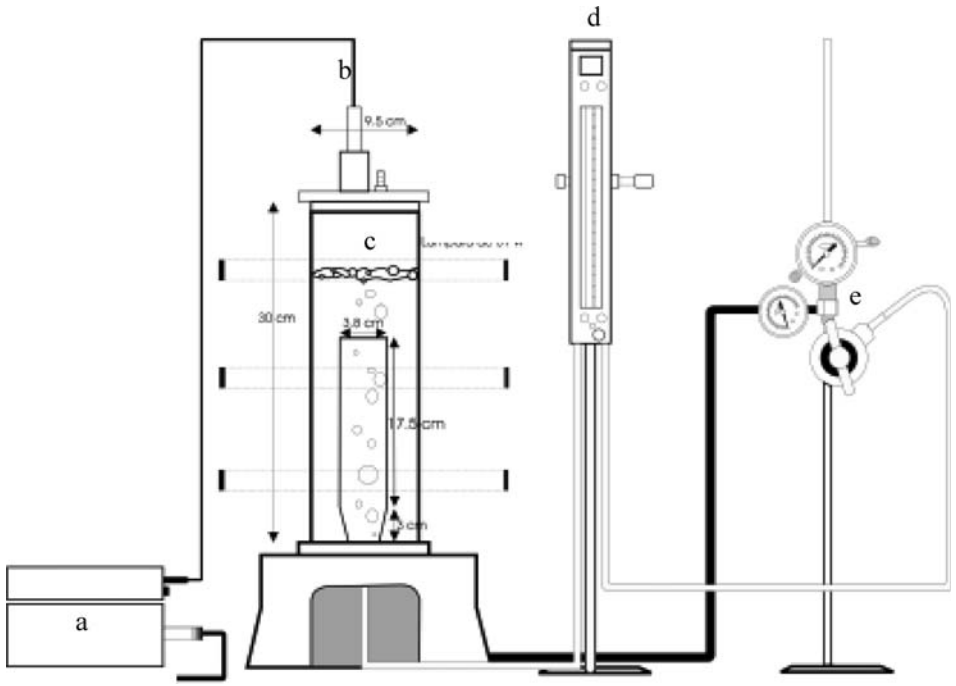

Fig. 1 Airlift bioreactor design. (a) Spectrophotometer, (b) Optic fiber, (c) Bioreactor, (d) Flow meter and (e) Manometer

Effect of plant growth regulators on callus induction

A factorial design was used to evaluate the effect of plant growth regulators on callus induction. Explants (5 mm) were disinfected with chlorine 1% 15 s + iodine 1% 15 s + antibiotics 24 h, which left over 90% of the explants contamination-free. Axenic conditions were tested by culturing explants in marine agar (García-Jiménez et al., 1999). The auxin 1-naphtalene acetic acid (N) (0, 1 mg L^{-1}), kinetin (K), 6-benzylaminopurine (B) (0, 1 mg L^{-1}) and the polyamine spermine (S) (0, 0.018 mg L^{-1}) were used in an experimental design with 16 combinations. Each Petri dish contained four explants and each treatment consisted of five Petri dishes. All culture procedures were done under a sterile laminar flow hood. Solid media were used at an agar concentration of 8% (w/v) and prepared with 0.2 μm filtered, sterilized seawater (33 psu). Cultures were kept for 30 days at 5 μmol photon m^{-2} s^{-1} at 26°C. Weekly evaluations of explants were done to quantify callus induction and regeneration patterns. Explant morphology was documented with an Axiocam® camera, using a Zeiss Stemi SV6 stereoscope and Axiovision® v. 4.1 software. The resulting explants were transferred to an airlift bioreactor, Fernbach and balloon flasks ($n = 3$) at a density of 2 g L^{-1}. Culture conditions were 90 μmol photon m^{-2} s^{-1}, 26°C, salinity 33 and 1 L min^{-1} air flow rate. Every 15 days, a 24 h nutrient pulse (PES) was applied and the bioreactor medium replaced. The number of axes per explant was quantified and photographed with Axiovision® v. 4.1 software.

Bioreactor design and mixing time

An airlift bioreactor with an effective working volume of 1.5 L was constructed with 10 cm inner diameter acrylic pipe at 30 cm height and a 3.8 cm inner diameter draught tube at 22.5 cm height (Fig. 1). Air was pumped into the bioreactor with a Cole-Parmer™ flow meter and it was externally lit with fluorescent lights. Mixing time (T_m), defined as the time needed to reach a given mixing intensity at a given scale (Ca°Caval et al., 2001), was determined using a colorimetric method. An optic fiber spectrophotometer (EPP/2000 StellarNet Inc.™) was placed 5 cm below water surface, and a tracer of 1.0 ± 0.3 mL saturated aniline blue aqueous solution was injected over the optic fiber through a Pasteur pipette. Mixing times were measured from T_0, when maximum absorbance values were recorded, to T_f, when minimum absorbance values were recorded,

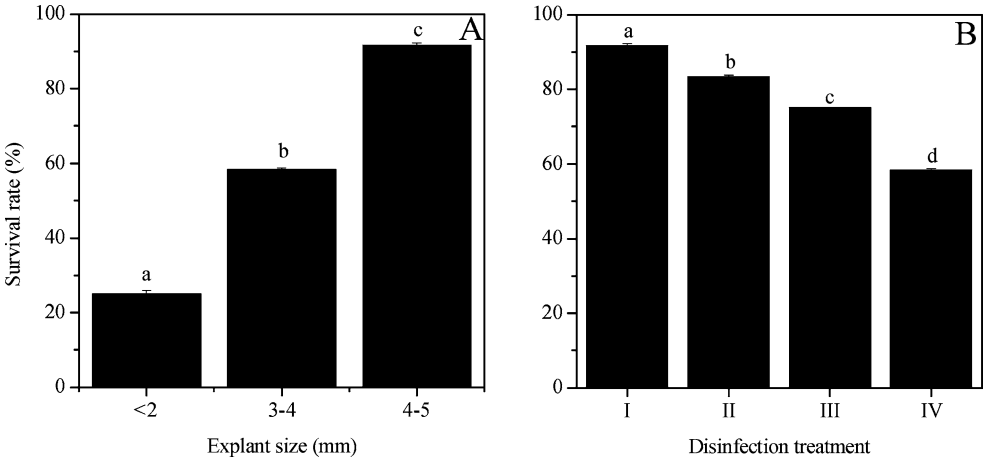

Fig. 2 *K. alvarezii* survival rate (%). A) Effect of explant size. B) Effect of disinfection treatment. I. Chlorine 1 % 15 s + iodine 1% 15 s + antibiotics 24 h; II. Chlorine 1% 25 s + antibiotics 24 h; III. Iodine 1% 25 s + antibiotics 24 h; IV. Osmotic shock 10 s + iodine 1% 10 s + antibiotics 24 h. Different letters indicate significant differences ($p < 0.05$) (±S.D.)

assuming a completely homogenized medium. Each experiment was done at five air flow rates: 200, 400, 600, 800 and 1000 mL min^{-1} ($n = 14$). Mixing times were also determined for Fernbach and balloon flasks, which have traditionally been used for macroalgae culture.

Liquid medium absorbance was recorded automatically every second with the Spectra Wiz™ (EPP 2000-ISA 2000) software. Data corresponding to 643 nm of visible spectra were used in the analyses, after being normalized and adjusted for the volume of each culture system: bioreactor (1.5 L), Fernbach (3 L) and balloon (1 L). Results were adjusted to a decay exponential function ($y = y_0 + Ae^{-(x-x0)/t}$) with the best fit to R^2. The expected absorbance values of each equation at the point when $x = 100$ s were calculated and treated as the time when the liquid medium was expected to be completely homogenized. The times T_{90} and T_{95} correspond to the times when 90% and 95% of homogenization were reached, respectively.

Statistical analysis

Data were tested for normality and variance homogeneity. A one-way analysis of variance (ANOVA) ($\alpha < 0.05$) was used to establish the differences in survival rate for explant sizes and disinfection treatments, as well as the callus induction rates for the plant growth regulator combinations. This was followed by a LSD posteriori analysis. Differences in the number of axes per explant were determined with a Kruskal-Wallis analysis ($\alpha = 0.05$). All statistical analyses were done using Stat-graphics Plus 4.1, and fitting of the exponential function of mixing times was done with the Origin 7.5 software.

Results

Survival increased as *Kappaphycus alvarezii* explant size increased. Significant differences in survival rate were observed between sizes ($p < 0.05$), with 5 mm explants having 91% survival and those <2 mm having 25% survival (Fig. 2A). The chlorine 1% 15 s + iodine 1% 15 s + antibiotics 24 h disinfection treatment had the highest survival rate (92%). The chlorine 1% 25 s + antibiotics 24 h treatment had 83% survival and the iodine 1% 25 s + antibiotics 24 h had 75% survival. The osmotic shock 10 s + iodine 1% 10 s + antibiotics 24 h treatment had a 58% survival rate. Differences between these disinfection treatments were significant ($p < 0.05$) (Fig. 2B).

Callus induction percentages in *K. alvarezii* cultured with plant growth regulators showed that the plant growth regulators N and B induced more than 100% more calluses than the control (Fig. 3A). Other combinations exhibited no significant differences in callus induction versus the control. The N+K, N+B and B+K combinations had the lowest callus induction rates. The S+N+K combination induced more calluses than the control (Fig. 3B), although the other combinations with S showed no significant difference

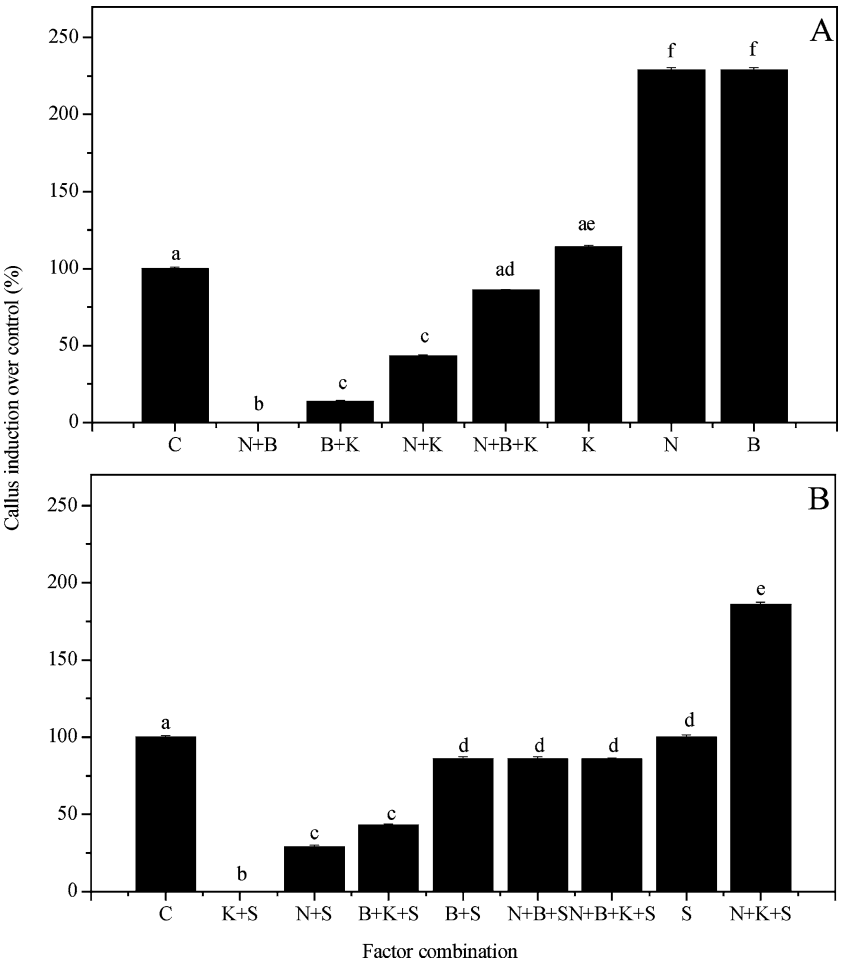

Fig. 3 Effect of plant growth regulators on callus induction in *K. alvarezii* explants using 16 factorial combinations: C) control; N) 1-naphtalene acetic acid; B) 6-benzylaminopurine; K) kinetin; S) spermine. Different letters indicate significant differences ($p<0.05$) (±S.D.)

in callus induction rate. The *K. alvarezii* explants produced filamentous calluses, crystalline filaments and pigmented buds (Fig. 4). The calluses were amorphous cell masses beginning from the medullar region, at one or both cut ends of the explant, and were pinkish to brown in color. Isolated crystalline filaments were observed developing along cortical and medullar regions or from the amorphous cell mass, while pigmented buds developed from the explant cortex. Callus structure developed faster (7 days) in treatments containing spermine (e.g. N+K+S) than in those with auxins and cytokinins. K and S produced more buds than calluses. Calluses produced with N+K+S transferred into the bioreactor grew for more than 30 days, although crystalline filaments disappeared during cultivation.

After 45 days cultivation, *K. alvarezii* explants grew significantly faster in Fernbach flasks than in the airflow bioreactor and balloon flasks (Fig. 5A). However, explants cultured in the bioreactor developed multiple axes (Fig. 5B) whereas those in balloon or Fernbach flasks did not (Fig. 4E, F).

Mixing times varied between the three culture systems (Fig. 6). The times T_{90} and T_{95} were shorter in the bioreactor (4.6–10.3 s) than in the Fernbach flasks (5.2–13.4 s) and balloon flasks (6.3–17.3 s) at all tested airflow rates. In addition, standard deviations were lower for the bioreactor, especially at flow rates from 200 to 800 mL min^{-1}.

Discussion

The results show that filamentous callus production from axenic *K. alvarezii* explants was effectively promoted with the N and B plant growth regulators. Filamentous and compact callus development has been described in *K. alvarezii* by Polne and Gibor (1987). The present results suggest that development of filamentous

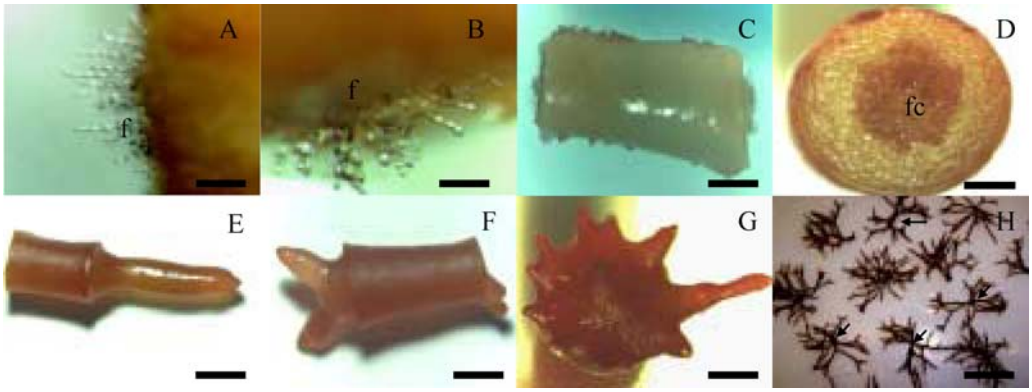

Fig. 4 *K. alvarezii* calluses produced after 16 days cultivation with polyamines and plant growth regulators. (A) Crystalline filaments (f) produced with 1-naphtalene acetic acid; (B) Buds and calluses developed with spermine (f) (scale bar 0.5 mm); (C) Basal, intercalary and apical growth developed in explant cultures with spermine; (D) Section of the filamentous callus (fc), (scale bar 1 mm); (E) Single-axis growth in Fernbach culture; (F) Multiple-axis growth in bioreactor culture without plant growth regulators; (G) Multiple-axis grown in bioreactor culture with plant growth regulators (scale bar 2 mm). (H) Spherical plantlets produced in bioreactor after 45 days cultivation (scale bar 5 mm), arrows indicate original explant

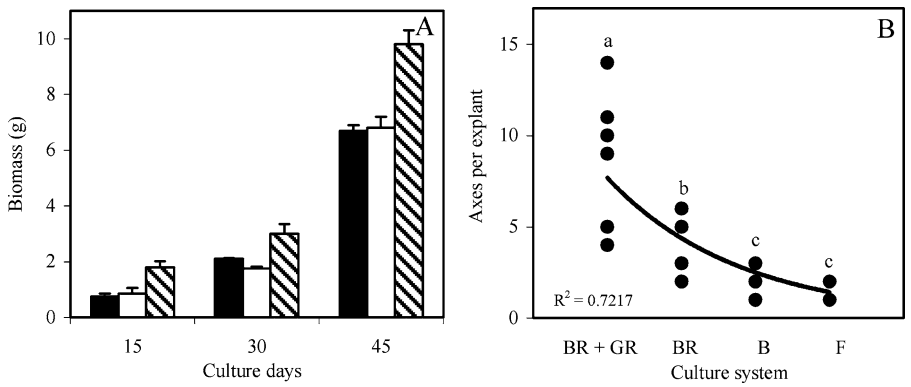

Fig. 5 A) Biomass increase of *K. alvarezii* explants after 15, 30 and 45 days cultivation in balloon flasks (black bars), airlift bioreactor (white bars) and Fernbach flasks (lined bars). B) Axes produced per explant in the three culture systems after 30 days cultivation. Explants cultured *in vitro* with plant growth regulators transferred to bioreactor (BR+GR); Explants cultured without plant growth regulators transferred to bioreactor (BR); Explants cultured in balloon flasks (B); and Explants cultured in Fernbach flasks (F). Different letters indicate significant differences ($p<0.05$)

callus is more common than of compact callus, but this may be in response to agar concentration. Dawes and Koch (1991) observed mostly sponge callus growing in media at 3–8% agar, whereas production of isolated crystalline filaments and pigmented buds in this study was obtained in substrates containing 8% agar. The callus pigmentation observed in the present study (light pink to brown) coincides with that reported in previous studies (Polne & Gibor, 1987; Dawes & Koch, 1991; Dawes et al., 1993).

Filamentous calluses developed from medullar cells in the present study. Polne and Gibor (1987) reported that calluses originated from cortical tissue in *K. alvarezii* and *Eucheuma uncinatum* Setchell and Gardner, while Dawes and Koch (1991) observed calluses originating from medullar tissue. These differences in callus growth and development have been attributed to the osmolality and agar concentration in the culture media. Robaina et al. (1990) reported a strong correlation between media solidity and callus and bud production in *Laurencia spp.* and *Grateloupia doryphora* (Montagne) M.A. Howe, with higher callus induction at 8 and 15 g L^{-1} agar concentration. In another study, Robaina et al. (1992) reported that an increase in agar concentration from 0.8 to 1.5% promoted callus development in *Laurencia spp.* and

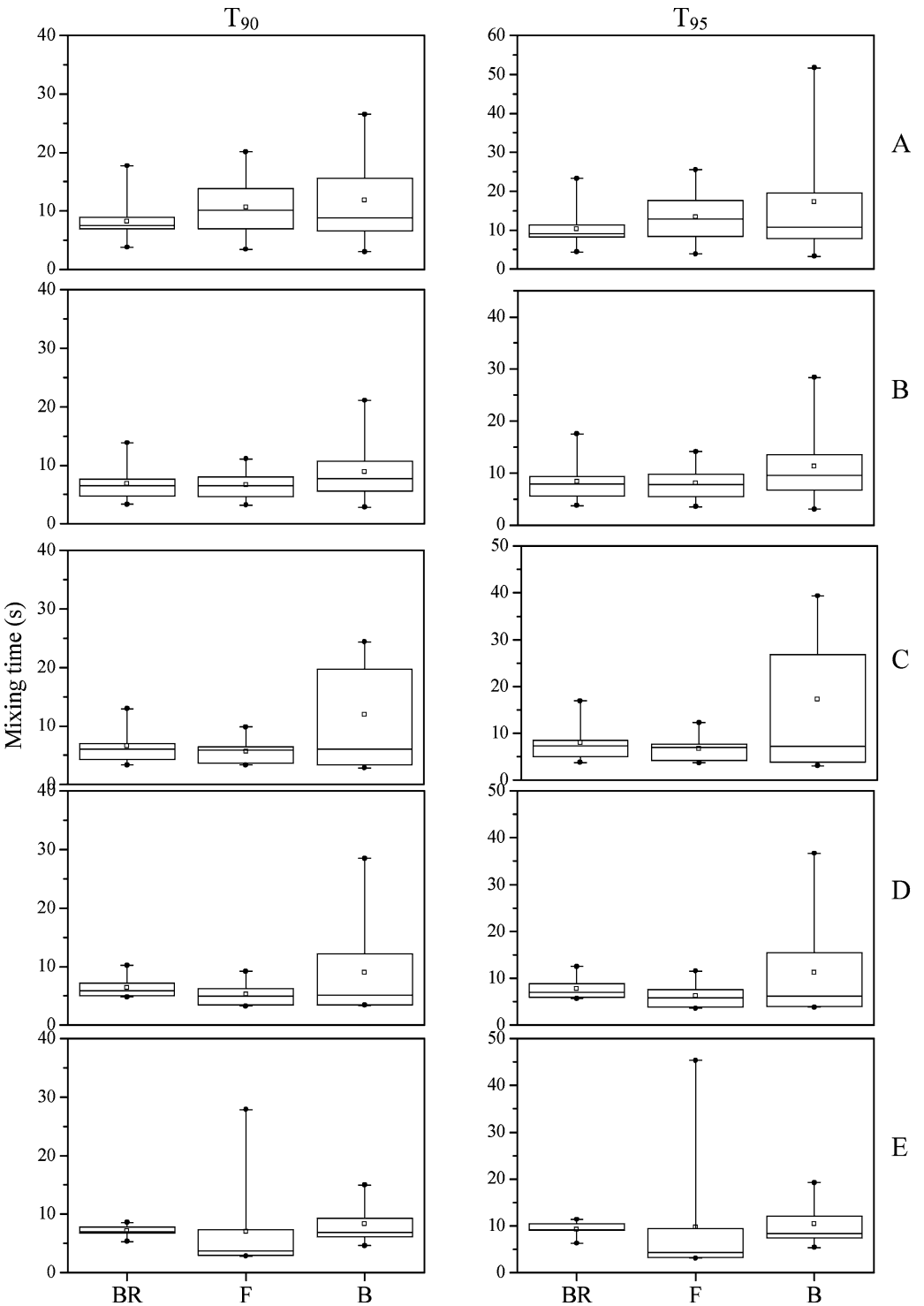

Fig. 6 Mixing times at T_{90} and T_{95} for airlift bioreactor (BR), Fernbach (F) and balloon flasks (B) at five working flow rates: (A) 200; (B) 400; (C) 600; (D) 800 and (E) 1000 mL min^{-1} ($\pm$ S.D.)

inhibited morphogenesis. For *K. alvarezii*, Reddy et al. (2003) reported no effect of agar concentration on callus induction, except at 0.8%, when bud development surpassed callus development.

The present results showed callus induction to be strongly effected by the tested plant growth regulators, although this does not coincide with some previous studies (Evans & Trewavas, 1991; Bradley, 1991). Some data indicate the usefulness of plant growth regulators for *in vitro* culture, for example, Jennings (1969) and Dawes (1971) reported that auxins and cytokinins stimulated growth, whereas Davison (1950) and Buggeln (1976) observed no significant effects. More recently, Reddy et al. (2003) reported 80% callus induction for *K. alvarezii* using solid substrates (0.8, 1, 1.5, 2 and 3% agar) enriched with PES, but without plant growth regulators. In this study use of healthy, recently cultivated and acclimated thalli was more important than the use of N or B in *K. alvarezii* callus induction. In the present study, however, N and B were observed to have a strong affect on callus induction and development of multiple structures (filaments and pigmented buds). This coincides with results for *Grateloupia dichotoma* J. Agardh (Yokoya & Handro, 1996) in which auxins and B, alone or in combination, promoted callus growth and elongation of apical and intercalary segments. Dawes and Koch (1991) also reported that N and B strongly induced callus growth in *K. alvarezii*. The effects of auxins have been linked to increases in protein synthesis, morphogenesis and changes in genetic expression (Yokoya et al., 2004) while cytokinins effects have been tied to increases in cell division and metabolic activities (Evans, 1984).

This is the first report on the effect of S in *K. alvarezii* callus induction. Spermine caused simultaneous callus growth in apical, intercalary and basal zones of the explant. Callus development on basal zones is the most common observation in red macroalgae, although Reddy et al. (2003) reported callus growth in apical zones for *K. alvarezii*. In *Gracilaria perplexa* Byrne and *Gracilariopsis tenuifrons* (C.J. Bird and E.C. Oliveira) Fredericq and Hommersand, apical callus development was induced by exogenous application of plant growth regulators, however, in *Gracilaria tenuistipitata* C.F. Chang and B.M. Xia exposition to air induced callus development (Yokoya et al., 2004). Loss of filaments during explant transfer to the bioreactor was also mentioned by Robaina et al. (1992), who reported filament disappearance after calluses were removed from *Laurencia spp*. explants.

Callus induction time in *K. alvarezii* in the present study is shorter than in other reports. Robaina et al. (1990) reported callus induction in *Grateloupia doryphora* (Montagne) M.A. Howe after 15 days, in *Laurencia spp*. after 30 days and in *Gelidium versicolor* (S.G. Gmelin) J.V. Lamouroux after 45 days. Callus induction time in *K. alvarezii* generally occurs after 14 days (Dawes & Koch, 1991; Dawes et al., 1993; Reddy et al., 2003).

Before the present study, mixing time had not been used to determine differences in the hydrodynamic behavior between an airlift bioreactor and two traditional culture systems (i.e. Fernbach and balloon flasks). Although common culture vessels have been used extensively for bacteria, yeast and algae culture, their hydrodynamic behavior has been ignored under the assumption that they are adequate for macroalgae cultivation. Increases in the areas of gas and nutrient exchange in these cultivation systems is another important aspect to consider (Sajc et al., 2000). The mixing times T_{90} and T_{95} and their standard deviations indicated that mixing efficiency in the airlift bioreactor had only minor static areas within the system. Liquid circulation is very sensitive to bioreactor geometry (i.e. height, diameter) and thus the geometry of the bioreactor tested here allowed adequate liquid circulation of the culture media (Sánchez-Mirón et al., 2004). Mixing time and other important parameters such as liquid circulation and mass transfer establish a baseline for better bioreactor performance and scalability of the bioreactor system. Use of an optic fiber spectrophotometer is a novel and rapid method for evaluating mixing time in bioreactor characterization. It also has several advantages over other methods: faster response without data loss; minimizes measurement error; allows use of several solutions as tracers; *in situ* measurements are faster and it reduces friction resistance.

The present study is an initial approach to establishing suspension culture of *K. alvarezii* in airlift bioreactors. Use of spermine together with other plant growth regulators reduced time to callus induction in *K. alvarezii*, and calluses produced with plant growth regulators can be cultured in an airlift bioreactor. This also demonstrates the potential use of bioreactors in biomass production and maintenance under axenic controlled conditions, although further research is needed on other aspects of callus growth and

development under different culture conditions in a bioreactor.

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References

Ask EI, Azanza RV (2002) Advances in cultivation technology of commercial eucheumatoid species: A review with suggestions for future research. Aquaculture 206: 257–277

Barahona LF, Rorrer GL (2003) Isolation of monoterpenes from bioreactor cultured microplantlets of the macrophytic red algae *Ochtodes secundiramea* and *Portiera hornemanii*. J Nat Prod 66: 743–751

Bixler HJ (1996) Recent developments in manufacturing and marketing carrageenan. Hydrobiologia 326/327: 35–57

Bradley PM (1991) Plant hormones do have a role in controlling growth and development of algae. J Phycol 27: 317–321

Buggeln RG (1976) Auxin, an endogenous regulator of growth in algae? J Phycol 12: 355–358

Ca°Caval D, Oniscu C, Galaction AI, Dumitru IF (2001) Characterization of mixing efficiency in bioreactors. Roum Biotechnol Lett 6: 281–291

Cohen E, Shoshana A, Heimer YH, Mizrahi Y (1984) Polyamine biosynthetic enzymes in the cell cycle of *Chlorella*. Plant Physiol 74: 385–388

Davison FF (1950) The effects of auxins on the growth of marine algae. Am J Bot 37: 502–510

Dawes CJ (1971) Indole-3-acetic acid in the green algae coenocyte *Caulerpa prolif*era (Chlorophyceae, Siphonales). Phycologia 10: 375–379

Dawes CJ, Koch EW (1991) Branch, micropropagules and tissue culture of the red alga *Eucheuma denticulatum* and *Kappaphycus alvarezii* farmed in the Philippines. J Appl Phycol 6: 21–24

Dawes CJ, Trono GC Jr, Lluisma AO (1993) Clonal propagation of *Eucheuma denticulatum* and *Kappaphycus alvarezii* for Philippine farms. Hydrobiologia 260/261: 379–383

Evans ML (1984) Functions of hormones at the cellular level of organization. In Scott TK (ed), Encyclopaedia of plant physiology. New series, Vol. 10. Springer-Verlag, Berlin, pp. 23–80

Evans LV, Trewavas AJ (1991) Is algal development controlled by plant growth substances? J Phycol 27: 322–326

García-Jiménez P, Rodrigo M, Robaina RR (1998) Influence of plant growth regulators, polyamines and glycerol interaction on growth and morphogenesis of carposporelings of *Grateloupia* cultured *in vitro*. J Appl Phycol 10: 95–100

Garcia-Jimenez P, Marian FD, Rodrigo M, Robaina R (1999) Sporulation and sterilization method for axenic culture of *Gelidium canariensis*. J Biotech 70: 227–229

Guzmán-Urióstegui A, García-Jiménez P, Marián F, Robledo D, Robaina R (2002) Polyamines influence maturation in reproductive structures of *Gracilaria cornea* (Gracilariales, Rhodophyta). J Phycol 38: 1169–1175

Huang Y, Rorrer GL (2002) Optimal temperature and photoperiod for the cultivation of *Agardhiella subulata* microplantlets in a bubble column photobioreactor. Biotech Bioeng 79: 135–144

Jennings RC (1969) Cytokinins as endogenous plant growth regulators in the algae *Ecklonia* and *Hypnea*. Aust. J Biol Sci 22: 621–627

Muñoz J, Freile-Pelegrín Y, Robledo D (2004) Mariculture of *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae) color strains in tropical waters of Yucatán, México. Aquaculture 239: 161–177

Ohno M, Largo DB, Ikumoto T (1994) Growth rate, carrageenan yield and gel properties of cultured kappa carrageenan producing red alga *Kappaphycus alvarezii* (Doty) Doty in the subtropical waters of Shikoku. Jpn J Appl Phycol. 6: 1–5

Paula EJ, Pereira RTL, Ohno M (2002) Growth rate of the carrageenophyte *Kappaphycus alvarezii* (Rhodophyta, Gigartinales) introduced in subtropical waters of São Paulo State, Brazil. Phycol Res 50: 1–9

Polne M, Gibor A (1987) Calluses and callus-like growth in seaweeds: Induction and culture. Hydrobiologia 151/152: 131–138

Polzin JP, Rorrer GL (2003) Halogenated monoterpene production by microplantlets of the marine red alga *Ochtodes secundiramea* within an airlift photobioreactor under nutrient medium perfusion. Biotechnol Bioeng 82: 415–428

Provasoli L (1968) In Watanabe, Hattori (eds), Culture and collections of algae. Jpn Soc Plant Physiol Tokyo pp. 63–75

Reddy CRK, Raja Krishna Kumar G, Siddhanta K, Tewari A (2003) *In vitro* somatic embryogenesis and regeneration of somatic embryos from pigmented callus of *Kappaphycus alvarezii* (Doty) Doty (Rhodophyta, Gigartinales). J Phycol 39: 610–616

Robaina R, García-Reina P, Luque A (1990) The effects of the physical characteristics of the culture medium on the development of red seaweeds in tissue culture. Hydrobiologia 204/205: 137–142

Robaina R, García-Jiménez, Luque A (1992) The growth pattern and structure of callus from the red alga *Laurencia sp.* (Rhodophyta, Ceramiales) compared to shoot regeneration. Bot Mar 35: 267–272

Rorrer GL, Tucker MP, Cheney DP, Maliakal S (2001) Bromoperoxidase activity in microplantlet suspension cultures of the macrophytic red alga *Ochtodes secundiramea.* Biotech Bioeng 74: 389–395

Rorrer GL, Cheney DP (2004) Bioprocess engineering of cell and tissue cultures for marine seaweeds. Aquacult Eng 32: 11–41

Sajc L, Grubisic D, Vunjak-Novakovic G (2000) Bioreactors for plant engineering: An outlook for further research. Biochem Eng J 4: 89–99

Sacramento A, García-Jiménez P, Alcázar R, Tiburcio A, Robaina R (2004) Influence of polyamines on the

sporulation of *Grateloupia* (Halymeniaceae, Rhodophyta). J Phycol 40: 887–894

Sánchez Mirón A, Cerón MC, García F, Molina E, Chisti Y (2004) Mixing in Bubble Column and Airlift Reactors. Chem Eng Res Des 82: 1367–1374

Yokoya NS, Handro W (1996) Effects of auxins and cytokinins on tissue culture of *Grateloupia dichotoma* (Gigartinales, Rhodophyta). Hydrobiologia 326/327: 393–400

Yokoya NS, West JA, Luchi AE (2004) Effects of plant growth regulators on callus formation, growth and regeneration in axenic tissue cultures of *Gracilaria tenuistipitata* and *Gracilaria perplexa* (Gracilariales, Rhodophyta). Phycol Res 52: 244–254