

Sustainable use of *Cryptocoryne wendtii* and *Echinodorus cordifolius* in the aquaculture industry of Sri Lanka by micropropagation

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Abstract

In the aquaculture industry of Sri Lanka, *Cryptocoryne* and *Echinodorus* species are important aquatic plants. In the absence of a regular supply due to lack of effective propagation methods, *Cryptocoryne* species are indiscriminately harvested from the wild to supply to the export market. The threat on the species is further compounded by the loss of their rainforest habitats. Out of the ten endemic *Cryptocoryne* species that occur in Sri Lanka, nine are classified as “Highly Threatened” species in the Red List of International Union for the Conservation of Nature. *Echinodorus cordifolius* is not found in the wild but is popular in aquaria. In order to overcome the problems of species loss and inadequate supply to the local and foreign markets, an *in vitro* micro-propagation method was developed for both these species. Due to the difficulty in obtaining axenic cultures from these species, rhizome segments were used to induce axillary bud growth and subsequent shoot multiplication. Shoot buds were induced from rhizome segments of *C. wendtii* cultured on a Murashige and Skoog (MS) basal medium supplemented with 11 – 133 μM benzyladenine (BA) and 13.4 μM naphthalene acetic acid (NAA). The induced shoots were separated after 21 days and sub-cultured twice every 14 days on MS medium, which increased the shoot multiplication. *E. cordifolius* responded positively to a combination of 24.6 μM N-isopentenyladenine (2iP) and 2.68 μM NAA, while 2iP alone did not induce shoots. Two sub-cultures at 14 day intervals increased the shoot multiplication. Rooting was induced in both species by culturing the shoots in $\frac{1}{2}$ MS liquid medium with indole butyric acid (IBA). Acclimatization was done in a humid growth chamber for one week and the plantlets were gradually transferred to the green house. All the plantlets rooted and survived in the green house.

Introduction

The aquatic plants *Cryptocoryne wendtii* and *Echinodorous cordifolius* belong to the Araceae family. Cryptocorynes grow in running water and each species is adapted to its habitat. More than fifty species are found to occur in the tropical lowland rainforests in South Asia. Ten species are endemic to Sri Lanka (Jacobsen 1987) and they are found in springs, streams and rivers of rainforests in the lowlands and midlands. The loss of natural rainforests to agriculture and human settlements as well as indiscriminate harvesting of cryptocorynes from their natural habitats have brought nine cryptocoryne species in Sri Lanka to a "Highly Threatened" status in the IUCN Red List (IUCN 2000). The cryptocoryne species in Sri Lanka show polyploidy and have chromosome numbers (2n) of 28, 36 and 42. Plants with 42 chromosomes are triploid derivatives of plants with 28 chromosomes. These plants are sterile and therefore reproduce only through vegetative means (Jacobsen 1987). Seven species have plants with diploid and triploid chromosome numbers, including *C. wendtii*. Such plants are additionally endangered due to their inability for seed production. Six of the species are popular in the ornamental trade with a high export potential and therefore under threat of depleting population size. *E. cordifolius* which is an introduced plant from Singapore is not found in the wild but it is popular in aquaria.

Alongside the ornamental fish industry in Sri Lanka, a high demand exists for fresh water aquatic plants, and US \$ 2.4 million worth of aquatic plants have been exported in 2001 (Anon 2001). In response to the high demand in European countries, five large aquatic plant exporting companies have emerged in Sri Lanka in recent years, exerting tremendous pressure on the natural aquatic ecosystems (Yapabandara and Ranasinghe 2000). Most of the exported plants are collected from the wild due to the lack of rapid methods for their multiplication. Such exploitation could eventually restrict the biodiversity and even endanger these species. Since they propagate vegetatively, cryptocorynes are very sensitive to habitat destruction (Bastmeijer et al. 1984; Jacobsen 1976; Jacobsen 1987). Jacobsen (1976) with his study of cryptocorynes of south east Asia and Sri Lanka has concluded that deforestation and forest conversion to agricultural lands could severely threaten their survival. This was further proved by the IUCN study of the Sri Lanka's fauna and flora (IUCN 2000). The major exporters in the industry purchase their requirement from local collectors in undisclosed areas (Mee 1993), resulting in a threat to survival for many aquatic plant genera including *Cryptocoryne*, *Aponogeton* and *Lagenandra* that are endemic to Sri Lanka. Legislation has now been introduced prohibiting the collection of any ornamental aquatic plant from the wild. This is a major restriction on the Sri Lankan ornamental plant and fish industry that depends on wild stocks of which no quantitative data on standing stock densities are available at present. Assistance to the exporters and growers in artificial propagation of aquatic plants can reduce the

pressure on the wild populations (Mee 1993). In order to meet the demands of the aquatic plant and fish industry while conserving the biodiversity of cryptocorynes in Sri Lanka, an alternative method for their propagation is urgently required. Mass propagation of these species through tissue culture technology may provide a solution to the issue of maintaining a steady supply of aquatic plants and fish for exportation and local use, without depleting natural stocks.

Most aquatic plants, including *C. wendtii*, do not produce seeds nevertheless they propagate vegetatively in the wild. The extent to which vegetative propagation takes place is inadequate to meet the commercial demand. *In vitro* propagation of *C. wendtii* was reported from shoot tips by Kane et al. (1999). The endemic cryptocoryne species, *C. wendtii* and *C. nevilli*, have been propagated *in vitro* from their rhizomes (Ranasinghe et al 2000, 2004). The endemic aquatic species *Lagenandra thwaitesii* has also been propagated *in vitro* by Ranasinghe et al (2001). No records are available on propagating *E. cordifolius* by tissue culture methods. Micropropagation by tissue culture techniques provides a means of large-scale production of uniform and high quality plants, free of pests and diseases that will also facilitate obtaining quarantine clearance for export and import purposes. Furthermore, it would lead to a reduction in collecting plants from the wild. This paper presents the results of an experimental *in vitro* attempt made to propagate *C. wendtii* collected from the wild and *E. cordifolius* obtained from a commercial supplier.

Materials and Methods

Plant material

C. wendtii was collected from a natural habitat of running water in Kurunegala, and *E. cordifolius* from a commercial grower in Padukka, Sri Lanka. Complete plants with leaves, rhizome and roots were potted in plastic basins (diam 35 cm x 13 cm) in a mixture of mud, topsoil and sand (2: 1: ½). They were maintained as the parent plant stock in the greenhouse at the IFS, Hantana where the daytime temperature was 26° - 30 °C and relative humidity was 70%. The experiments were carried out from 2001 to 2003.

Explant preparation and surface sterilization

For tissue culture, the leaves and roots were removed from selected plants of both species and the rhizomes were thoroughly washed in tap water. Cleaned rhizomes were kept in tap water with two drops of a commercial detergent (Teepol) for 30 min and then in running water for one hour. The rhizomes were cut into segments and immersed in 5% (w/v) Clorox (active ingredient – 5.25% sodium hypochlorite) for 15 min with vacuum infiltration. Under the laminar flow hood, the rhizome segments

were immersed in 95% ethanol for 1 min and finally in 0.1% (w/v) mercuric chloride for 30 sec and rinsed three times in sterile distilled water.

In vitro culture

Rhizome segments were cultured on Murashige and Skoog (1962) culture medium (MS) with 2% sucrose. The pH was adjusted to 5.8 using 1N NaOH or 1N HCl, agar was added at 0.7% (w/v) and the medium was autoclaved at 121 °C for 20 min at 1.06 kg cm⁻² pressure. Approximately 25 ml of medium was poured into 120 ml-baby food jars. *In vitro* culture of the rhizome segments was carried out in two stages. In stage I (induction of axillary shoots), the surface sterilized rhizome segments of both species were cultured in MS medium with different combinations of hormones to identify the appropriate concentration to induce maximum axillary shoot development. In stage II (multiplication of shoots), the induced shoots were separated and sub-cultured in two cycles in the best hormone combination determined at stage I. Segments of 0.5 cm in length, were cut from rhizomes of both species and they were cultured on the MS medium. The cytokinin, N6-benzyladenine (BA), was used to induce bud-break in the rhizome segments of *C. wendtii*. A range of hormone concentrations (11.0, 22.0, 44.0, 66.0 and 133.0 µM) of BA with (concentration of 13.4µM) and without naphthalene acetic acid (NAA) was added to the MS medium, and 21 replicates were used. Rhizome segments of *E. cordifolius* were cultured in media with NAA concentrations, 0, 2.6 and 26.8µM in combination with 0, 2.4 and 24.4 µM N-isopentynyladenine (2-iP) and 12 replicates were used in the experiment. The multiplied shoots were separated under aseptic conditions after 21 days. Subsequent sub-cultures were prepared every 14 days, with 8 replicates, and the number of new shoots was recorded. The separated shoots were cultured in hormone free MS medium for stem elongation. All the cultures were incubated at 25±2 °C under 16/8 h light/dark photoperiod. Light was provided by fluorescence bulbs (Thorn, 40 W tropical daylight). Control cultures in both species were cultured in hormone free MS basal medium.

Rooting and acclimatization

To induce rooting in both species, 12 shoots of *E. cordifolius* and 15 shoots of *C. wendtii*, were cultured in ½ strength MS liquid media with 0.5 µM and 1 µM indole butyric acid (IBA) for 1 day and transferred to IBA free liquid medium with 2% sucrose in test tubes. Rooting was scored after 21 days. The plants were acclimatized in a growth chamber, maintained at 24 °C and 80% relative humidity, by keeping the rooted plantlets in sterile tap water for 1 week and thereafter potting the plants in mud, topsoil and sand (2: 1: ½).

Data analysis

The culture tubes were completely randomized and the experiments were repeated. The non-parametric statistical procedure, Kruskal-Wallis One Way ANOVA on Ranks was used to analyse the rate of shoot multiplication at different combinations of hormones and sub-culture levels. Multiple comparisons between the treatment means were performed using the Dunn's method at $P < 0.05$ significance level (SigmaStat 2.0, 1995). The effect of IBA on rooting was determined by Fisher's Exact two tail test (SigmaStat 2.0, 1995).

Results

Obtaining contamination free rhizome explants was difficult in both species. Surface sterilizing procedure was adopted and 65% and 60% of contaminant-free rhizome explants of *C. wendtii* and *E. cordifolius* were obtained respectively. In the absence of mercuric chloride this declined to 35% and 30% respectively. Sterility of the rhizome segments cultured was verified by the presence or absence of fungal and bacterial contaminants, in the culture medium. Although different surface sterilizing agents were used (data not given), best results were obtained by immersing the rhizome segments in 5% Clorox for 15 min with vacuum infiltration, followed by 95% ethanol for 1 min and finally in 0.1% mercuric chloride for 30 seconds.

Shoot induction in *C. wendtii*

In the absence of hormones in the control cultures, the mean number of shoots produced by the rhizome segments was 0.8 (Figure 1). The rhizome segments that were cultured in media containing BA in concentrations of 44 μM and 66 μM in the presence of NAA in the basal medium, induced a maximum of 4 shoots per cultured rhizome segment, while in the absence of BA, most rhizome segments produced 1 or 2 shoots with a few producing 3 shoots (Table 1). The Kruskal-Wallis one-way ANOVA on ranks showed that the differences between treatments were significant ($P = 0.001$). While BA alone in the basal medium induced the maximum number of shoots at 22 μM and the addition of NAA produced an interaction with BA at 44 μM concentration produced the most number of multiple shoots (Figure 1). However, this was not significantly different from the treatment of 22 μM BA alone.

Shoot induction in *E. cordifolius*

Out of the various combinations of auxins and cytokinins tested, shoots were induced only with the combination of NAA and 2iP. In the absence of both hormones the explants produced on average a single shoot and in the absence of 2iP, NAA induced a maximum of 1.8 shoots per explant (Figure 2).

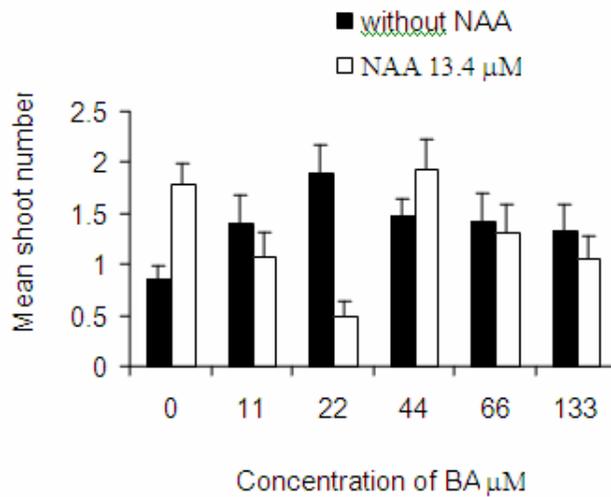


Figure 1. Mean number of shoots induced on rhizome explants of *Cryptocoryne wendtii* by BA alone and in combination with 13.4 μM NAA (n=21). Bars represent standard errors.

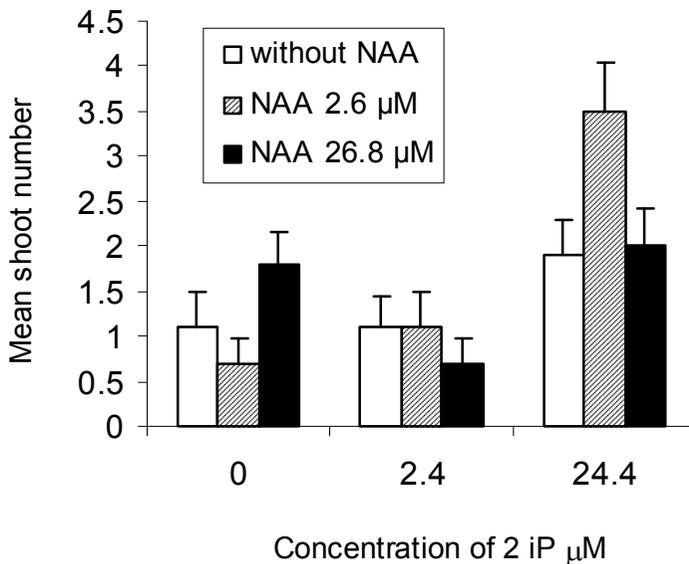


Figure 2. Mean number of shoots induced on rhizome explants of *Echinodorus cordifolius* by combinations of NAA and 2-iP (n=12). Bars represent standard errors.

Table 1. Number of shoots per rhizome segment and mean number of shoots (\pm standard error) induced on *Cryptocoryne wendtii* rhizome explants cultured on MS medium with 13.4 μ M NAA and different concentrations of BA. Control medium did not contain both the hormones.

BA (μ M)	Replicates	Number of shoots per rhizome segment					Mean no. of shoots/explants
		None	1	2	3	4	
0	19	1	6	8	4	-	1.7 \pm 0.19
11	15	5	6	2	2	-	1.0 \pm 0.25
22	14	7	7	-	-	-	0.5 \pm 0.13
44	18	2	6	4	3	3	1.94 \pm 0.29
66	13	2	7	3	-	1	1.3 \pm 0.27
133	17	5	7	4	1	-	1.0 \pm 0.21
Control	14	3	10	1	-	-	0.85 \pm 0.13

The presence of 2iP at 2.4 μM concentration did not have a significant difference in shoot production. Increasing 2iP concentration to 24.4 μM in combination with NAA (2.6 μM), increased the mean shoots produced per explant to 3.5. This interaction was significantly different from the rest ($P < 0.05$). Increasing NAA concentration to 26.8 μM with the same concentration of 2iP, reduced the shoot production (Figure 2).

Sub-culture cycles

After 21 days of culture, the proliferated shoots of *C. wendtii* were separated from the rhizome segment. The shoots were sub-cultured in separate media containing 22 and 44 μM of BA, with and without NAA at 13.4 μM concentration (Table 2). In *C. wendtii* shoot multiplication increased significantly through two sub-cultures to produce 6-7 shoots per rhizome segment cultured. The shoots of *E. cordifolius* were separated after 28 days and sub-cultured in medium containing NAA (2.6 μM) and 2iP (24.4 μM). After two sub-cultures, rhizome segments of *E. cordifolius* produced nearly 8 shoots (Table 2). The shoots of both species elongated after the multiple shoots were separated and transferred to hormone free MS medium for seven days. Elongation was very low when the separated shoots remained in the MS medium with hormones.

Table 2. The effect of sub-cultures, at three week intervals, under selected hormone combinations, *in vitro* shoot production by rhizome explants of *Cryptocoryne wendtii* and *Echinodorus cordifolius*. Means in a column with a different superscript are significantly different from each other at 5%.

Culture	Mean number of shoots/explant		
	<i>C. wendtii</i>		<i>E. cordifolius</i>
	BA 22 μM , NAA 0 μM	BA 44 μM , NAA 13.4 μM	2iP 24.4 μM NAA 2.6 μM
Initial culture	2.6 ^a	2.6 ^a	4 ^a
1 st subculture	4 ^b	5 ^b	4.9 ^b
2 nd subculture	7.1 ^c	6 ^c	7.9 ^c

Rooting and hardening

Shoots of *C. wendtii* and *E. cordifolius* were cultured in separate MS media with IBA (0.50 μM and 1.0 μM) and without IBA (control) to induce roots. Only few of the shoots of both species produced roots in the control cultures, whereas the application of IBA (0.5 μM) induced 86% and 73% rooting in *C. wendtii* and *E. cordifolius*, respectively (Table 3). Increasing IBA to 1.0 μM reduced rooting and transfer to IBA free $\frac{1}{2}$ MS medium increased the rate of growth of the plantlet. Rooted plantlets were successfully acclimatized by transfer from $\frac{1}{2}$ MS to sterile tap water for one week and finally to an aquatic tank. *C. wendtii* was acclimatized and

showed 100% survival while *E. cordifolius* showed 90% survival after acclimation.

Table 3. Number of shoots produced by rhizomes of *Cryptocoryne wendtii* and *Echinodorus cordifolius*, following culture in MS medium containing 0 (control), 0.50 μ M and 1.0 μ M of indole butyric acid (IBA) for 1 day and transferred to IBA free MS medium

IBA treatment μ M	Species	
	<i>C. wendtii</i> (n=15)*	<i>E. cordifolius</i> (n=12)*
0	3 ^a	2 ^a
0.50	13 ^b	11 ^b
1.0	9 ^c	6 ^c

* Shoots per treatment

Means in a column with a different superscript are significantly different from each other.

Discussion

Most aquatic plants grow submerged in water and their wet surfaces retain high levels of microbes. Under warm tropical conditions a higher number of microorganisms are present in the aquatic environments than under temperate conditions. Rhizome explants of both species used in this study have an uneven and a hairy surface preventing contact between the explant surface and the sterilant. Thus, microorganisms easily enter the culture medium which is an inherent problem with rhizome explants of tropical aquatic plants. This is a serious obstacle that discourages the use of hydrophytes for micro-propagation (MohanRam and Agrawal, 1999). Use of mercuric chloride successfully sterilized 60 - 65% of the explants of both species in this study whereas Kane et al (1999) reported successful surface sterilization of *C. wendtii* using ethanol and NaOCl. In the absence of mercuric chloride, surface sterilization was reduced to 30 – 35%. Using nodal explants of *Nymphoides indica*, Jenks et al. (2000) also reported difficulties in establishing axenic cultures, with a success rate of 3%. MohanRam and Agrawal (1999) have reviewed the inherent difficulties in obtaining aseptic cultures from aquatic plants, which dissuades investigators from using hydrophytes as experimental material for in vitro propagation.

Rhizome segments with dormant axillary buds at the nodes of *C. wendtii* and *E. cordifolius* were used as explants. The general hypothesis that the axillary buds could be induced to form shoots in the absence of the apical meristem and hence apical dominance was amply supported by the results of the present study. In both species, the number of shoots induced from rhizome explants increased after two sub-cultures. Kane et.al. (1999) used rhizome shoot tips from *C. wendtii* to establish *in vitro* cultures for

multiplication. The shoot tip explants were sub-cultured for uniform shoot multiplication from which shoots proliferated seven-fold with 20 μ M of BA after 4 weeks of culture. Ranasinghe et al. (2000) using rhizomes of *C. wendtii* obtained the highest shoot multiplication with 5 mg L⁻¹ BA. Based on this, rhizome segments of *C. wendtii* in this study were treated with 0 to 133 μ M of BA and with and without NAA to identify the optimum hormonal combination to induce bud-break. In the absence of previous records over *in vitro* propagation of *E. cordifolius*, different combinations of cytokinins (BA, 2 iP, kinetin, TDZ), and auxins (2,4 D, NAA) were tested to identify a suitable combination of hormones to induce bud-break. However, only the combination of 2 iP and NAA, induced an increase in shoot development after two sub-cultures of the rhizome segments. Similarly in the water-lily *Nymphaea* species, 2 iP, BA and NAA induced shoot proliferation from rhizome tips (Laksmanan 1994) and a combination of 2 iP and IAA produced aerial leaves with epiphyllous plantlets (Jenks et al. 1990). Multiple shoots also were regenerated in the wetland plant *Juncus effuses* by the cytokinins BA and 2 iP (Sarma and Rogers 2000). From the hormone combinations tested in the present study *E. cordifolius* requires the cytokinin 2iP in a specific combination with NAA to induce multiple shoots. This indicates that there are species-specific hormonal requirements for an *in vitro* propagation of aquatic plants.

Bud break is commonly induced using a cytokinin. In three species of the water reed *Phragmites*, BA was the most effective cytokinin for inducing adventitious shoot regeneration (Poonawala et al 1999) for most species and also for *Phragmites communis* too (Guo et al., 2004). In this study BA and NAA significantly induced multiple shoot production in rhizome explants of *C. wendtii* than in their absence, and certain combinations of BA and NAA decreased the number of shoots. However, a ratio of BA to NAA of 4:1 was similar to BA alone at its concentration of 22 μ M in the basal medium. Ranasinghe et al (2000) too have found no significant difference to occur in shoot induction in the presence of NAA and IAA. Kane et al (1990) showed similar results with *C. lucens* using an MS basal medium with 0.45 mg l⁻¹ of BA and 0.1 mg l⁻¹ of NAA. The effective hormone concentration is also species dependent. Some species require a low cytokinin concentration for shoot regeneration as in *Baccopa monnieri* (Shrivastava and Rajani, 1999) while *P. communis* required a very high level (53.4 μ M) of BA (Guo et al. 2004). For *E. cordifolius*, the combination of cytokinin BA, kinetin and 2 iP did not induce shoot buds. A specific combination of 2 iP with NAA induced *in vitro* shoot buds and shoot multiplication.

Since rhizomes can be segmented into many sections, they were used to induce shoots. The difference in hormone requirements for *C. wendtii* (Kane et al. 1999) indicates species that have been conditioned in different environments have altered sensitivity to hormones for shoot induction and proliferation. While Kane et al (1999) were able to produce

rooted plantlets of *C. wendtii* without hormones, in the present study it required low concentration of IBA for the purpose. Ranasinghe et al. (2000) obtained 100% rooting in hormone free medium, while addition of 0.2% activated charcoal improved rooting. Thus specific hormonal treatments need to be determined under different environments for *in vitro* propagation of aquatic plants. The need to develop propagation protocols for plants that occur in specific local conditions has become a challenge due to the requirement of surface sterilization using mercuric chloride under tropical conditions.

Nine out of the ten endemic cryptocoryne species in Sri Lanka have been classified under “highly threatened” while *C. wendtii* under “threatened” Although not listed as threatened conservation status in the IUCN Red List (IUCN 2000). Six of these species are popular ornamental plants in the export trade. They are found as solitary colonies confined to upper parts of rivers and streams and seldom downstream, indicating that they are hardly dispersed by seeds (Yapabandara and Ranasinghe 2000). The triploid species of *C. wendtii* reproduce only vegetatively (Jacobsen 1987) and hence it is susceptible to extermination. In the absence of a viable alternative to supply plants in large scale to meet the demands of the exportation, unscrupulous exploitation of the wild populations and destruction of natural habitats of these species may contribute to their extinction.

Present study shows that *C. wendtii* can be propagated *in vitro* on MS basal medium supplemented with BA and NAA followed by sub-culturing to multiply the shoots. *E. cordifolius* requires a combination of 2iP and NAA to induce shoots followed by sub-culturing for shoot multiplication. Micro-propagation on a large scale is desirable to support the export industry since quarantine standards are easily met and production can be planned according to the demand, independent of seasons and months (Yapabandara and Ranasinghe, 2000). The most significant benefit however, would be the contribution it makes towards conservation of the species. The protocol developed for *C. wendtii* could be extended to *in vitro* propagation of the other nine “Highly Threatened” species in Sri Lanka. This would indirectly contribute to their conservation in the wild, by ensuring a continuous supply of high quality plants for the aquatic plant export industry.

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