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HIGH EFFICIENCY PLANT REGENERATION FROM SHOOT TIP EXPLANTS OF *STAUROGYNE REPENS* (NEES) KUNTZE

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Abstract

A method for tissue culture of micropropagation of aquatic plant *Staurogyne repens* (Nees) Kuntze has been developed. Different phytohormones were investigated to optimize the growth of explants. The shoot tip explants of *S. repens* were placed in MS nutrient medium including BAP (0.30-1.50 mg/l) + TDZ (0.10 mg/l) + NAA (0.10 mg/l) and Kn (0.30-1.50 mg/l) + TDZ (0.10 mg/l) + NAA (0.10 mg/l). Shoot regeneration frequencies were changed as 77.77-100% and 66.66-100% in cultivation medium augmented with BAP-TDZ-NAA and Kn-TDZ-NAA, respectively. In the growing media with BAP, the maximum shoot number (14.44 shoot/explant) was obtained in MS medium with 1.20 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA. In Kn applications, the highest shoot number (9.50 shoot/explant) was recorded in growing medium with 0.30 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA. The longest shoots (2.33 cm) was reached in nutrient medium with 0.90 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA. Regenerated plants rooting at 0.25 mg/l IAA have been effectively acclimated to aquarium conditions.

Introduction

The ornamental plant industry has become an important agricultural activity that generates significant income in many developed and developing countries all over the world (van Heezik *et al.* 2013, Idrovo-Novillo *et al.* 2018). Nevertheless, the plants are collected in large quantities from their habitat due to the increasing demand for ornamental plants. The intensive collection of plants from nature has revealed the possibility of extinction of the species (Seydi *et al.* 2019). For these reasons, many countries have emphasized that culturally important ornamental plants should be cultivated and given priority (Chowdhuri and Deka 2019).

Biotechnological methods, especially plant tissue cultures come to the fore in order to support breeders and producers in meeting the demands of the ornamental plants industry in the next century (Nawrot-Chorabik *et al.* 2019). Compared to traditional production methods, tissue culture techniques have many advantages such as mass production in a short time, disease-free plant production, plant production independent from seasonal conditions (Shahzad *et al.* 2017). In recent years, many valuable ornamental plants such as *Echinopsis chamaecereus* H. Friedrich & Glaetzle cv. 'Aurea' (Téllez-Román *et al.* 2020), *Limnophila aromatica* (Lamk.) Merr. and *Rotala rotundifolia* (Buch-Ham. ex Roxb) Koehne (Dogan 2020) have been successfully produced with tissue culture techniques.

Staurogyne repens (Nees) Kuntze belongs to Acanthaceae. 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. *Staurogyne* was being cultivated recent years and now it is of a commercial interest in the market of decorative plants designated for landscaped aquariums (Sereda *et al*. 2017).

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In vitro propagation techniques are an important approach for establishing rapid replication protocols and genetic manipulation studies. In the present study, effects of different plant hormones on *in vitro* multiple shoot regeneration of the valuable ornamental plant *S. repens* were investigated. For this purpose, different cytokine and auxin combinations were tested for multiple regeneration and effective combinations were determined for optimum propagation. This comprehensive study can make an important contribution to the literature and can offer an effective alternative to propagation methods to meet the increasing demand for *S. repens.*

Materıals and Methods

Experimental applications of the present study were done in Karamanoğlu Mehmetbey University (Turkey), Department of Biology. *S. repens* plants were collected from the aquarium store with barcodes. The plants were surface-sterilized before being cultured. Surface sterilization of the plants was provided according to Kose *et al.* (2021). Then, the shoot tip explants were isolated and placed to glass tubes including MS (Murashige and Skoog 1962). Sterile and intact shoots were formed in the fourth week. Shoot tip explants from these regenerated shoots were used in reproduction trials.

The MS essential nutrient salts containing 3% sucrose (Duchefa) 0.65% agar (Duchefa) were used for *in vitro* propagation experiments. Different combinations of BAP (0.30-1.50 mg/l) + TDZ (0.10 mg/l) + NAA (0.10 mg/l) and Kn (0.30-1.50 mg/l) + TDZ (0.10 mg/l) + NAA (0.10 mg/l) were added. pH was regulated to 5.7 ± 1 via NaOH (1 N) and HCI (1 N). The prepared nutrient media were sterilized in an autoclave (1.2 atm pressure, for 20 min). Experiments were carried out in a temperature (24°C) and light controlled cabin (Sanyo) for eight weeks.

For *in vitro* root formation, an elongated regenerated shoots were cut approximately 3 cm long and placed in MS media with 0.25 mg/l IAA. Root-formed plants were planted to aquarium with water at the end of the fourth week. The aquarium was designed to be 24ºC and 16 hrs of illumination.

In trials, glass tubes were used in six replicates. SPSS 21 for Windows package was used for all statistical analysis. The data were analyzed via One-way ANOVA. Then, Duncan test was used to identify significant differences.

Results and Discussion

In the present study, the explants of *S. repens* were cultivated in the growing medium including diverse densities of BAP-TDZ-NAA and Kn-TDZ-NAA. Shoot tip are highly favored in micropropagation works. This is due to the fact that the shoot-end meristem region has a small cell population that is capable of continuously producing organs and tissues (Murray *et al.* 2012). Similarly, many plant species such as *Woodfordia fruticosa* (Linn.) Kurz (Neha *et al.* 2019), *Ceratophyllum demersum* L. (Dogan 2019) and *Cannabis sativa* L. (Wróbel *et al.* 2020) were produced *in vitro* using the shoot tip explant.

The emergence of new shoots from the meristematic area at the shoot apex is directly related to the presence of cytokines and auxins (Ahmad *et al.* 2012). In the present study, both cytokinin and auxin hormones were used together and were used together to determine shoot regeneration capacities. The first variations on the explants were observed on the 5th day and the first shoot creations on the 12th day in MS medium fortified with BAP-TDZ-NAA. The first shoots in nutrient media with Kn were detected on the 16th day. In general, the emergence of the first shoots was higher in the nutritive medium including BAP. After eighth week, the trials were completed as the shoots in the nutritive medium supported with BAP-TDZ-NAA (Fig. 1a,b) and Kn-TDZ-

NAA (Fig. 1c, d) reached a certain size. After the regeneration data were obtained, they were subjected to analysis of variance (Table 1).

When the analysis data in Table 1 was examined, shoot regeneration rate (%) (R^2: 0.783; F: 7.943), the count of shoots (R^2: 0.948; F: 39.896) and shoot lengths (R^2: 0.931; F: 29.827) were found to be statistically valid at $p < 0.05$. The Duncan test was performed (Table 2).

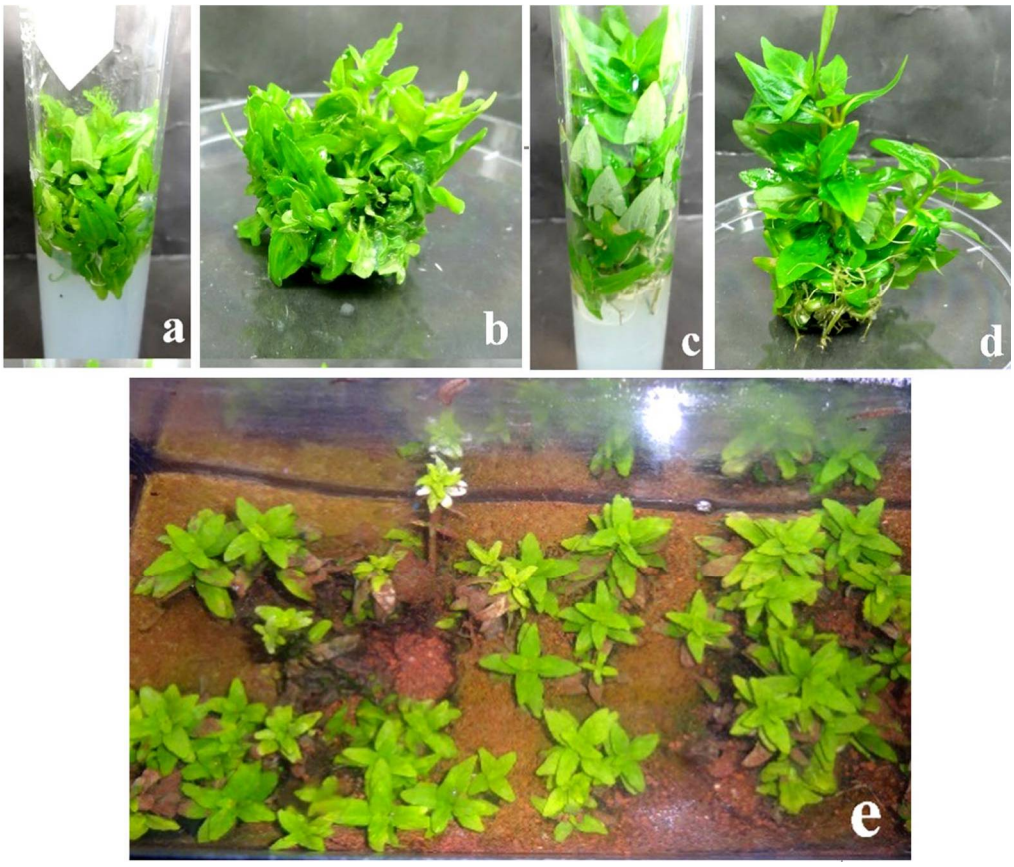

Fig. 1. Plant formation from the shoot tip meristem explant of *S. repens*. (a, b) Multiple shoot regenerations in growth medium including 1.20 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA (c, d) Regenerated shoots in growth medium supported with 30 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA combination. (e) Acclimation of plants produced in the growth medium to aquarium conditions.

Regeneration frequencies were determined as 77.77-100% and 66.66-100% in cultivation medium augmented with BAP-TDZ-NAA and Kn-TDZ-NAA, respectively. In general, higher shoot regeneration percentages were obtained in the MS medium augmented with BAP. 100% shoot creations (LB: 90.807 - UB: 109.193) were reached in cultivation media including 1.20 and 1.50 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA and 0.30 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA. Regeneration ability of cells also increased in environments with high BAP. On the other hand, high regeneration levels in Kn containing environments were obtained with the low Kn application. The lowest shoot creation was recorded as 77.77% (LB: 68.581- UB: 86.966) in nutrient medium with 0.30 ve 0.60 mg/l BAP + 0.10 mg/*l* TDZ + 0.10 mg/l NAA and 66.66%

(LB: 57.467- UB: 75.853) in nutritive medium augmented with 1.50 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA. As can be seen from the percentage regeneration results, although BAP and Kn cytokines are hormones, they have shown almost the opposite effect. This may be related to the mechanism of action of hormones in cells, or they may have different effects due to their interaction with TDZ and NAA. Accordingly, Kaviani *et al*. (2019) and Karakas (2020) have shown various impacts of growth regulators.

Table 1. Results of variance analysis of between-subjects effects for BAP-TDZ-NAA and Kn-TDZ-NAA.

Source	Dependent variable	Type III sum of squares	df	Square mean	F
Corrected Model	Shoot reformation	4682.007[a]	10	468.201	7.943
	Shoot counts	395.730[b]	10	39.573	39.896
	Shoot extents	5.379[c]	10	0.538	29.827
Intercept	Shoot reformation	240395.957	1	240395.957	4078.231
	Shoot counts	2328.144	1	2328.144	2347.127
	Shoot extents	105.986	1	105.986	5877.230
Medium	Shoot reformation	4682.007	10	468.201	7.943
	Shoot counts	395.730	10	39.573	39.896
	Shoot extents	5.379	10	0.538	29.827
Error	Shoot reformation	1296.815	22	58.946	
	Shoot counts	21.822	22	0.992	
	Shoot extents	0.397	22	0.018	
Total	Shoot reformation	246374.778	33		
	Shoot counts	2745.696	33		
	Shoot extents	111.762	33		
Corrected Total	Shoot reformation	5978.822	32		
	Shoot counts	417.552	32		
	Shoot extents	5.776	32		

a R Squared = 0.783 ; b R Squared = 0.948 ; c R Squared = 0.931.

In the growth media with BAP, the greatest shoots count were obtained in 1.20 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA application as 14.44 shoot/explant (LB: 13.248 - UB: 15.632), followed by 1.50 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA application as 13.39 shoot/explant (LB: 12.194 - UB: 14.579). The lowest shoot count was determined in the growing media including 0.30 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA as 7.71. The results revealed that high BAP application positively affects the number of shoots.

The shoot count in cultures with Kn varied between 5.22 and 9.50 shoot/xplant. The application of a low level of Kn resulted in a higher shoot count. The greatest shoots count (9.50 shoot/explant; LB: 8.304 - UB: 10.689) was obtained in growth medium including 0.30 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA, followed by culture medium with 0.60 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA (8.25 shoot/explant; LB: 7.061- UB: 9.446). A small shoot counts were

identified as 5.22 shoot/explant (LB: 4.024 - UB: 6.409) in MS growing medium with 1.50 mg/l Kb + 0.10 mg/l TDZ + 0.10 mg/l NAA.

Table 2. Effect of different concentrations and combinations of auxin and cytokinin on multiple shoot formation of *S. repens*.

Growth regulators (mg/l)				Shoot reformation (%)		
				Average	95% trust region	
BAP	KIN	TDZ	NAA		Lower boundary	Upper boundary
-	-	-	-	72.22^{c}	63.024	81.409
0.30	-	0.10	0.10	77.77^{bc}	68.581	86.966
0.60	-	0.10	0.10	77.77^{bc}	68.581	86.966
0.90	-	0.10	0.10	94.44^{a}	85.251	103.636
1.20	-	0.10	0.10	100.00^{a}	90.807	109.193
1.50	-	0.10	0.10	100.00^{a}	90.807	109.193
	0.30	0.10	0.10	100.00^{a}	90.807	109.193
	0.60	0.10	0.10	88.89^{ab}	79.694	98.079
	0.90	0.10	0.10	88.89^{ab}	79.694	98.079
	1.20	0.10	0.10	72.22^{c}	63.024	81.409
	1.50	0.10	0.10	66.66^{c}	57.467	75.853

Right side of the table.

Shoot counts (shoots/explant)			Shoot extents (cm)		
	95% trust region			95% trust region	
Average	Lower boundary	Upper boundary	Average	Lower boundary	Upper boundary
2.20^{f}	1.004	3.389	0.97^{i}	0.806	1.127
7.71^{cd}	6.518	8.902	1.34^{h}	1.179	1.501
8.51^{c}	7.318	9.702	1.47^{gh}	1.309	1.631
11.23^{b}	10.038	12.422	1.59^{fg}	1.429	1.751
14.44^{a}	13.248	15.632	1.75^{ef}	1.589	1.911
13.39^{a}	12.194	14.579	1.82^{cde}	1.659	1.981
9.50^{c}	8.304	10.689	1.94^{cd}	1.779	2.101
8.25^{c}	7.061	9.446	2.06^{bc}	1.896	2.217
6.47^{de}	5.274	7.659	2.33^{a}	2.169	2.491
5.49^{e}	4.294	6.679	2.27^{ab}	2.109	2.431
5.22^{e}	4.024	6.409	2.18^{ab}	2.019	2.341

Mean values followed by different letters are statistically significant at the $p < 0.05$ level.

While low doses of Kn were more efficient on the shoot count, high doses of BAP were more efficient on the shoot count. In general, in cultures using BAP, more shoot numbers were obtained compared to Kn applications. Thus, when the greatest shoots counts of BAP and Kn applications were compared, 52% higher shoot count was recorded with BAP application. Similarly, Kaviani *et al.* (2019) cultivated the apical meristems of *Aglaonema widuri* in MS media with diverse growth regulating agent and obtained the most number of nodes in cultures with 4.0 mg/l BAP +

0.5 mg/l TDZ + 0.1 mg/l NAA. Joshi *et al*. (2020) cultivated the shoot tip explants of *Vernonia amygdalina* Delile in the nutrient media supported with BAP, TDZ and Kn individually and associated with NAA, and obtained a large number of shoots (25.42 ± 2.5 shoots/explant) in nutritional medium strengthened with 4.4 µM BAP + 1.59 µM NAA. Raghavendra *et al*. (2019) kept *Cicer arietinum* L. in an environment fortified with diverse doses of BAP, Kn, NAA and IAA for *in vitro* shoot regeneration and achieved the best shoot differentiation in a nutritional medium including 4.0 mg/l BAP + 2.0 mg/l NAA. The zygotic embryos isolated from *Amygdalus communis* L. cv. Nonpareil mature seeds were transferred in nutrient medium with diverse doses of BAP and Kn (0-4.0 mg/l) and the best shoot creation was detected in media including 1 mg/l BAP (Isikalan *et al.* 2008). The nodal explants of *Prunus persica* L. Batsch. cv. Garnem were cultivated in various doses of BAP (0.5-4 mg/l) single and along with 0.01 mg/l IBA (BAP + IBA) and 0.5 mg/l GA_3 (BAP + GA_3). In another experimental group, Kn was used in the same densities instead of BAP. It was noted that the nutrient media supported with 0.5 mg/l BAP together with 0.01 mg/l IBA and 0.5 mg/l GA_3 was a appropriate media for the shoot creations. The culture with 2 mg/l BAP + 0.01 mg/l IBA + 0.5 mg/l GA_3 was found to be a proper medium for the most shoot count per explant (Felek *et al.* 2017). These data revealed that BAP was an effectual growth regulating agent.

Length values was changed with the effects of BAP (1.34-1.82 cm; $p < 0.05$). When the cultures with BAP were examined, the tallest plants (1.82 cm; LB: 1.659 - UB: 1.981) were obtained in an environment with 1.50 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA. On the other hand, the shortest shoots (1.34 cm; LB: 1.179 - UB: 1.501) were identified in nutritional media fortified with 1.50 mg/l BAP + 0.10 mg/l TDZ + 0.10 mg/l NAA. Results demonstrated that the utilization of high densities of BAP promotes the extension of the shoots.

Shoot lengths in MS media with Kn were listed between 1.94-2.33 cm. The tallest plants was reached in nutrient medium fortified with 0.90 mg/l Kn + 0.10 mg/**l** TDZ + 0.10 mg/l NAA (2.33 cm). Using Kn higher or lower than 0.90 mg/l Kn reduced the shoot length. The shortest shoots (1.94 cm; LB: 1.779 - UB: 2.101) were recorded on MS media including 0.90 mg/l Kn + 0.10 mg/l TDZ + 0.10 mg/l NAA. When shoot lengths were evaluated, Kn application was found to be more successful on shoot length than BAP application.

The extended shoots were transplanted to the cultivation medium including 0.25 mg/l IAA for rooting trials. Root formations appeared in the second week and the roots were clearly observed at the end of four weeks. Then, the process of accustoming to external conditions was initiated for rooted plants. First of all, the nutrient medium on the plants has been carefully removed to prevent any pollution in the aquatic environment. Then plants were planted in aquariums with water. Rapid development was observed in the leaves and lengths of the plant during the first week. After four weeks, they were successfully accustomed to aquarium conditions (Fig. 1e). Similarly, successful adaptation of *in vitro* propagated aquatic plants such as *Cryptocoryne wendtii* (Unal *et al.* 2019), *Hemianthus micranthemoides* (Nuttall) and *Lilaeopsis brasiliensis* (Glaziou) Affolter (Ing *et al.* 2019) to aquarium conditions have been reported.

As a result, multiple reproductions of *S. repens* from shoot-end explants has been successfully achieved. While the most shoots were detected in the nutritive medium with BAP, the longest shoots were obtained in the medium with Kn. In general, use of high doses of BAP significantly increased the number of shoots. In experiments with Kn, the number of excess shoots was determined in nutrient media with low Kn. *S. repens* is a valuable ornamental plant. The interest in *S. repens* is constantly increasing. This comprehensive production protocol can contribute to large-scale propagation and genetic studies of *S. repens*. It also offers an important alternative in meeting growing plant demand.

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