Indian Journal of Experimental Biology
Vol. 57, May 2019, pp. 330-337

Improved *in vitro* propagation and direct acclimatization of *Cryptocoryne wendtii* in aquarium in the presence of aquarium fish *Puntius tetrazona* (Bleeker)

Sundus Unal[1], Gurel Turkmen[2], Bulent Yagmur[3], Meltem Bayraktar[4]* & Aynur Gurel[5]

[1]Biotechnology Department, Graduate School of Nature and Applied Sciences, Ege University, 35100 Bornova-Izmir, Turkey

[2]Faculty of Fisheries, Ege University, 35100 Bornova-Izmir, Turkey

[3]Soil Science Department, Faculty of Agriculture, Ege University, 35100 Bornova-Izmir, Turkey

[4]Genetic and Bioengineering Department, Faculty of Engineering and Architecture, Kırşehir Ahi Evran University, 40100 Kirsehir, Turkey

[5]Bioengineering Department, Faculty of Engineering, Ege University, 35100 Bornova-Izmir, Turkey

Received 10 March 2017; *revised 13 February 2018*

Cryptocoryne wendtii is an important ornamental aquatic plant and has difficulties in conventional cultivation. In the present study, we propose an efficient *in vitro* propagation and acclimatization protocols for *C. wendtii*. The highest number of shoots was achieved on Murashige and Skoog (MS) medium containing 4.0 mg/L BA+1.0 mg/L IBA. In the second subculture, the number of shoots per explant increased from 7.2 to 51.8 in this medium. The shoot length per explant, the percentage of shoots rooted, and the number of roots per shoot were maximum in MS medium supplemented with 1.0 mg/L IBA. The *in vitro* rootless and rooted shoots were acclimatized in aquariums containing different substrates [Calcite (Ca), River Sand (RS), Zeolite (Ze) and Shell Grit (SG)] and tetrazona fish *Puntius tetrazona* (Bleeker). One third of the water in the aquarium was replaced with fresh tap water every two weeks and the water analyses were carried out before replacement of aquarium water every time. All shoots were successfully acclimatized for a period of 3 months. The RS and rooted shoot explants were found to be more effective than the other substrates, and rootless shoots in terms of all parameters that were tested.

Keywords: Calcite, Ornamental aquatic plants, River sand, Shell grit, Wendt's cryptocoryne, Zeolite

Aquatic plants are of great importance for aquatic organisms (fish, aquatic insects, etc.) by providing habitat and food. Apart from this, they are used in many areas for many purposes like medicine, phyto-remediation, food and biofuels. They are also used as ornamental purposes because of their beauty[1-3]. *Cryptocoryne* genus belongs to the family Araceae and has 50 species. Most of the *Cryptocoryne* species, as the highly popular ornamental aquarium plants, are native to Southeast Asia and Indonesia, grow in either submerged or emerged state in slow running rivers and streams, and seasonally inundated forest pools[4-9]. They provide nutrients for aquatic organisms and the occurrence of *Cryptocoryne* species in water is to some extent also an indication of a certain level of cleanness of the water[10]. *Cryptocoryne wendtii* is an herbaceous perennial, rhizomatous aquarium plant with a wide range of foliage colour, green or brown. Due to the specialty of its foliage, it has high market demand[7].

Since *Cryptocoryne* species do not produce sufficient seeds, and vegetative propagation by rhizome is slow, their production is limited to a small number of plants[4,6]. The plants produced in these ways are not adequate for meeting commercial demand[11]. Therefore, *in vitro* propagation is of great importance for the commercial production of the *Cryptocoryne* species[7]. Plant species, which have commercial importance and are difficult and slow in propagation using conventional methods, such as grafting and cutting, can be rapidly and uniformly mass proliferated by micropropagation throughout the year independent of seasonal changes in a small place.

*Correspondence:
Phone: +90 3862803821
E-mail: meltembayraktar5@gmail.com,
meltem.bayraktar@ahievran.edu.tr

Although the *in vitro* propagation and acclimatization of *C. wendtii* has been reported previously[6,7,11,12], there is a need to improve *in vitro* propagation and acclimatization protocols. Stanly *et al.*[7] transferred eight-week-old rooted *in vitro* plantlets of *C. wendtii* into plastic pots containing a mixture of organic soil and sand under greenhouse conditions. Kauth *et al.*[12] used soil substrate and hydroponic tank systems for the acclimatization of *C. wendtii*. They observed significant reductions in both leaf and root development because of water stress in soil condition. Dissanayake *et al.*[11] acclimatized the plantlets first to sterile top water for one week and finally into an aquatic tank. The acclimatization process where the *in vitro* plantlets were first transferred to the soil and then into water is time consuming and needs additional steps to be followed and additional costs. This process also can cause plant losses. Besides, aquatic plants are generally displayed together with the fish in aquariums. Therefore, the successful acclimatization of *in vitro* regenerated aquatic plantlets directly in aquarium conditions is important. In the present study, we tried to improve up on the *in vitro* propagation and acclimatization of *C. wendtii*. To create natural aquarium conditions, tetrazona aquarium fishes [*Puntius tetrazona* (Bleeker)] were used, and the effects of different substrates on plant growth in aquarium conditions were examined in the acclimatization studies.

Material and Methods

Plant material and micropropagation studies

The *in vitro* propagated *C. wendtii* at the Ege University Bioengineering Department were used as plant material. For the establishment of the micropropagation studies, the numbers of donor plants were first increased by subculturings on Murashige and Skoog medium (MS, 1962)[13] supplemented with 0.5 mg/L BA. Shoot tips (0.5-1.0 cm long) with their roots and leaves removed were used as the explant. To increase the number of shoots and shoot length per explant and obtain rooted plantlets, micropropagation studies were conducted. The shoot tip explants were cultured into 210-mL glass culture jars containing 25 mL half-strength basal MS media supplemented with 0.5, 1.0, 2.0, 4.0 and 6.0 mg/L 6-benzyladenine (BA) or 0.5, 1.0 mg/L Indole-3-butyric acid (IBA), applied separately, or BA+IBA combinations at the concentrations of 2.0+1.0, 4.0+1.0 and 6.0+1.0 mg/L, respectively, plus 3% (w/v) sucrose and 0.7% (w/v) Plant agar (Duchefa®, Haarlem, Netherlands). The MS medium without plant growth regulators (PGRs) was used as a control (Table 1). The experiments were conducted in three replications, and 14 explants were used for each replication. The data was recorded 40 days after the beginning of culture. The induced shoots from all media were subcultured every 40 days in MS medium containing 4.0 mg/L BA+1.0 mg/L IBA (the best proliferation medium), 3% (w/v) sucrose and solidified with 0.7% (w/v) Plant agar.

Media and culture conditions

The pH of all the media was adjusted to 5.8 with 1 N HCl or 1 N NaOH prior to the addition of 0.7% (w/v) Plant agar. They were autoclaved at 121ºC at 1.04 kg cm^{-2} for 15 min. The *in vitro* cultures were incubated in a growth room at 26±1ºC under a 12/12 h

Table 1 — Effect of BA, IBA and BA + IBA combinations on multiple shoot regeneration, shoot growth, and rooting in shoot tips culture of *Cryptocoryne wendtii* after 40 days

Plant growth regulators (mg/L)		% explants producing multiple shoots	Number of shoots/explant	Shoot length (cm)/explant	Percentage of shoots rooted	Number of roots/shoot
BA	IBA					
-	-	28.57±4.12^{c}	1.33±0.09^{d}	2.20±0.14^{c}	92.86±4.13^{a}	5.36±0.41^{b}
0.5	-	76.19±10.38^{ab}	3.00±0.27^{c}	2.24±0.09^{c}	88.10±2.38^{a}	3.95±0.34^{c}
1.0	-	92.86±7.14^{a}	3.02±0.24^{c}	2.74±0.11^{bc}	42.86±14.87^{b}	1.26±0.29^{d}
2.0	-	100.00±0.00^{a}	5.43±0.31^{b}	2.72±0.07^{bc}	11.90±2.38^{c}	0.26±0.13^{d}
4.0	-	100.00±0.00^{a}	5.83±0.42^{ab}	3.05±0.08^{b}	0.00±0.00^{d}	0.00±0.00^{d}
6.0	-	100.00±0.00^{a}	6.02±0.42^{ab}	3.21±0.11^{b}	0.00±0.00^{d}	0.00±0.00^{d}
-	0.5	57.14±8.25^{b}	1.93±0.18^{cd}	4.14±0.19^{a}	92.86±4.13^{a}	5.52±0.45^{b}
-	1.0	61.91±2.38^{b}	2.21±0.20^{cd}	4.44±0.20^{a}	95.30±2.38^{a}	6.88±0.56^{a}
2.0	1.0	97.62±2.38^{a}	6.14±0.45^{ab}	2.74±0.09^{bc}	0.00±0.00^{d}	0.00±0.00^{d}
4.0	1.0	100.00±0.00^{a}	7.21±0.50^{a}	3.00±0.12^{b}	0.00±0.00^{d}	0.00±0.00^{d}
6.0	1.0	100.00±0.00^{a}	7.07±0.46^{a}	3.13±0.12^{b}	0.00±0.00^{d}	0.00±0.00^{d}

[Mean values ± SE followed by the same letters in each column are not significantly different (P=0.05) according to the Tukey HSD test.]

(light/dark) photoperiod with light supplied by cool white fluorescent tubes at 4000 lx intensity.

Acclimatization

Calcite (Ca), river sand (RS), zeolite (Ze) and shell grit (SG) were used as substrates for acclimatization studies. The substrates were soaked in 10 L of tap water containing 100 mL sodium hypochlorite (Merck, 6-14% active chlorine) for 5 min and then rinsed with tap water to remove the residues of the sodium hypochlorite from the surface of the substrates.

The roots of the plantlets were cut and shortened to a size of 1.0 cm. The multiple shoots were separated into single shoots without roots. Both the rooted and rootless *in vitro* shoots were transferred into small pots containing Ca, RS, Ze or SG, individually, and then were placed into 50 L aquariums (50 cm length × 35 cm width × 35 cm height). Before placing the rooted and rootless *in vitro* shoots to the substrates, the shoot weights (g), shoot lengths (cm), leaf lengths (cm), leaf widths (cm), root lengths (cm), and leaf numbers were recorded. After 3 months of acclimatization of the shoots into the aquariums, the above mentioned parameters were recorded again, and the differences between the two time periods in terms of parameters were determined. The substrates were placed separately into four aquariums. A total of 60 *in vitro* shoots (30 rooted and 30 rootless) were used for each aquarium. The experiments were conducted in three replications and 10 explants were used for each replication. A natural environment was provided using tetrazona (*P. tetrazona*) aquarium fish. Thirty tetrazona fishes were placed into each aquarium and fed with daily fish feeds as *ad libitum*. The initial water sample used for acclimatization belongs to the C_2S_1 irrigation water group.

Aquarium conditions

All of the aquariums were kept at 26±1ºC under day light (6500 K) provided by fluorescent tubes (Sylvania). Ventilation (200 L flow/h) and waterfall type filters were used. The photoperiod was 12-/12 h (light/dark).

One third of the water in the aquariums was replaced with fresh tap water every two weeks. The water analyses were carried out before each replacement of aquarium water. The pH was determined with a glass electrode pH-meter[14]; and the electrical conductivity with an EC-meter[15]. The Na^+ and K^+ cations were analyzed with a flame photometer by modifying the absorbance values via a curve of the standard solution absorbance[16]; the calcium + magnesium (Ca^{2+} + Mg^{2+}) was determined with a erichrom T black indicator and titration with 0.01 N EDTA[15]; the anions of Cl^- was determined accompanied by a potassium bichromate ($K_2Cr_2O_7$) indicator via a titrating with 0.05 N $AgNO_3$[15]; carbonate (CO_3^{2-}) and the hydro carbonate phenolphtalein, (HCO_3^-) analyses were identified as titrimetric with 0.1 N HCl accompanied by phenolphtalein and methyl orange indicators respectively[14]; total phosphorus (P) was determined according to Murphy-Riley method[17]; Nitrate-N (NO_3-N) was determined with 1-2-4 xylenol method[18]; Nitrite-N (NO_2-N) was determined according to Griess Hosway method[19]; Ammonium-N (NH_4-N) was identified as colorimetric in the presence of phenolate solution[20]. Total inorganic nitrogen was determined by calculating the sum of NO_2-N, NO_3-N and NH_4-N.

Statistical analysis

The data of the micropropagation studies were recorded after 40 days of culture. The micro-propagation experiments were set up in a completely randomized design. Fourteen cultures were raised for each treatment, and all the experiments were repeated three times (a total of 42 cultures per treatment). All data were analyzed using standard ANOVA procedures. The significant differences among the mean values were compared by the Tukey's HSD test at P = 0.05 using SPSS Version 16.0 (SPSS Inc., Chicago, USA) (Table 1).

The data of the acclimatization studies were recorded 90 days after acclimatization. The acclimatization experiments were set up in a completely randomized factorial block design. All data were analyzed by the GLM (General Linear Model) univariate analyses procedure. The significant differences among the mean values were determined by the Tukey's HSD test (P = 0.05) using SPSS statistical package (version 16.0).

Results and Discussion

Micropropagation studies

In order to determine an efficient micropropagation medium, the shoot tip explants were cultured on 11 different media (Table 1). The induction of the multiple shoots in the shoot tips varied with the concentration and type of PGRs used. The percentage of the explants that formed multiple shoots reached 100% on the MS containing 2.0, 4.0 and 6.0 mg/L BA,

applied separately, and the BA+IBA combinations at the concentrations of 4.0+1.0 and 6.0+1.0 mg/L, respectively (Table 1). The BA and BA+IBA combinations at different concentrations induced more shoots per explant compared to IBA (Table 1). Between the BA and BA+IBA combinations, the latter were more effective than the BA for multiple shoot regeneration in *C. wendtii*.

The highest number of shoots (7.21 shoots/explant) was obtained on the MS supplemented with 4.0 mg/L BA+/1.0 mg/L IBA followed by 6.0 mg/L BA+1.0 mg/L IBA (7.07 shoots/explant). They were placed in statistically same group (Table 1). As in our study, the positive effect of various concentrations of cytokinin+auxin combinations on the induction of multiple shoots has been reported for many aquatic plants, for example; *C. lucens*[4], *Ludwigia repens*[21], *Echinodorus cordifolius*[11], *C. bogneri*[2], *Lysimachia christinae*, *L. rubinervis*, and *L. nummularia* 'Aurea'[22], *C. wendtii*, *C. becketti*[7], *Bacopa monnıeri* L. PENNELL[23] and *Ceratophyllum demersum*[24]. Dissanayake *et al*.[11] obtained 7.1 shoots per rhizome segment of *in vitro* grown *C. wendtii* shoots on the MS medium containing 22 µM BA from the second subculture. This result is in agreement with the results of the initial culture from our study. Kane *et al*.[6] observed the maximum shoot proliferation (sevenfold increase) on the agar-gelled medium containing 20 µM BA in *C. wendtii*. This result concurs with the results of the second subculture of our study. In the present study, when the *in vitro* shoots obtained from the primer culture (from all media used) were subcultured on the MS supplemented with 4.0 mg/L BA+1.0 mg/L IBA (the best proliferation medium), the number of shoots per explant increased from 7.2 to 51.8 (approximately sevenfold) in the second subculture (data not shown). Among the studies conducted regarding the micropropagation of *C. wendtii*, it is, to date, the best result obtained. With an increasing concentration of BA in the media, the number of shoots per explant, as well as the shoot length (cm) per explant, increased. This is in accordance with the results found by Stanly *et al*.[7], who reported that the number of shoots per shoot explant of the *C. wendtii* rose as the concentration of the BA added into the MS medium increased. Stanly *et al*.[7] obtained 4.5 shoots per explant on the agar-gelled MS medium supplemented with 0.5 mg/L BA+0.2 mg/L IBA, and increased the shoot number to 9.4 with a liquid medium which had the same content. These results indicated that the MS supplemented with 0.5 or 1.0 mg/L IBA showed no significant differences in terms of the above mentioned parameters (Table 1).

The shoot length per explant was the maximum (4.44 cm/explant) in the MS medium containing 1.0 mg/L IBA followed by 0.5 mg/L IBA (4.14 cm/explant). They were placed in statistically same group (Table 1).

The PGRs-free basal MS medium (control medium) (92.86%) and 0.5 mg/L BA (88.10%), 0.5 mg/L IBA (92.86%) or 1.0 mg/L IBA (95.30%) containing MS media showed the highest rooting percentage and were placed in the same statistical group (Table 1). The highest number of roots per shoot (6.88 roots/shoot) was recorded for MS medium containing 1.0 mg/L IBA followed by 0.5 mg/L IBA supplemented medium (5.52 roots/shoot) and the control medium (5.36 roots/shoot). The control medium and 0.5 mg/L IBA supplemented medium showed no significant differences, and were placed in the same statistical group (Table 1).

Acclimatization

Effects of substrate on plant growth

For acclimatization of the *C. wendtii* plantlets, different methods like transferring of shoots with or without roots to greenhouse[6,7], aquatic[11] or hydroponic tanks[12] have been used in previous studies. Differently from these studies, in the present study, the acclimatization of *C. wendtii* plantlets into the aquarium conditions is detailed with different applications, such as using different substrates and tetrazona aquarium fish (*P. tetrazona*), and this acclimatization procedure is pioneering for such studies. All the *in vitro* shoots were 100% acclimatized for a period of three months (Fig. 1). Our result regarding survival rate is consistent with Kane *et al*.[6] and Dissanayake *et al*.[11] as well as higher than the results obtained by Stanly *et al*.[7] and Kauth *et al*.[12].

When the results were evaluated regarding the substrate types and explant types, the RS and rooted shoots were found to be more effective than the other substrates and rootless shoots in terms of all the parameters observed. Between the beginning and end of the acclimatization, the maximum difference in the shoot length (5.1 cm), the leaf length (3.6 cm), the leaf number (4.5), and the fresh weight (0.74 g) were The difference in the leaf width (cm) and the root

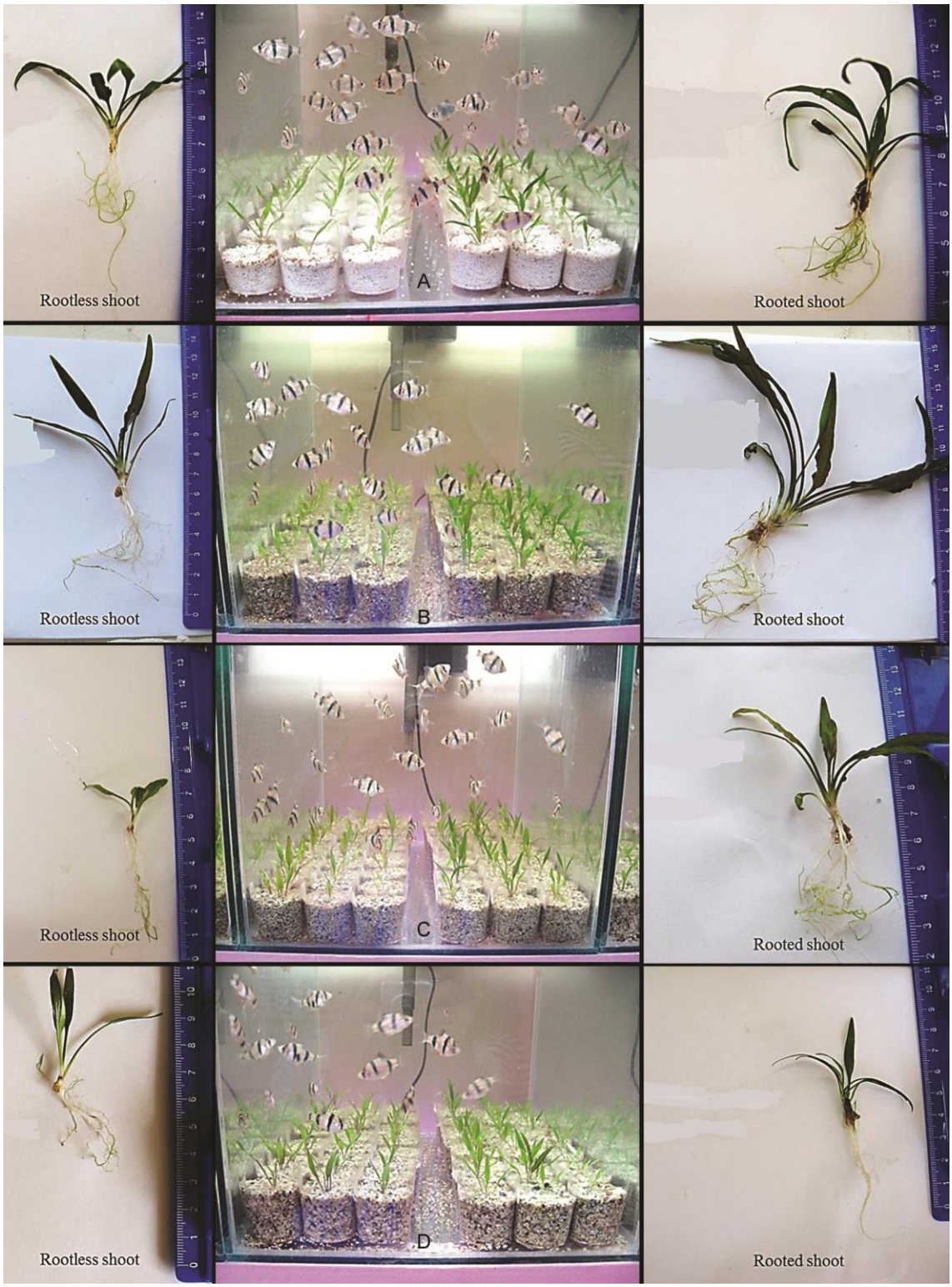

Fig. 1 -—The growth and development of *in vitro* propagated rootless and rooted shoots in aquarium containing tetrazona fishes and different substrates: (A) Ca; (B) RS; (C) Ze; and (D) SG substrates

observed in the rooted shoots acclimatized to the RS. length (cm) were not statistically affected by either the substrate types or the explant types (Table 2). The reason why the RS was more effective could be related to the neutral pH of the RS, the water circulation in the aquarium and the water permeability among the particles. In addition, because of the granule structure of RS, the plants were able to hold firmly on the RS substrate and thus, feed better. Besides this, to be rich in silicon (Si) and oxygen brings the RS into the forefront a little more. It has been reported that Si is absolutely necessary for unicellular organisms such as marine algae[25] and has beneficial effects on the plant growth and yield[26]. Having more available pH (neutral) than the other substrates, the existence of available nutrients (without any fix) resulted in the support of plant growth.

By using tetrazona fish in the acclimatization studies, we tried to create natural aquarium conditions. The tetrazona fish in the aquariums didn't damage the acclimatized plantlets since they were fed with daily fish feeds as *ad libitum*. Therefore, tetrazona fish can be used in acclimatization studies of aquatic plants provided they are fed *ad libitum*.

Effects of substrates on water quality

The pH, electrical conductivity, total cations (Na^+, K^+, Ca^{++} + Mg^{++}), total anions (Cl^-, HCO_3^-), sodium adsorption ratio (SAR), irrigation water class, total N (NO_3-N, NO_2-N, NH_4-N), phosphor and N/P values were determined separately, and the results are shown in Table 3. The values obtained from the water analyses differed according to the substrates and

Table 2 — Effect of different substrates on shoot growth after 90 days following the transfer of *Cryptocoryne wendtii* plantlets to aquariums containing tetrazona fish

Substrate types	The differences between the beginning and the end of the acclimatization											
	Differences in leaf length (cm)		Differences in leaf width (cm)		Differences in leaf number		Differences in shoot length (cm)		Differences in root length (cm)		Differences in fresh weight (g)	
	Explant type		Explant type		Explant type		Explant type		Explant type		Explant type	
	Rootless shoot	Rooted shoot	Rootless shoot	Rooted shoot	Rootless shoot	Rooted shoot	Rootless shoot	Rooted shoot	Rootless shoot	Rooted shoot	Rootless shoot	Rooted shoot
Ca	0.7±0.1^{c}	2.4±0.2^{b}	0.2±0.0	0.2±0.0	2.1±0.2^{c}	3.3±0.3^{abc}	0.9±0.1^{c}	2.8±0.2^{b}	11.6±0.7	10.9±0.4	0.12±0.0^{c}	0.16±0.0^{c}
RS	1.7±0.1^{b}	3.6±0.1^{a}	0.3±0.0	0.4±0.0	3.4±0.2^{abc}	4.5±0.2^{a}	2.9±0.2^{b}	5.1±0.2^{a}	10.8±0.5	12.4±0.3	0.29±0.0^{bc}	0.74±0.1^{a}
Ze	0.6±0.1^{c}	2.1±0.2^{b}	0.2±0.0	0.3±0.0	0.9±0.2^{d}	3.6±0.3^{abc}	0.6±0.1^{c}	2.6±0.2^{b}	11.5±0.6	11.7±0.4	0.18±0.0^{c}	0.46±0.0^{b}
SG	1.1±0.1^{c}	2.0±0.1^{b}	0.2±0.0	0.2±0.0	2.6±0.2^{bc}	3.8±0.2^{ab}	1.2±0.1^{c}	2.4±0.2^{b}	10.7±0.7	12.7±0.7	0.17±0.0^{c}	0.31±0.0^{bc}

Mean values ± SE followed by the same letters in each column are not significantly different (p=0.05) according to the Tukey HSD test.

Table 3 — The results of the water samples analyses after 14 weeks in acclimatization

Analyses	Symbol	Unit	Initiation water	Ca	RS	Ze	SG
pH		(25 ºC)	7.54	6.70	7.05	7.53	8.12
Electrical conductivity		(ECx10^6, µmhos/cm)	615	741	746	750	721
Cations							
Sodium	(Na^+)	(me/L)	1.20	1.60	1.65	1.50	1.45
Potassium	(K^+)	(me/L)	0.05	0.10	0.10	0.10	0.10
Calcium + Magnesium	(Ca^{++} + Mg^{++})	(me/L)	4.90	5.80	5.65	5.95	5.65
Total cations		(me/L)	6.15	7.50	7.40	7.55	7.20
Anions							
Chlorine	(Cl^-)	(me/L)	1.10	1.60	2.35	2.05	2.00
Hydrocarbonate	(HCO_3^-)	(me/L)	5.00	5.85	5.10	5.40	5.25
Total anions		(me/L)	6.10	7.45	7.45	7.45	7.25
Sodium adsorption ratio	SAR		0.76	0.94	0.98	0.87	0.86
Irrigation water class			C_2S_1	C_3S_1	C_2S_1	C_2S_1	C_2S_1
Nitrate-N (NO_3-N)		(mg/L)	0.073	0.112	0.075	0.103	0.108
Nitrite-N (NO_2-N)		(mg/L)	0.01	0.02	0.01	0.01	0.02
Ammonium-N (NH_4-N)		(mg/L)	0.001	Trace	Trace	Trace	Trace
Total N		(mg/L)	0.084	0.132	0.085	0.113	0.128
Phosphor		(mg/L)	0.008	0.013	0.008	0.011	0.009
N/P			15.60	10.15	10.63	10.27	14.22

almost all parameters examined showed increase. These differences were reported to be due to structures of the substrates.

In a biological system like aquarium containing plants, the pH level of the water affects the plant nutrients uptake and therefore it is a significant factor for the plant growth[27]. With the exception of SG application, pH was seen to decrease in the Ca, RS, and Ze applications. The pH of the acclimatization water reduced in the Ca substrate application compared with initiation water (Table 3). The effect of water pH on the acclimatization of *Bacopa monnıeri* L. PENNELL and *Ceratophyllum demersum* has been studied by Karatas *et al.*[23,28]. They acclimatized 100% plantlets of *B. monnıeri* L. PENNELL and *C. demersum* in water of various pH levels between 4.0-10.0 and found that alkaline pH was more suitable for plant growth of these aquatic plants under aquatic conditions. In our study, neutral pH (7.05=RS application) was found to be more suitable for *C. wendtii*.

Electrical conductivity (EC) gives an idea about the amount of dissolved minerals in water and it is a good indicator of the presence of some minerals like sodium, potassium, chloride or sulphate[29]. In the present study, EC increased in all of the substrate applications compared to initiation water sample. EC values varied between 721 and 750 µmhos/cm in SG and Ze, respectively. In parallel with the increase of EC values, total cations (Na^+, K^+, Ca^{++} + Mg^{++}) increased in all of the substrate applications compared to initiation water. The total cation values ranged between 7.20 and 7.55 me/L in SG and Ze, respectively (Table 3). The highest sodium (1.65 me/L) and calcium + magnesium (5.95 me/L) amounts were observed in the RS and Ze substrates, respectively. Potassium values were found to be same in all of the substrate applications.

In parallel with the increase of EC values, total anions (Cl^-, HCO_3^-) also increased in all of the substrate applications. The total anion values ranged between 7.25 (SG) and 7.45 me/L (Ca, RS, Ze) (Table 3). The highest chlorine (2.35 me/L) and hydrocarbonate were evaluated in RS and Ca, respectively.

The initial water sample used for acclimatization belongs to the C_2S_1 irrigation water group. This water group is used for salt resistant plants in medium level with any disadvantages in the medium leveled leaching conditions without any need for salt control and soil management. However, it may be needed to leach in low permeable soils for salt susceptibility. This group water can be used in nearly all soil types as irrigation water without any exchangeable sodium borne problems. In addition, it doesn't originate unfavorable changes because of sodium. The water analyses were carried out every two weeks, but the results from the fourteenth week are given in Table 3 only. The irrigation water class changed only in the Ca substrate application as C_3S_1.

Nitrate (NO_3), Nitrite (NO_2) and Ammonium (NH_4) are three forms of nitrogen. Total nitrogen (NO_3-N, NO_2-N, NH_4-N) increased in all of the substrate applications compared to initiation water. The total nitrogen values ranged between 0.085 (RS) and 0.132 mg/L (Ca). The highest nitrate (0.112 mg/L) was evaluated in the Ca substrates. Ammonium values were found to be same (trace amount) in all of the substrate applications. Nitrite amounts were also same in RS and Ze applications (0.01 mg/L) or Ca and SG applications (0.02 mg/L).

Phosphor concentrations were found to increase in Ca, Ze and SG applications and be same in RS application compared to initiation water. The phosphor values varied between 0.008 (RS) and 0.013 mg/L (Ca). In contrast to the other measured parameters, N/P values decreased in all of the substrate applications compared to initiation water. N/P values were found to change between 10.15 (Ca) and 14.22 (SG).

Conclusion

The present study emphasizes a successful micropropagation protocol for *Cryptocoryne wendtii*. The multiple shoot regeneration was achieved best on the MS medium containing 4 mg/L BA + 1 mg/L IBA (7.21 shoots per explant). The shoot number per explant showed a considerable increase in the second subculture on the same medium (51.8 shoots per explant). Although the use of PGRs at high concentrations could increase the cost of *in vitro* propagation, we compensated high PGRs cost with a quite high regeneration rate (51.8 shoots per explant). As well as the IBA containing media, the PGRs-free control medium showed a high rooting percentage and well-developed roots. This facilitates more economical rooting protocol. The direct acclimatization of the *in vitro* plantlets in aquariums provides a reduction in the costs and in the duration of the micropropagation process. *In vitro* propagation cycle could be completed

within 170 days (40 days for *in vitro* propagation, 40 days for *in vitro* rooting and 90 days for acclimatization). Besides, a natural environment was established by using tetrazona fish in the aquariums. Thus, the present study may also help those studies related to the interactions between fish and aquatic plants and the effect of water quality and supplying CO_2 on aquatic plants growth.

Conflict of Interest

The authors declare no conflict of interest.

References

1 Micheli M, De Gasperis A, Prosperi F & Standardi A, Micropropagation of three species of aquatic plants. *Agricoltura Mediterr*, 131 (2006) 46.

2 Herath HMI, Krishnarajah SA & Wijesundara DSA, Micropropagation of two endemic threatened *Cryptocoryne* species of Sri Lanka. *Trop Agric Res Ext*, 11 (2008) 19.

3 Dogan M, Karatas M & Aasim M, *In vitro* shoot regeneration from shoot tip and nodal segment explants of *Pogostemon erectus* (Dalzell) Kuntze, a multipurpose ornamental aquatic plant. *Fresen Environ Bull*, 25 (2016) 4777.

4 Kane ME, Gilman EF, Jenks MA & Sheehan TJ, Micropropagation of the Aquatic Plant *Cryptocoryne lucens*. *Hort Sci*, 25 (1990) 687.

5 Kane ME, Davis GL, Hoffner TD & Henny RJ, Gibberellins promote flowering in two *Cryptocoryne* species. *Hort Sci*, 30 (1995) 380.

6 Kane ME, Davis GL, McConnell DB & Gargiulo JA, *In vitro* propagation of *Cryptocoryne wendtii*. *Aquat Bot*, 63 (1999) 197.

7 Stanly C, Bhatt A & Keng CL, An efficient *in vitro* plantlet regeneration of *Cryptocoryne wendtii* and *Cryptocoryne becketti* through shoot tip culture. *Acta Physiol Plant*, 33 (2011) 619.

8 Bambaranda BVASM & Peiris SE, *Cryptocoryne wendtii* can successfully be grown in river sand enriched with nutrients. *Sri Lanka J Aquat Sci*, 21 (2016) 67.

9 Ahmad A, Ismun A & Taib M, Effects of salinity stress on carbohydrate metabolism in *Cryptocoryne elliptica* cultures. *J Trop Plant Physiol*, 9 (2017) 1.

10 Ipor I, Yahya Md. D & Tawan C, Response of *Cryptocoryne pallidinervia* Engler (Araceae) on light intensity and water depth. *J Trop Biol Conserv*, 14 (2017) 1.

11 Dissanayake C, Hettiarachchi M & Iqbal MCM, Sustainable use of *Cryptocoryne wendtii* and *Echinodorus cordifolius* in the aquaculture industry of Sri Lanka by micropropagation. *Sri Lanka J Aquat Sci*, 12 (2007) 89.

12 Kauth P, Kane ME & Vendrame W, Greenhouse irrigation method influences growth and quality of *in vitro* propagated *Cryptocoryne wendtii* plantlets. *Plant Cell Tissue Organ Cult*, 87 (2006) 219.

13 Murashige T & Skoog F, A revised medium for rapid growth and bioassay with tobacco tissue cultures, *Physiol Plant*, 15 (1962) 473.

14 Merck E, Die Untersuchung von Wasser: Eine Auswahl chemischer Methoden für die Praxis (Merck, Darmstadt), 1973, 111.

15 Richards LA, U.S. Salinity Lab. Staff, Diagnosis and Improvement of Saline and Alkali Soils (U.S. Government Printing Office, Washington), 1954, 160.

16 Jackson ML, Soil chemical analysis (Prentice-Hall Inc., Engle Wood Cliff, New Jersey, America), 1958, 485.

17 Kurmies B & Bredeney B, Zur Fraktionierung der Bodenphosphate. *Die Phosphorsaure Arbeiten über Phosphorsaurefragen*, 29 (1972) 118.

18 Balks R & Reekers I, Bestimmung des Nitrat und Ammoniakstickstoffs im Boden. *Land Forschung*, 8 (1955) 7.

19 Riele G & Jung J, Zur Vorgang der Nitritbildung im Spinat. *Landwirt Forsch*, 19 (1966) 231.

20 Wehrmann J & Scharpf HC, Der Mineralstickstoffgehalt des Bedens als Maβstab für den stickstoffdüngerbedarf. *Plant Soil*, 52 (1979) 109.

21 Öztürk M, Khawar KM, Atar HH, Sancak C & Özcan S, *In vitro* micropropagation of the aquarium plant *Ludwigia repens*. *Asia Pac J Mol Biol Biotechnol*, 12 (2004) 21.

22 Zheng W, Xu XD, Dai H & Chen LQ, Direct regeneration of plants derived from *in vitro* cultured shoot tips and leaves of three *Lysimachia* species. *Sci Hortic*, 122 (2009) 138.

23 Karatas M, Aasim M, Dogan M & Khawar KM, Adventitious shoot regeneration of the medicinal aquatic plant water hyssop (*Bacopa monnıeri* L. PENNELL) using different internodes. *Arch Biol Sci*, 65 (2013) 297.

24 Karataş M, Aasim M & Dogan M, Efficacy of *in vitro* propagated coontail (*Ceratophyllum demersum* L.) on quality of different water samples, *Fresen Environ Bull*, 25 (2016) 5113.

25 Kacar B & Katkat AV, Bitki besleme (Plant Nutrition) (Nobel Yayınları, Ankara, Turkey) (In Turkish), 2006.

26 Etesami H, Can interaction between silicon and plant growth promoting rhizobacteria benefit in alleviating abiotic and biotic stresses in crop plants? *Agric Ecosyst Environ*, 253 (2018) 98.

27 Kuzovkina YA, Schulthess CP & Zheng D, Influence of soil chemical and physical characteristics on willow yield in Connecticut. *Biomass Bioenergy*, 108 (2018) 297.

28 Karataş M, Aasim M & Dogan M, Multiple shoot regeneration of *Ceratophyllum demersum* L. on agar solidified and liquid mediums. *Fresen Environ Bull*, 24 (2014) 3.

29 Nazir R, Khan M, Masab M, Rehman HU, Rauf NU, Shahab S, Ameer N, Sajed M, Ullah M, Rafeeq M & Shaheen Z, Accumulation of heavy metals (Ni, Cu, Cd, Cr, Pb, Zn, Fe) in the soil, water and plants and analysis of physico-chemical parameters of soil and water Collected from Tanda Dam kohat. *J Pharm Sci Res*, 7 (2015) 89.