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Callus induction and micropropagation improved by colchicine and phytoregulators in *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae)

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Abstract Tissue culture techniques were applied for micropropagation of the red alga *Kappaphycus alvarezii* in order to select the best strain and experimental system for *in vitro* culture. Five strains were tested: brown (BR), green (GR) and red (RD) tetrasporophytes, brown female gametophyte (BFG), and a strain originating from tetraspore germination ("Edison de Paula", EP). The effects of three culture media were tested on callus formation, regeneration from explants and from callus in the three tetrasporophytic and EP strains: seawater enriched with half-strength of von Stosch's (VS 50) and Guillard & Ryther's (F/2 50) solutions, plus synthetic ASP 12-NTA medium, with or without gelling agent. Explants of the EP strain were treated with glycerol and the phytoregulators indole-3-acetic acid (IAA); 2,4-diclorophenoxyacetic acid (2,4-D); and benzylaminopurine (BA), alone or in combination. The effects of colchicine (0.01%) during 24, 48, 72 hours and 14 days were analyzed in the BFG and EP strains. The EP strain showed the highest percentage of explants forming callus and regeneration from explants in VS 50, indicating its high potential for micropropagation in comparison to the other strains. Regeneration from callus was very rare. Treatments with glycerol and IAA:BA (5:1 mg L^{-1}) stimulated the regeneration from explants. Significant differences were observed in the percentages of regeneration of EP strain explants treated with colchicine for 14 days. Our results indicate that IAA and BA stimulated the regeneration process, and that colchicine produced explants with high potential for regeneration, being useful for improving the micropropagation of *K. alvarezii*.

Keywords Colchicine · *Kappaphycus alvarezii* · Micropropagation · Strain selection · Tissue culture

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Introduction

Since the beginning of its commercial cultivation in the 1970s, *Kappaphycus alvarezii* (Doty) Doty ex P. C. Silva has been propagated vegetatively. Repeated clonal propagation reduces genetic variability which may be the reason for the observed decrease in growth rates, carrageenan yield and gel strength, and the increase of susceptibility to diseases (Trono and Lluisma 1992; Hurtado and Cheney 2003). According to Ask and Azanza (2002), those problems could be mitigated by strain selection, genetic manipulation and transgenic production. The cultivation could also be improved by careful selection of specimens from previous cultivated generations or in seed stocks from wild strains (van der Meer 1987).

The application of micropropagation in commercially important seaweeds may help to multiply selected strains, to increase seed stock production for cultivation and to produce a high number of uniform specimens with desired characteristics in a short period of time. Previous studies showed that micropropagation of *K. alvarezii* can be successful, with production of propagules within 4 to 8 weeks (Dawes and Koch 1991). Reddy et al. (2003) observed that plants originating from tissue culture had

growth rates 1.5 to 1.8 times higher than field plants when cultivated in sea farms in India. According to these authors, the high potential of development of adventitious plantlets is a promising tool to the fast clonal production of stocks for commercial cultivation.

Despite the advantages, seaweed tissue culture has faced several problems including the lack of good protocols to obtain axenic cultures, callus induction and differentiation of whole plants with the application of plant growth regulators (Collantes and Melo 1995).

Organic carbon sources and plant growth regulators play an important role on *in vitro* propagation of macroalgae, although the optimum concentrations of these compounds vary from species to species. The carbon sources support growth and development, while the plant growth regulators control the growth pattern and development (Garcia-Jimenez et al. 1998). Several studies reported the occurrence of hormones in seaweeds, *e.g.* auxins (Jacobs et al. 1985; Bradley 1991; Jacobs 1993; Stirk et al. 2004), and cytokinins (Bradley 1991; Zhang et al. 1991; Stirk and van Staden 1997; Stirk et al. 2003). The effects of exogenous applications of auxins and cytokinins in seaweeds are documented in literature (Dawes and Koch 1991; Huang and Fujita 1997; Yokoya et al. 1999; Yokoya 2000; Yokoya et al. 2004).

Based on several techniques, including intensive cultivation and selection, mutation induced by X-rays and treatments with colchicine, new and improved strains of *Laminaria* J.V. Lamouroux were produced, resulting in higher production and geographic expansion of cultivation industries in China (Cheney 1984). The effect of colchicine is well known as an inhibitor of spindle fibers formation during cell division, giving rise to cells with double the number of chromosome, stimulating polyploidy (De Robertis and De Robertis 1980).

In this study we tested the technique of tissue culture in the micropropagation of *K. alvarezii* aiming to select the best strain and experimental system for *in vitro* culture, to assess the effects of phytoregulators on micropropagation, and to produce new strains by application of colchicine.

Materials and methods

Strains studied

Five strains of *Kappaphycus alvarezii* were tested: three tetrasporophytic strains (brown, BR, green, GR, and red, RD), a brown female gametophyte (BFG) and a brown strain derived from tetraspore germination isolated by Paula et al. (1999), named "Edison de Paula" strain (EP). All were cultivated in sterilized seawater (32 psu) enriched with 25% Guillard & Ryther's solution (F/2), according to McLachlan (1973), photoperiod of 14 h, temperature of 25°C (±1°C) and irradiance of 200 (±10) μmol photons m^{-2} s^{-1}. Cultures were aerated in alternating periods of 30 minutes.

Axenic cultures

Axenic cultures of the five strains of *Kappaphycus alvarezii* were obtained according to the methodology described by Yokoya (1996). Branches of *K. alvarezii* (5 cm) were incubated in an antifungal and antibiotic solution (including 10,000 units penicillin G, 10 mg streptomycin sulphate and 25 μg amphotericin B *per* liter, Sigma) and 0.1 μg mL^{-1} nystatin (Sigma) added to sterilized seawater enriched with 50% von Stosch's solution (VS 50, according to Edwards 1970) for 48 h. After this period, branches were transferred to an aseptic chamber and washed in sterilized seawater with 0.05% sodium hypochlorite and 2% organic detergent (Amway), followed by successive washes with autoclaved seawater. Thallus segments 3 mm in length were cut and inoculated into the culture medium described in each experiment.

Axenic cultures were grown under the following culture conditions: salinity of 30 psu, photoperiod of 14 h, temperature of 23°C (±2°C) and irradiance of 40 (±10) μmol photons m^{-2} s^{-1}.

Effects of culture medium

Three media were tested: autoclaved seawater (30 psu) enriched with 50% of von Stosch's solution (VS 50) or 50% Guillard & Ryther solution (F/2 50), and synthetic ASP 12-NTA medium (ASP 12, according to Iwasaki 1961, Sigma), with addition of 0.5% agar (Merck). For each treatment, 3 replicates with 8 to 10 explants from the BR, GR, RD and EP strains were inoculated into 30 mL of culture medium. Calluses were induced in 45 days, and the culture medium was renewed at intervals of 15 days.

After the period of callus induction, explants were transferred to liquid culture medium (30 mL) under the same culture conditions. Each treatment had three replicates with the surviving explants from the previous experiment (from 1 to 9 explants *per* replicate). Explants were cultured during 35 days and culture medium was renewed weekly.

Effects of phytoregulators on direct regeneration

In our study, direct regeneration is defined as the regeneration originating from explant cells. The experimental design for testing the phytoregulators followed the methodology described by Yokoya (1996) and the phytoregulator concentrations were chosen based on results of Yokoya et al. (1999) and Yokoya (2000). Explants from the EP strain were cultured in VS 50 medium with glycerol

(90 mM, Synth) previously autoclaved, with the addition of 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma) and 6-benzylaminopurine (BA, Sigma) in concentrations of 0.5 and 5.0 mg L^{-1}. Combination of IAA:BA (indole-3-acetic acid, IAA - Sigma) was tested in concentration of 5:1 (mg L^{-1}). Culture medium without phytoregulators was used as the control. For each treatment, three replicates with 5 explants in each were inoculated into 30 mL of culture medium. Explants were cultured for 61 days and the renewal of culture medium was made fortnightly.

Effects of colchicine on direct regeneration

Explants from the BFG and EP strains were cultured for 24, 48, 72 h and 14 days in VS 50 medium with glycerol (90 mM), previously autoclaved, and 0.01% colchicine (Sigma). VS 50 culture medium without colchicine was used as the control.

Three replicates with 5 explants in each were used in the control, while 10 replicates with 6 to 8 explants in each were used in the treatments with colchicine. Explants were inoculated into 30 mL of culture medium and cultured for 73 days. The culture medium was renewed weekly.

Data and statistical analysis

The percentage of explants forming callus and the number of explants with direct or indirect regeneration (i.e. developing upright axes from the explant or callus) was recorded.

Data were submitted to one-way, many effects or repeated measurements analysis of variance followed by *a posteriori* tests of Tukey (one-way), Duncan (many effects), and Unequal N (repeated measurements with different n) considering $p<0.05$. Analysis was using *Statistica* Software (release 6.0).

Results

Effects of culture medium

Two types of callus were observed in the four strains of *Kappaphycus alvarezii* studied: a) filamentous callus, observed in the explant surface exposed to the air, composed of filaments derived from divisions of cortical and medullary cells (Fig. 1a); and b) compact callus, growing on the explant surface in contact with culture medium, formed by cortical and medullary cells and having organized medullary and cortical regions (Fig. 1a and b). The EP strain also presented filament proliferation growing from the explant region in contact with culture medium (Fig. 1c). In all strains, callus formation was observed after 15 days of cultivation, and callus growth was observed during the entire experimental period.

Explants of the BR strain had the highest percentage of explants forming callus when cultured in F/2 50 in comparison to others culture media. The EP strain showed higher levels of explants forming callus in VS 50 and F/2 50 media

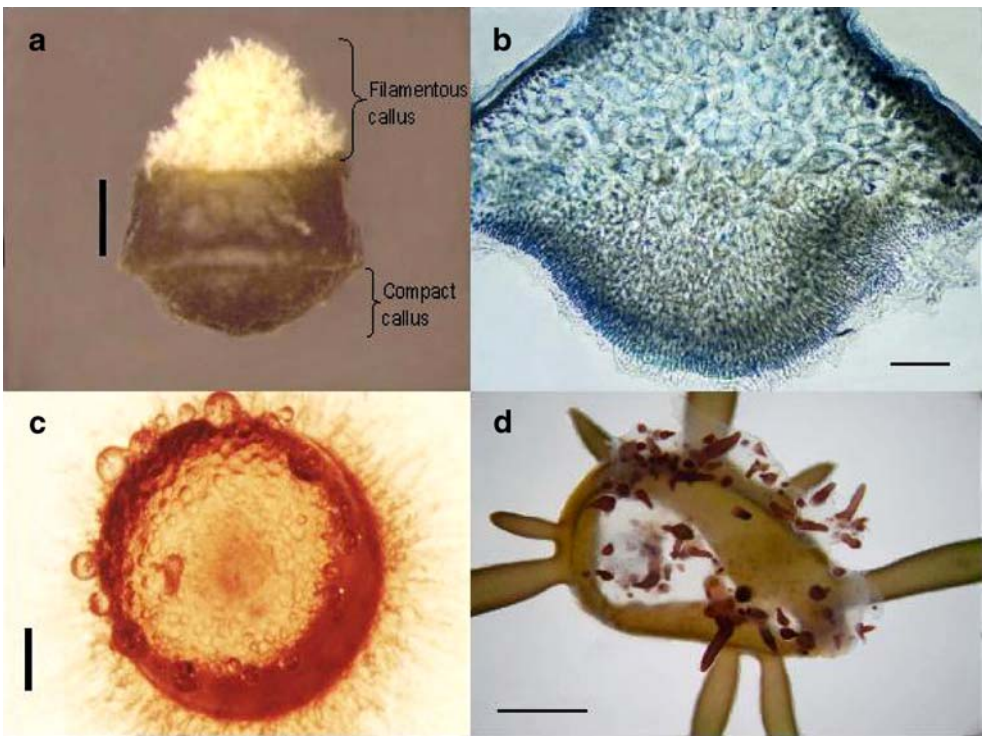

Fig. 1 Callus formation and indirect regeneration in *Kappaphycus alvarezii*. **a)** Filamentous and compact calluses from green tetrasporophytic strain induced by VS 50 medium with 0.5% agar after 45 days (scale bar= 1 mm); **b)** Longitudinal section of compact callus from green tetrasporophytic strain (scale bar =200 μm); **c)** Callus from "Edison de Paula" strain growing on the surface of the explant in contact with VS 50 medium with 0.5% agar after 45 days (scale bar=1 mm); **d)** Indirect regeneration from filamentous callus of the brown tetrasporophytic strain cultured at VS 50 liquid medium and agitation during 30 days (scale bar=500 μm)

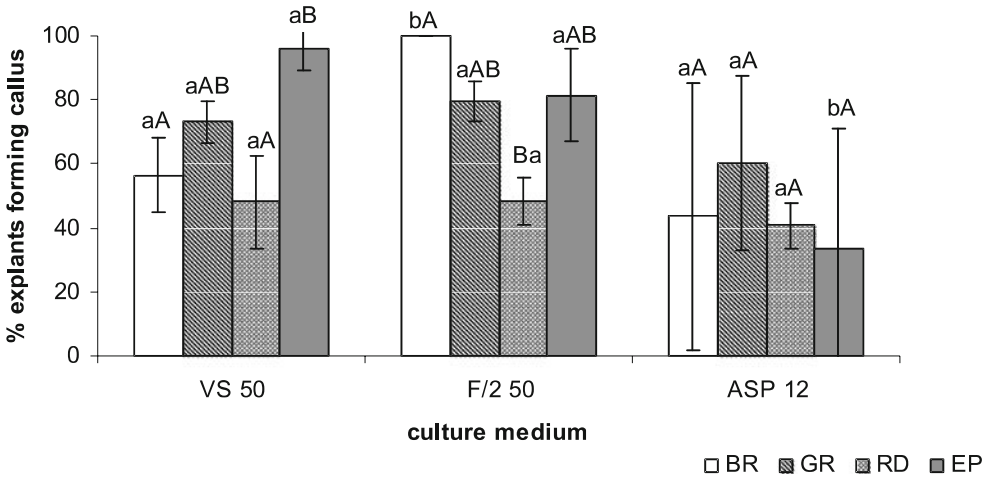

Fig. 2 Effects of culture medium on callus formation in brown (BR), green (GR) and red (RD) tetrasporophytic strains, and "Edison de Paula" (EP) strain of *Kappaphycus alvarezii* cultured in VS 50, F/2 50 or ASP 12 with 0.5% agar for 45 days. Values presented as average (n=3) and vertical bars represent confidence intervals. The uppercase letters indicate significant differences among culture medium for each strain and the lowercase letters, the significant differences among strains, according to Duncan's *a posteriori* test ($p<0.05$)

than in ASP 12 medium. For the other strains, significant differences were not observed among the treatments (Fig. 2).

Callus production from the EP strain was also significantly different than those presented by the BR and RD strains when cultured in VS 50 medium, and significantly different than the RD strain when cultured in F/2 50 medium, while the BR strain showed significant differences only in relation to the RD strain when cultured in F/2 50 medium (Fig. 2).

During the period of callus induction, direct regeneration occurred in some explants independently of strain or type of culture medium. In these explants, calluses ceased from growing.

Despite the calluses transferring to liquid medium, indirect regeneration was very rare, in all culture medium tested, with the exception of one explant among approximately 100 from all strains tested in our study. The explant of BR strain showed several micropropagules (approximately 90) regenerating from filamentous callus, when cultured in VS 50 medium with glycerol and mechanical agitation for 30 days (Fig. 1d).

Effects of phytoregulators on direct regeneration

Explants cultured in VS 50 medium with glycerol and IAA:BA 5:1 (mg L^{-1}) showed significant differences in the number of upright axes *per* explant than the control or treatment with 2,4-D 0.5 (mg L^{-1}) (Fig. 3). In the others treatments, no significant differences were observed. The same explants, when transferred to VS 50 without glycerol and cultured for 23 days, presented significant differences only in treatment with IAA:BA (5:1 mg L^{-1}) in comparison to the control (Fig. 3).

Effects of colchicine on direct regeneration

Explants of EP strain of *Kappaphycus alvarezii* cultured in VS 50 medium with glycerol, in different incubation periods with colchicine, showed direct regeneration in all treatments. No significant differences in the number of upright axes *per* explant among treatments with colchicine were observed. However, the level of direct regeneration

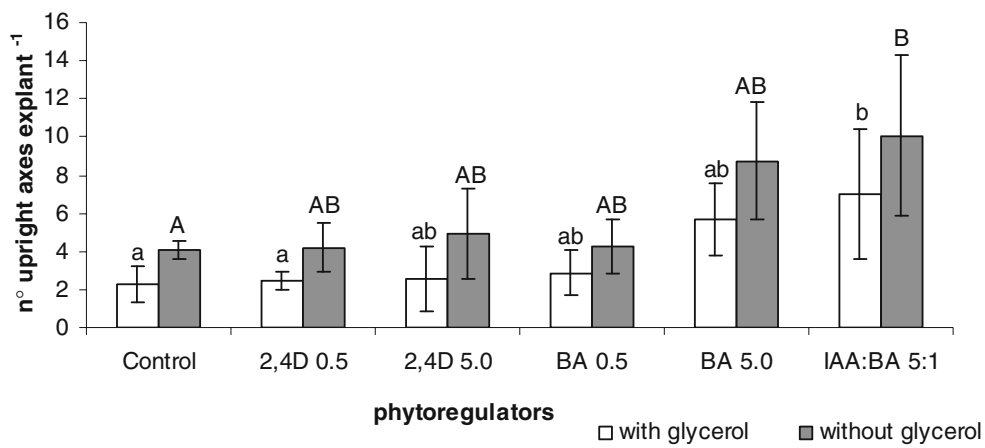

Fig. 3 Effects of glycerol and phytoregulators in direct regeneration of "Edison de Paula" (EP) strain of *Kappaphycus alvarezii* cultured for 58 days in liquid VS 50 medium. Values presented as average (n=3) and vertical bars represents confidence intervals. The uppercase letters indicate significant differences among treatments with glycerol and the lowercase letters, the significant differences among treatments without glycerol, according to Tukey's test ($p<0.05$)

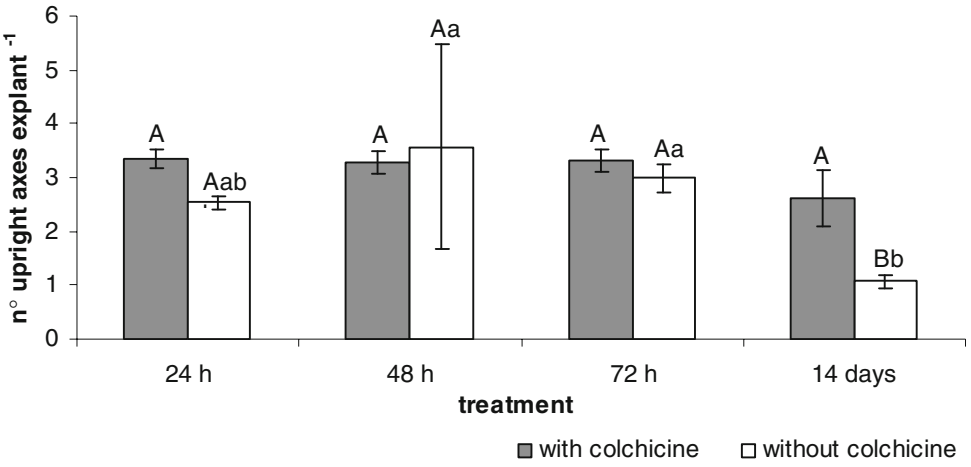

Fig. 4 Effects of colchicine on direct regeneration of "Edison de Paula" (EP) strain of *Kappaphycus alvarezii* cultured in liquid VS 50 medium with glycerol for 67 days. Values presented as average (n=3 for control; n=10 for treatments) and vertical bars represent confidence intervals. The uppercase letters indicate significant differences in the treatments and the lowercase letters, the significant differences among treatments, according to Unequal N's *a posteriori* test ($p<0.05$)

was significantly different in explants incubated for 14 days with colchicine than the control (Fig. 4).

Explants of BFG strain incubated with colchicine for 24, 48, 72 h and 14 days died after 15 days.

Discussion

Callus formation in *Kappaphycus alvarezii* strains was observed after 15 days in all culture media tested, corroborating data obtained by Reddy et al. (2003) for the same species. These authors observed percentages of callus induction higher than 80% in explants cultured in Provasoli culture medium with 1.5% of bacto-agar, without addition of other compounds, as phytoregulators or carbon organic compounds. In our experiments, the suitable medium for callus induction was F/2 50 in the *K. alvarezzi* BR strain, while in the EP strain higher levels of callus induction were presented in VS 50 and F/2 50 media. Although no significant differences were observed among the culture media, explants cultured in ASP 12 medium had lower percentage of explants forming callus and presented thallus bleaching. Based on these results, VS 50 medium was selected for subsequent experiments.

Some explants cultured in 0.5% agar showed direct regeneration from the explant surface in contact with the medium. When this occurred, the growth of filamentous callus ceased. The EP strain showed the formation of filamentous callus in the same region, corroborating the observation made for the same species by Polne-Fuller and Gibor (1987).

In general, the regeneration occurred directly from explant cells or in the compact callus region, as observed by Azanza-Corrales and Dawes (1989), who studied the wounding process in thallus fragments of *K. alvarezii* var. *tambalang*. Reddy et al. (2003) observed percentages of indirect regeneration lower than 10%, while Polne-Fuller and Gibor (1987) observed regeneration from calluses and explants in *Eucheuma* and *Kappaphycus* cultured in medium with agar as well as in liquid medium, although they did not quantify the number of regenerations.

According to Yokoya et al. (2004), the physical state of culture medium may affect the indirect regeneration process depending on the species. These authors observed that calluses of *Gracilaria tenuistipitata* (Chang & Xia) regenerated when cultured in solid medium, while the regeneration of *G. perplexa* (Byrne, Zuccarello, West, Liao & Kraft) occurred only after transferring from medium with agar to liquid medium, indicating a morphogenetical function of physical state of the medium. In *Solieria filiformis*, the solid medium induced filament growth, while adventitious plantlets were regenerated in liquid medium (Yokoya and Handro 2002). In the present work, the physical state of the medium did not induce the regeneration from calluses, and indirect regeneration was very rare. The exception was the BR strain, which showed indirect regeneration from only one callus. Despite Reddy et al.'s (2003) observation that mechanical agitation could stimulate the regeneration of adventitious plantlets, which gave rise to micropropagules after 40 days, this response was not observed in the present study. Only one callus among 15 (6.7%) of the BR strain regenerated when cultured under mechanical agitation (data not shown).

In relation to the effects of phytoregulators on direct regeneration, explants treated with IAA:BA (5:1 mg L^{-1}) regenerated up to 7 upright axes per explant when cultured with glycerol for 35 days, and up to 10 upright axes per explant when cultured without glycerol for 23 days. These results are not in agreement to the ones obtained by Yokoya (2000), where the direct regeneration of *Gracilariopsis tenuifrons* (Bird & Oliveira; Fredericq & Hommersand) was promoted by IAA:BA (1:5 mg L^{-1}). Probably there are some interactions of exogenous phytoregulators and en-

dogenous substances that promote similar effects in some species and different effects in other species.

Explants incubated with colchicine for 14 days showed more direct regeneration than the control. However, in our experiment, the chromosome numbers were not checked, so this response could not be related to a possible doubling of chromosomes. The chromosome number of *K. alvarezii* requires further investigation. Kapraun and Lopez-Bautista (1997) described the presence of 10 bivalents in prophase I (n=10) of *Kappaphycus alvarezii* from the Philippines, while Contador (2001) observed up to 6 bivalents in some cells and more than 10 in others from the same material studied in the present work. Further studies should be conducted in order to verify the effects of colchicine on the ploidy of *K. alvarezii*.

Based on results obtained in the present study, the EP strain is more tolerant and showed higher potential for micropropagation than the other strains tested in our experiments. Previous studies with the same strains of *K. alvarezii* demonstrated that EP strain had a lower growth rate than the tetrasporophytic strain when cultivated in the sea, but produced higher carrageenan content (Hayashi 2001; Hayashi et al. 2007).

In conclusion, the micropropagation of EP strain of *Kappaphycus alvarezii* was stimulated by IAA:BA (5:1 mg L^{-1}), and treatments with colchicine also stimulated the regeneration of upright axes. Both factors could be useful to enhance the micropropagation of *K. alvarezii*.

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