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Research Article
A Method for Microclonal Propagation of *Staurogyne repens* in Tissue Culture

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Abstract

Objective: Staurogyn (*Staurogyne repens* (Nees) Kuntze) is a perennial herb, widely used in aquarium landscape design. Representatives of the *Staurogyne* species are sources of unique glycosides in their chemical composition, which are taste stimulators. However, a method of the tissue culture of *S. repens* was not described in the literature until now. In this study, such a method is proposed. **Materials and Methods:** Node bines with axillary buds were taken as explants. Surface sterilization with mercuric chloride ($HgCl_2$) was found to be the most effective (60% survivors of sterile explants). Solid and liquid media contained 3% sucrose and various combinations of plant hormones: Benzyladenine (BA), thidiazuron (TDZ) and indole-3-acetic acid (IAA) were applied. **Results:** It was shown that the intensive multiplication of shoots can be induced using a liquid Murashige-Skoog (MS) medium with the following combination of phytohormones: Benzyl adenine (2 mg L^{-1}) and indole-3-acetic acid (0.5 mg L^{-1}). The MS concentration of 0.2 mg L^{-1} was the most effective for the rhyzogenesis. The adaptation of plants was successful enough under conditions of regular liquid media changeout and under maintaining overwater air humidity of 90% when the plants were grown in emergent conditions. **Conclusion:** The developed tissue culture method for micropropagation of aquatic plant *Staurogyne repens* can used be for commercial purposes.

Key words: Aquatic plants, *Staurogyne repens*, plant tissue culture, micropropagation, phytohormones

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Staurogyn (*Staurogyne repens* (Nees) Kuntze) belongs to the family acanthus (Acanthaceae), order lamiales, class dicotyledonous. Taxonomically, this species are close to its parent type-Hygrophila. In nature these perennial ground-covering plants are found in the countries of the South American continent with a hot climate as a part of coastal aquatic communities. Ecologically, they are typical hydrophytes[1]. Having a number of decorative properties, staurogyn was being cultivated recent years and now it is of a commercial interest in the market of decorative plants designated for landscaped aquariums. Traditional breeding techniques can be used to keep up staurogyn in collection. However, the accompanying flora of lower plants, such as different types of filamentous green algae could endanger the development of the entire artificial aquarium ecosystem. Therefore, the development of technologies of mass reproduction of this plant represents a profound interest. The composition of biologically active compounds of staurogyn is an object of recent studies. Thus, Hiura *et al.*[2] have identified five new glycosides in *Staurogyne merguensis* Wall.

Staurogyn also have a food value. After adding of milled leaves of *S. merguensis* the water becomes a pleasant taste. This plant grows wild and native people often cook rice with its leaves to give a pleasant taste to the rice.

It should be noted here, that the relatively close parentage between *S. repens* and *S. merguensis* allowed to hope that the *S. repens* propagation protocol, would it be developed can be applied for a more valuable *S. merguensis*. In general, the very fast speed of micropropagation, high rate of multiplication of bines would make *S. repens* a successful model object for experiments in plant physiology, cellular engineering purposes, etc. Reproduction of staurogyn *in vitro* tissue culture seems to be the most effective way of its mass propagation, in which the plants are devoid of any detrimental effects of concomitant biota.

In spite of that some microclonal propagation protocols for aquatic and semi-aquatic plants[3-7] were developed, the tissue culture of *S. repens* was not described in the study until now. In this study it described a method for the microclonal propagation of *S. repens* as well as the results of the studies on the adaptation of *S. repens* grafts (obtained in tissue culture) to the aquarium conditions.

MATERIALS AND METHODS

The *S. repens* grafts were obtained using the plants (Fig. 1a) from the collection of Botanical garden of Southern Federal University. They were dissected so that each cutting has one node and small fragments of clipped laminas (Fig. 1b). A several-step sterilization of grafts was performed in accordance with our previous experience and the known methodological principles of aquatic tissue culture[8-10].

Before sterilization, the grafts were washed with 0.01% tween-80 for 15 min with constant shaking and then running water. The subsequent steps were carried out in a laminar box. The grafts were immerged in water for 30 sec. Then they were treated with 70% ethanol followed by washing with gnotobiotic water. Further, the following reagents were attempted in the process of sterilization-5% solution of chloramine B, 1% sodium hypochlorite solution, 0.1% mercuric chloride solution. Surfaces of the sterilized grafts were washed three times in gnotobiotic water for 15 min.

Grafts (explants) were placed on a solid agar (0.6% agar) with the Murashige-Skoog (MS) medium[11] or on a liquid MS. Solid and liquid media contained 3% sucrose and various compositions of plant hormones: Benzyladenine (BA), thidiazuron (TDZ) and indole-3-acetic acid (IAA) and were adjusted to pH 5.7. IAA was applied in the following concentrations in attempts to induce rhizogenesis; 0.1, 0.2 and 0.5 mg L^{-1}. In the cases of both solid and liquid media, the cultivation was carried out at 25±1°C, 16 h photoperiod under illuminance of 50 mmol photons m^2 sec^{-1}. If the explants were placed into a liquid medium, they were cultured on a shaker with a speed of 50 rpm min^{-1}.

The regenerants obtained in the course of cultivation and having an extensive root system were transferred to *ex vitro* conditions to the bottom of a glass reservoir of 10 L with different levels of water. A solid mixture containing clay, quartz sand and mineral wool were used as a substrate.

RESULTS AND DISCUSSION

As a result of the present study, the staurogyn (*Staurogyne repens*) plants were micropropagated for the first time using tissue culture method which was optimized for this purpose.

Surface sterilization of explants gave the best results with 0.1% mercuric chloride (5 min). In this case, about 60% of the explants appeared to be not contaminated. The usage of a sterilizing reagents: 5% chloramine B, 1% sodium, hypochlorite brought to damage the plants (Table 1) or didn't prevent the development of micro-organisms.

On the 2nd week of cultivation the explants which were placed on a solid agar medium demonstrated indications of growth; elongation of bine and leaf development. About 60% of the bines were rooted.

Fig. 1(a-d): Micropropagation of *S. repens*, (a) Mother plants of *S. repens*, (b) Explant with axillary buds, (c) Multiple shoot formation on MS medium treated with 2 mg L^{-1} BAP and 0.5 mg L^{-1} IAA and (d) Multiple shoot formation on liquid MS medium

Table 1: Effectiveness of influence of the sterilizing agents on staurogyn explants, percentage of survived plants

	Sterilization time (min)*			
Sterilizing agent	5	10	15	20
Mercuric chloride (0.1%)	61±5.1	22±2.4	21±2.2	-
Sodium hypochlorite (1%)	23±2.7	33±4.2	22±2.4	-
Chloramine B (5%)	5±0.7	15±1.8	5±0.7	5±0.7

*Mean±SD, n = 20

Cultivation of explants in a liquid medium gave better results. There were observed the emergence of new bines on the third week, under the influence of a particular combination of plant hormones (Fig. 1c).

The mix of BA (2 mg L^{-1}) and IAA (0.5 mg L^{-1}) gave the largest multiplication factor. In this case, one bine formed 7-9 adventitious bines (Table 2). The alone BA usage showed the worst results. About 1 mg L^{-1} BA induced formation of 2-3 bines from one explants in a longer period of time. The higher concentrations of BA caused the vitrification of staurogyn tissues. The usage of thidiazuron was not effective. Its influence led to plant growth arrest.

Table 2: Effect of different concentrations and combinations of auxins and cytokinins on multiple shoot formation and rooting shoots of *S. repens* (liquid media)

BA (mg L^{-1})	IAA (mg L^{-1})	TDZ (mg L^{-1})	No. of shoots per explant (pieces)*	Proportion of rooted shoots (%)*
0	0	0.05	0.0±0.0	0
0	0	0.1	0.0±0.0	0
0.5	0	0	0.8±0.3	0
1	0	0	2.2±0.1	0
1	0.1	0	3.1±0.2	0
1	0.2	0	3.4±0.2	0
2	0.1	0	5.3±0.3	0
2	0.2	0	5.2±0.3	30±10.3
2	0.5	0	8.1±0.3	35±10.5
3	0	0	0.3±0.1	0
3	0.5	0	0.3±0.3	0
0	0.1	0	0.0±0.0	45±11.0
0	0.2	0	0.0±0.0	80±9.1
0	0.5	0	0.0±0.0	80±9.1

*Mean±SD, n = 20

There were significant differences in characteristic of *S. repens* bines developed on solid and a liquid media. Basically they relate with the shape and dimensions of the leaf. In the first case, the leaves have a relatively well-developed blade in the form close to natural. In the second case the leaves appear to have 7-9 pieces per explants on average, with a relatively small and rounded leaf blade.

Duration of a single subcultivation was 2 weeks. About 30% of the explants spontaneously formed roots at the end of each subcultivation-5 pieces/explant on average. The remaining (non-rooted) explants were transferred on an IAA-contained medium where they rooted on the 10-12th day, while the most effective concentration of IAA for rooting was 0.2 mg L^{-1}.

In general our results support the observation[3,4] that the micropropagation of plants in the liquid media (without agar) gives better results than on the solid media. Thus, in our study, the coefficient of propagation of *S. repens* was increased from 1:5 on the solid media to 1:15 on the liquid media.

In addition, the time period between subcultivations was found to be increased when using liquid media compared with solid media. The entire period of *S. repens* development lasted longer than 8 weeks.

The results of this study have also showed that the application of the alone IAA phytohormone for aquatic plants gives a higher proportion of rooted bines than the application of its mix with BA (Table 2) or than the alone BA (Table 2) which most frequently was used for the aquatic plants cultivation without other phytohormones[12-15].

Under *ex vitro* conditions the regenerants were successfully developed on a quartz sand substrate. There were some difficulties to optimize submersion depth of in aquarium. In a fully submerged condition the regenerants continued to grow and but then turned into a resting phase. Most of these plants remain viable for several weeks. In attempt to cultivate them on a solid substrate, the regenerantes quickly died. It was found that the regenerants should be submerged so that to remain leaves on the surface. Furthermore, the adaptation of plants was effective at a regular changeout of MS and maintaining the overwater air humidity in the aquarium about 90-100%. After the appearance of well-developed leaves, the plants were being able to successfully adapt to both subwater and overwater conditions.

CONCLUSION

A method for tissue culture of micropropagation of aquatic plant *Staurogyne repens* has been developed. The most effective mode sterilization of explants was found to be applied using 0.1% mercuric chloride. Various phytohormone compositions were tested to optimize growth of explants. The maximum multiplication factor of bines (7-9 shoots explant^{-1}) was achieved on a liquid Murashige-Skoog medium with addition of benzyladenine (2 mg L^{-1}) and indole-3-acetic acid (0.5 mg L^{-1}). Massive formation of roots was observed with use of a liquid MS media with addition of indole-3-acetic acid at concentration of 0.2 mg L^{-1}.

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