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RESPONSE OF DIFFERENT NODAL AND INTERNODAL EXPLANTS ON *IN VITRO* SHOOT REGENERATION OF AQUATIC PLANT-*SHINNERSIA RIVULARIS* WEISS-GRÜN

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ABSTRACT

Aquatic plants are the primary and secondary producers of the ecosystem and also important part of aquatic industry. *Shinnersia rivularis* is an important ornamental plant of aquarium industry due to presnece of white veins on the leaves. *S. rivularis* is mainly vegetatively propagated. This study is the first report of propagating *S. rivularis* under *in vitro* conditions. Five different explants divided into two groups of nodal segments (shoot tip, 1st and 2nd nodal segment) and internodes (1st and 2nd internode) were cultured on agar solidified MS medium without any plant growth regulators (PGR) or enriched with 0.05-0.80 mg/l BA+0.20 mg/l NAA for eight weeks. 50-75 % shoot regeneration frequency was recorded irrespective of explant type or plant growth regulators (PGR). Shoots per explants ranged for shoot tip (4.33-12.71), 1st nodal segment (6.24-15.61), 2nd nodal segment (4.99-12.50), 1st internode (4.67-11.37) and 2nd internode (5.03- 11.93). In general, maximum and minimum number of shoots per explants with average shoot length above 1 cm were recorded on MS medium containing 0.20 mg/l BA+0.20 mg/l NAA irrespective of explant type. *In vitro* regenerated shoots were rooted on agar solidified MS medium enriched with 1.0 mg/l IBA followed by successful adaptation in the aquarium provided with tap water (pH ≈8.5) and oxygen. 80 % of plants transferred to aquarium survived and continued their growth. This work can be employed for commercial propagation of *S. rivularis* and for further biotechnological studies

Key words: Aquatic plant, Explant, *In vitro*, Internodes, Nodal segments.

INTRODUCTION

Aquatic plants are the important member of of water ecosystem due to provision and maintaining continous oxygen in the water bodies (Cirik *et al.* 2011). The efficiency of any water body is dependant on aquatic plants due to source of primary and secondary producers of the ecosystem (Oyedeji *et al.* 2012). Aquatic plants provide favourable medium for reproduction of some fish species in water environment. Fish use these aquatic plants for egg laying, living and feeding and shelter of off-springs on leaves (Yenice 2010). In recent years, aquatic plants are also used for phytoremediation studies (Rai 2009; Singh *et al.* 2011) to remove heavy metals from polluted water bodies by absorbing in thier bodies. Besides that, aquatic plants are used for biomonitor (m pollution) of water bodies (Zurayk *et al.* 2001) depending on the availibility of these plants.

Shinnersia rivularis Weiss-Grün is an important aquatic plant of *Asteraceae* family used in the aquariums as an ornamental plant (Zwerin 2010). The leaves have white veins which make the plant more attractive (Elias *et al.* 2009). The plant gain height of 10-50 cm and spread in the water environment upto 5-15 cm. Plant growth and developement especially leaf structure is associated with the light availibility and intensity like leaf length and internodal distance. The ideal pH for plant growth and developement is 5.0-8.0 with ideal temperature range of 15-30 °C.

Aquariums and ponds become popular hobby in most of the countries all around the World (Maki and Galatowitsch 2014). Only in USA, around 16 millions houses have ponds (Crosson 2010) and around 400 different aquatic species are in use all over the World (Petroeschevsky and Cahampion 2008). The aquarium industry is growing rapidly in Turkey and rest of the World. The material for aquarium industry especially plants, are imported from Far East Asian countries and import bill of the aquarium industry in Turkey in 2011 was estimated 137.1 million euro and increasing gradually due to high demand.

Aquatic plants are propagated through traditional vegaetative means but limited to material available for commercial production. In recenet years, aquatic plants are propagated through micropropagations under *in vitro* conditions (Karataş *et al.* 2013a; Karataş *et al.* 2014a). To date, no effort has been made to propagate *S.rivularis* under *in vitro* conditions. This study presented the efficacy of different nodal and internodal explants on shoot proliferation. This study could be helpful for maintaining the genetic stability and effective conservation of the plant.

MATERIALS AND METHODS

The plants of *S. rivularis* were purchased through local aquarium store in Karaman, Turkey and these plants were maintained in the aquariums containing water prior to *in vitro* studies. For surface sterilization, the twigs were continuously washed under running tap water for 10 min followed by treatment with 0.5% NaOCl for 5 min and rinsed thrice for 5 min. Therafter, twigs were dried on sterilized filter paper followed by culture of twigs on MS (Murashihe and Skoog 1962) medium without growth variants in Magenta GA7 vessels for 2 weeks for screening to obtain contamination free explants.

Following sterilization, the different nodal segments (shoot tip, 1st and 2nd nodal segment) and internodes (1st and 2nd internode) were excised from the twigs and cultured for *in vitro* multiplication on agar solidified MS medium provided with 0.05-0.80 mg/l 6-Benzylaminopurine (BA) and 0.20 mg/L Naphthalene acetic acid (NAA). All explants were also cultured on MS medium as control.

Multiplied regenerated shoots were used for the rooting using MS basal medium containing 1.0 mg/l Indole-3-butyric acid (IBA) for 4 weeks. Thereafter, *in vitro* rooted plantlets were taken from the culture medium and washed under running tap water to remove any agar adhering to the roots. Thereafter, the plantlets were transferred to aquariums containing sand as substrate and filled with water and provided with continous aeration.

All culture media were supplemented with 30 g/l (w/v), solidified with 0.65% (w/v) agar and the pH of all culture media was adjusted to 5.7 ± 1 with 1 N NaOH or 1 N HCl followed by autoclaving for 20 min at 121 °C (1.5 kg cm^{-2} pressure). All cultures were grown at 23 ± 2 °C under 16/8-h light/dark photoperiod using cool white fluorescent lamps. All chemicals used in this study were purchased from Duchefa Biochemie B.V. (The Netherlands).

The treatments were triplicates and post hoc tests were performed using Tukey's or Duncan's multiple range test at the $p \leq 0.01$ level of significance. The data on each type of explant and proliferation medium were subjected to analysis of variance (ANOVA), using SPSS 20 for Windows (USA). All experimental data taken as percentages were arcsine transformed (Snedecor and Cochran 1967) prior to statistical analysis.

RESULTS

Application of 0.5 % NaOCl for 5 min was found sufficient for surface sterilization with no contamination on all explants used in this study under *in vitro* conditions. After two weeks of culture. all explants were cultured on MS medium containing different concentrations of BA-NAA. Callus induction from shoot tip explant started within 10 days with single shoot induction followed by multiple shoot induction after 15 days (Figure 1a). Callus induction from both nodal segment explants (1st and 2nd nodal segment-Figure 2a) and internodal explants (1st and 2nd internode) started after 2 weeks. First shoot induction from these explants were observed after 3 weeks of culture which turned into multiple shoot induction after 4 weeks of culture (Figure 2b, 3a,b). Whereas, multiple shoot induction with rooting was also sobderved on 1st nodal segment explant after 4 weeks of culture (Figure 4a,b). At later stages, root induction from calli and regenerated shoots was also observed on some explants (Figure 1b, 4b). After 8 weeks of total culture, the data were subjected to statistical anlaysis. The results revealed the statisitcally significant effects on growth variants on shoot regeneration, shoots per explant and shoot length shoot.

Callus induction was recorded 100% on all culture media irrespective of explant type. The results on shoot regenration frequency (%) revealed the variable reponse of explants to BA-NAA and ranged 50.0-75.0% irrespective of explant type. Shoot regenertaion frequency ranged 50.0-75.0% for shoot tip, 1st and 2nd internoe explant and 58.33-75.0% for 1st and 2nd nodal segment explant (Table 1). MS medium containing 0.05 mg/l BA+0.20 mg/l NAA showed maximum shoot regeneration frequency (75.0%) on shoot tip, 1st and 2nd nodal segment and 1st internode. Whereas, 2nd internode explant generated maximum shoot regeneration frequency on medum enriched with 0.80 mg/l BA+0.20 mg/l NAA. On the other hand, shoot regeneration frequency on MS medium devoid of growth variants resulted in the range of 58.33-75.0%. 2nd node and internode explants were least responsive on MS medium without growth varinats and generated 58.33% shoot regeneration frequency (Table 1).

The results on shoots per explant also showed variable response of all explants to different BA-NAA concentrations (Table 2). Shoots per explants for different explants were recorded as 4.33-12.71 (shoot tip), 6.24-15.61 (1s nodal segment), 4.99-12.50 (2nd nodal segment), 4.67-11.37 (1st internode) and 5.03- 11.93 (2nd internode). In general, maximum number of shoots per explant were recorded on MS medium containing 0.20 mg/l BA+0.20 mg/l NAA irrespective of explant type. Contrarily, minimum number of shoots per explants were scored on MS medium containing 0.05 mg/l BA+0.20 mg/l NAA or on MS medium containing 0.40 mg/l BA+0.20 mg/l NAA (Table 2). Comparig explant type, 1st nodal segment explant was more responsive and generated maximum number of shoots per explant irrespective of BA-NAA concentration in the culture medium. Shoots per explants on MS medium were recorded as 5.20 (shoot tip), 6.78 (1s nodal segment), 5.0

(2nd nodal segment), 4.11 (1st internode) and 4.48 (2nd internode).

Response of different explants on mean shoot length was also variable to different BA-NAA concentrations. Mean shoot length ranged 0.83-1.39 cm for shoot tips, 0.79-1.25 cm for 1s nodal segment, 0.87-1.71 cm for 2nd nodal segment, 0.66-1.11 cm for 1st internode and 0.69-1.15 cm for 2nd internode explant (Table 3). In general, MS medium containing 0.20 mg/l BA+0.20 mg/l NAA induced longer shoots and resulted with average shoot length > 1 cm. Mean shoot length on MS medium ranged 0.83-1.65 cm. Shoot tip explants was least responsive (0.83 cm) and in contrast, 1st nodal segment was the most responsive and produced longest shoots (1.65 cm).

Approximately 1.0 cm long shoots were detached from their respective explants under *in vitro* conditions, followed by rooting them on MS medium containing 1.0 mg l^{-1} IBA. The results revealed root initiation after 2 weeks and 100% rooting was recorded after a total period of 4 weeks. After rooting, the plantlets were acclimatized in the aquariums filled with tap water (pH ≈8.5) provided with submersible pumping filters for aeration. Approximately 80% of these plant survived in the aquarium after 2 months.

Table 1. Response of different nodal explants of *S. rivularis* on shoot regeneration (%).

BA (mg/l)	NAA (mg/l)	Shoot tip	1st nodal segment	2nd nodal segment	1st internode	2nd internode
0.05	0.20	75.00^{a}	75.00^{ns}	75.00 ^{ns}	75.00^{a}	50.00^{b}
0.10	0.20	75.00^{a}	75.00	75.00	58.33^{b}	66.66^{ab}
0.20	0.20	66.66^{ab}	75.00	75.00	58.33^{b}	50.00^{b}
0.40	0.20	50.00^{c}	75.00	75.00	50.00^{a}	66.66^{ab}
0.80	0.20	58.33^{b}	58.33	58.33	50.00^{b}	75.00^{a}
MS		75.00^{a}	75.00	58.33	75.00^{a}	58.33^{ab}

Means followed by different small letters within columns are sigificantly different using DMRT test at P<0.005

Table 2. Response of different nodal explants of *S. rivularis* on shoots per explants.

BA (mg/l)	NAA (mg/l)	Shoot tip	1st nodal segment	2nd nodal segment	1st internode	2nd internode
0.05	0.20	4.33^{c}	6.24^{b}	8.35^{ab}	4.76^{cd}	5.76^{bc}
0.10	0.20	10.41^{a}	12.27^{ab}	8.65^{ab}	11.37^{a}	7.04^{b}
0.20	0.20	12.71^{a}	14.61^{a}	11.8^{a}	10.57^{a}	11.93^{a}
0.40	0.20	6.00^{bc}	6.12^{b}	4.99^{b}	5.84^{c}	5.03^{c}
0.80	0.20	9.00^{ab}	15.61^{a}	12.50^{a}	7.53^{b}	5.76^{bc}
MS medium (control)		5.20^{bc}	6.78^{b}	5.00^{b}	4.11^{d}	4.48^{c}

Means followed by different small letters within columns are sigificantly different using DMR test at P≤0.005

Table 3. Response of different nodal explants of *S. rivularis* on shoot length (cm).

BA (mg/l)	NAA (mg/l)	Shoot tip	1st nodal segment	2nd nodal segment	1st internode	2nd internode
0.05	0.20	1.39^{a}	0.79^{c}	1.71^{a}	0.83^{b}	1.15^{a}
0.10	0.20	0.83^{b}	0.98^{c}	0.87^{b}	0.86^{b}	1.02^{a}
0.20	0.20	1.28^{a}	1.06^{bc}	1.05^{b}	1.11^{a}	1.04^{a}
0.40	0.20	1.23^{a}	1.25^{bc}	0.88^{b}	0.83^{b}	0.69^{b}
0.80	0.20	0.84^{b}	1.22^{b}	0.91^{b}	0.66^{b}	0.87^{ab}
MS medium (control)		0.83^{b}	1.65^{a}	1.12^{a}	1.10^{a}	1.13^{a}

Means followed by different small letters within columns are sigificantly different using DMR test at P≤0.005

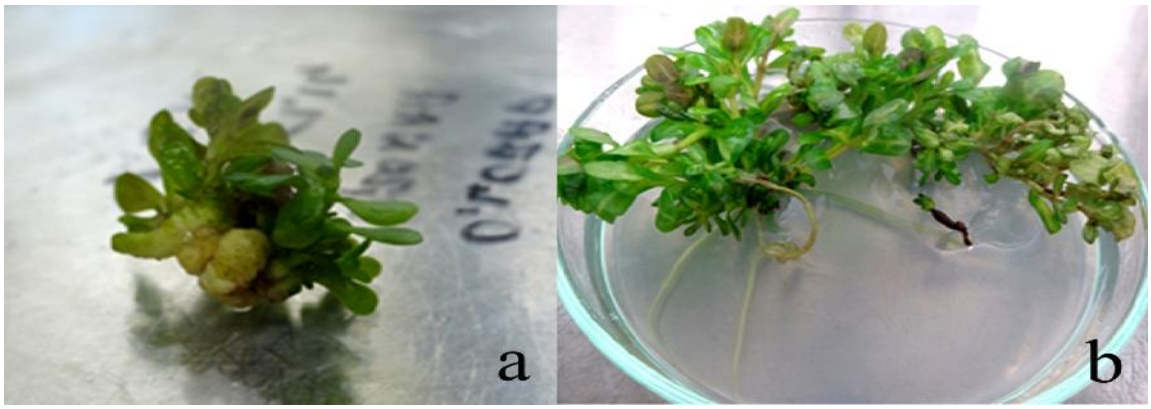

Figure 1: Multiple shoot regenration from shoot tip explant of *S. rivularis* (a) callus and shoots after 4 weeks and (b) well developed shoots with roots eight weeks of culture on medium enriched with BA+NAA

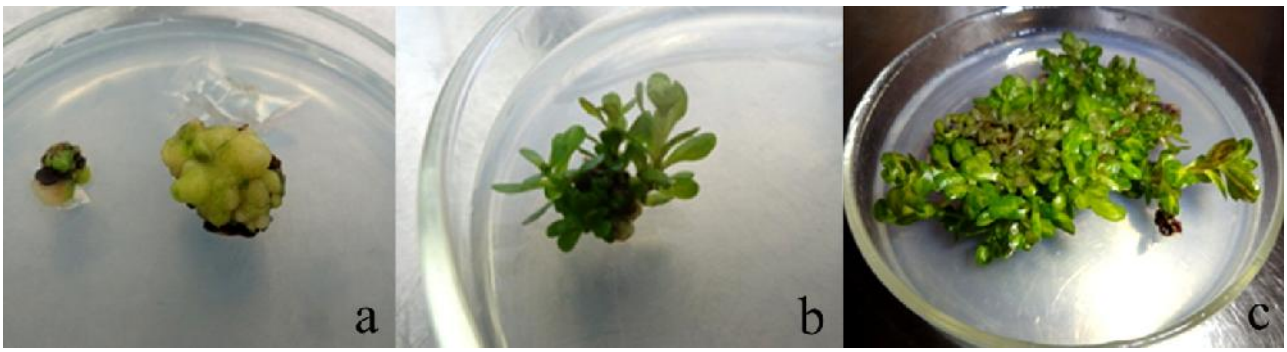

Figure 2: Multiple shoot regenration from 1st nodal segment explant of *S. rivularis* (a) callus induction after 2 weeks (b) multiple shoot initiation after 4 weeks and (c) eight weeks of culture on MS medium enriched with BA+NAA

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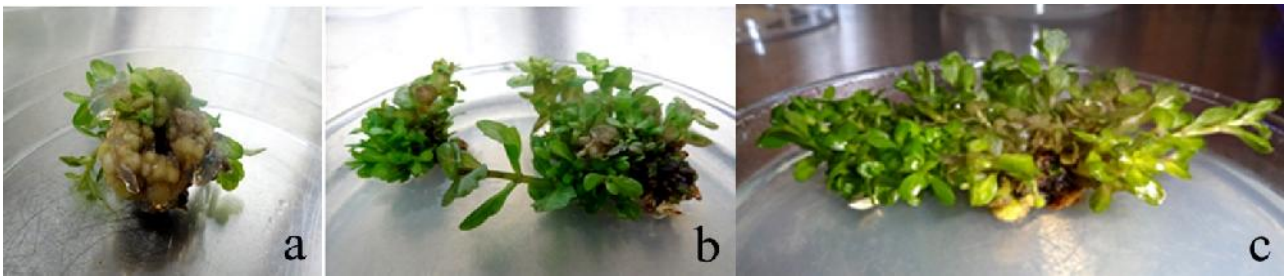

Figure 3: Multiple shoot regenration from 2nd nodal segment explant of *S. rivularis* (a) callus induction after 2 weeks (b) multiple shoot initiation after 4 weeks and (c) eight weeks of culture on medium enriched with BA+NAA.

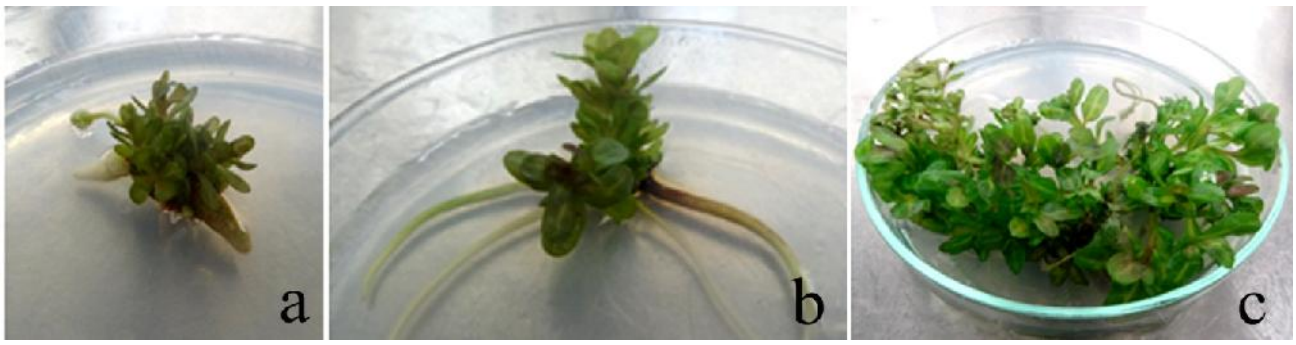

Figure4: Multiple shoot regenration from 1st internode explant of *S. rivularis* (a,b) multiple shoot induction with rooting after 4 weeks and (c) eight weeks of culture on medium enriched with BA+NAA

DISCUSSION

In vitro regeneration of water plants includes surface sterilization, regeneration, rooting and acclimatization. At first step, surface sterilization is very important and depends on selection of proper sterilization agent with proper concentration and sterilization timing. The main objective is to get explants without any cell damage. For aquatic plants, H_2O_2 is preferred for surface sterilization over commercial bleach due to less damage to plants or plant parts (Karataş *et al.* 2013b, 2014b; Barpete *et al.* 2015; Karataş and Aasim 2014, 2015a,b). Contrarily, Commercial bleach is not preferred for sterilization of green or fresh plant parts due to its damage to cells and loss of chlorophyll. In this study, very low concentration of commercial bleach with low exposure time of 5 min. was found sufficient for successful sterilization of twigs without any substantial damage to twigs. Commercial bleach has been reported for the sterilization of other aquatic plants like *Ludwiga repens* (Öztürk *et al.* 2004), water lily (Sumlu *et al.* 2010) and *P. Stratiotes* (Aasim *et al*. 2013, 2017).

In this study, different nodal segments and internodes were used for multiple shoot regeneration. Previously, these explants are also used for other aquatic plants like *Mentha viridis* (Raja and Arockiasamy 2008), *Stevia rebaudiana* (Janarthanam *et al.* 2009) and *Marsdenia brunoniana* (Ugraiah *et al.* 2011). The results revealed 100 % callus induction irrespective of BA-NAA concentrations and explant type. Karataş *et al.* (2013a) also reported 100% callus induction from different internodes and leaf explant of *B. monnieri* using BA-NAA in the culture emdium. The results also report more shoot induction from shoot tip explant compared to other explants which might be due to the presence of actively divided cells in the meristematic zones of the explants compared to other explants (Çeliktaş *et al.* 2006; Karataş *et al.* 2013a). Previously, low shoot regeneration frequency (%) of other aquatic plants has been reported for *B. monnieri* (Vijayakumar *et al.* 2010). Contrarily, 100% shoot regenration frequency has also been reported for aquatic plants like *B. monnieri* (Karataş and Aasim 2014) and *Hemianthus callitrichoides* (Barpete *et al.* (2015) using different explants. Results further revealed variable shoot regneration behaviour of all explants to growth variants (Karataş *et al.* 2013b). Results showed the need of low BA (0.05 mg/l) concentration with 0.20 mg/l NAA for maximum shoot regeneration frequency of all explants (Karataş and Aasim 2014) irrespective of 2nd internode explants which needed relatively high BA concentration (0.80 mg/l) with 0.20 mg/l NAA (Panigrahi *et al.* (2006).

Irrespective of higher shoot regeneration frequency, least number of shoots per explant were generated from 0.05 mg/l BA+0.20 mg/l NAA. Whereas, MS medium containing 0.20 mg/l BA+0.20 mg/l NAA was found more superior for shoots per explants with mean shoot length of above 1.0 cm confirmed the findings of Karataş *et al.* (2013a). They also achieved higher number of shoots per explants of *B. monnieri* using 1st, 2nd, 3rd internodes and leaf explant on MS medium containing 0.25 mg/l BA+0.25 mg/l NAA. Similarly, need of different growth variants concentrations for different explants has been reported for shoot apex and nodal segments of *Gloriosa superba* L. (Hasan and Roy 2005).

Comparasion of types of explants revealed that 2nd internode explants was most least responsive for shoot regeneration frequency to growth variants which confirmed the previous findings of Karataş *et al.* (2014a) for *C. demersum*. On the other hand, 1st nodal segment was more responsive and yielded more number of shoots per explants compared to other explants. Çınar *et al.* (2013) reported shoot tip explant more responsive compared to 1st nodal segment explant of *Hygrophila polysperma*. Relatively shorter shoots were obtained from shoot tip explants compared to other explants used in the study. Karataş *et al.* (2013a) gained maximum number of shoots with longer shoots from leaf explant compared to 1st, 2nd and 3rd internodes explants of *B. monnieri.* Öztürk *et al.* (2004) reported maximum number of 52.63 shoots per explant with longer shoots (5.63 cm) from 1st nodal segments explants compared to leaf and petiol explants of *Hygrophila difformis*.

Rooting and acclimatization is an important step for the development of successful *in vitro* regeneration protocols. Rooting of *in vitro* regenerated aquatic plants is relatively easy and reported for most of the commercial aquatic plants regenerated under *in vitro* conditions (Karataş *et al.* 2013a, 2014a; Karataş and Aasim 2015a, b). Results revealed that plants were rooted easily by the application of exogenous IBA confirmed the findings of previous work on other aquatic plants (Sharma *et al.* 2010; Karataş *et al.* 2013a,b, 2014a,b,c; Karataş and Aasim 2015a,b). Similarly, acclimatization of *in vitro* rooted plantlets in the aquariums using tap water has been reported for *Nymphoides indica* (Jenks *et al.* 2010), *Ludwigia repens* (Öztürk *et al.* 2004), *Veronica anagallisaquatica* (Shahzad *et al.* 2011), *A. sessilis* (Gananraj *et al.* 2011), *Cryptocoryne wendtii* and *Cryptocoryne beckettii* (Stanly *et al.* 2011), *Bacopa monnieri* (Karataş *et al.* 2013a, 2016; Karataş and Aasim 2014), *Higrophila polysperma* (Çınar *et al.* 2013; Karataş *et al.* 2013b, 2014c), *Limnophila aromatica* (Karataş and Aasim 2014) and with or without oxygen supply. On the other hand, a study on *B. monnieri* (Karataş *et al.* 2013a) and *R. rotundifolia* (Karataş *et al.* 2014b) suggested 100% acclimatization on pH between 6–8.

Conclusıon: This study presents the successful *in vitro* multiple shoot regeneration from different nodal segments and internode explants followed by rooting and

survival of explants (80%) in the auarium. This protocol can be helpful for commercial propagation of *S Rivularis* for aquatic industry of Turkey. Furthermore, this protocol can be used for the application of modern biotechnological techniques like genetic transformation.

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