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Multiple Shoot Regeneration from Shoot Tip and Nodal Explants of *Rotala rotundifolia* (Buch-Ham. ex Roxb) Koehne

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Rotala rotundifolia (Buch-Ham. ex Roxb) Koehne'nın Sürgün Ucu ve Boğum Eksplantlarından Çoklu Sürgün Rejenerasyonu

Abstract: *Rotala rotundifolia* (Buch-Ham. ex Roxb) Koehne, an aquatic plant belonging to the family Lythraceae, is used for the treatment of some diseases due to its medical and anti-microbial properties. This study presents multiple shoot regeneration from shoot tip and nodal explants of *R. rotundifolia* cultured on Murashige and Skoog (MS) nutrient medium containing 0.05-1.25 mg/L Kinetin (KIN) and 0.25 mg/L Gibberellic acid (GA_3) combinations for eight weeks. At the end of the second week, shoot formations began to be observed on the explants. High shoot regeneration frequencies were determined for both explants in the culture medium. The maximum number of shoots per explant was obtained from shoot tip (38.66) and nodal (30.77) explants cultured on MS medium containing 0.25 mg/L KIN + 0.25 mg/L GA_3. Whereas the minimum number of shoots per explant was determined on MS medium containing 1.25 mg/L KIN + 0.25 mg/L GA_3 for both explant types. The highest shoot lengths for shoot tip (1.87 cm) and nodal (1.79 cm) explants were obtained on MS culture medium containing 0.75 mg/L KIN + 0.25 mg/L GA_3 and 0.50 mg/L KIN + 0.25 mg/L GA_3, respectively. For *in vitro* rooting of the regenerated shoots, 2 cm long cut shoots were transferred to MS medium containing 0.25 mg/L indole-3-butyric acid (IBA). The rooted shoots were then successfully acclimatized to external conditions in the aquarium environment.

Keywords: *In vitro*, Kinetin, *R. rotundifolia*, Shoot regeneration, Tissue culture

Özet: Lythraceae familyasına ait bir su bitkisi olan *Rotala rotundifolia* (Buch-Ham. ex Roxb) Koehne, tıbbi ve antimikrobiyal özelliklerinden dolayı bazı hastalıkların tedavisinde kullanılır. Bu çalışma, 0,05-1,25 mg/L Kinetin (KIN) ve 0,25 mg/L Gibberellik asit (GA_3) kombinasyonları içeren Murashige ve Skoog (MS) besin ortamında sekiz hafta boyunca kültüre alınan *R. rotundifolia*'nın sürgün ucu ve boğum eksplantlarından çoklu sürgün rejenerasyonunu sunmaktadır. İkinci haftanın sonunda explantlar üzerinde sürgün oluşumları gözlemlenmeye başlanmıştır. Kültür ortamında her iki eksplant için yüksek sürgün rejenerasyon oranları belirlenmiştir. Maksimum eksplant başına sürgün sayısı, 0,25 mg/L KIN + 0,25 mg/L GA_3 içeren MS ortamında kültüre alınan sürgün ucu (38,66) ve boğum (30,77) eksplantlarından elde edilmiştir. Buna karşın, her iki eksplant türü için minimum eksplant başına sürgün sayısı 1,25 mg/L KIN + 0,25 mg/L GA_3 içeren MS ortamında belirlenmiştir. Sürgün ucu (1,87 cm) ve boğum (1,79 cm) eksplantları için en yüksek sürgün uzunlukları sırasıyla 0,75 mg/L KIN + 0,25 mg/L GA_3 ve 0,50 mg/L KIN + 0,25 mg/L GA_3 içeren MS kültür ortamında elde edilmiştir. Rejenere sürgünlerin *in vitro* köklendirilmesi için, 2 cm uzunluğunda kesilen sürgünler 0,25 mg/L indol-3-bütirik asit (IBA) içeren MS ortamına aktarılmıştır. Köklü sürgünler daha sonra akvaryum ortamında dış koşullara başarılı bir şekilde alıştırılmıştır.

Anahtar Kelimeler: *In vitro*, Kinetin, *R. rotundifolia*, Sürgün rejenerasyonu, Doku kültürü

1. Introduction

Aquatic plants, the primary producers of the aquatic environment, are a good source of nutrients for fish, invertebrates, and birds in tropical chains (Gross et al., 2001; Oyedeji and Abowei, 2012). They also provide habitats and refuges for periphyton, zooplankton, other invertebrate species, and vertebrates (Bornette and Puijalon, 2011). Moreover, many aquatic plants such as *Bacopa monnieri* (Linn.) Pennell, *Alternanthera sessilis* R. Brown ex DC., *Hydrolea zeylanica* Vahl, *Ipomoea aquatica* Forsskal, *Limnophila indica* (L.) Druce, *Ludwigia adscendens* (Linn.) Hara, *Nymphaea nouchali* N.L. Burman, *Pistia stratiotes* Linn. and *Trapa natans* Linn. have been reported for medical use in the treatment of diseases (Swapna et al., 2011).

Rotala rotundifolia (Buch-Ham. ex Roxb) Koehne (Lythraceae family) is an aquatic and amphibian plant of South and Southeast Asia, Japan, Africa, Australia, China, India and North America (Tan et al., 2009; Bhowmik et al., 2012). *R. rotundifolia* is reputed of antipyretic, detoxication, anti-swelling and diuresis properties and useful in treatments of cirrhosis ascetic fluids, gonorrhea, menstrual cramps and piles in the south of China (Zhang et al., 2011). The plant has also been used for the treatment of carbuncle, furuncle, rheumatism, and arthralgia (Tan et al., 2009). In a study to determine antioxidant and total phenolic content of 31 wetland plants, aqueous extracts of *R. rotundifolia* have been reported to have the highest antioxidant capacity (Ho et al., 2012).

The aim of this study is to investigate the efficient and rapid propagation from shoot tip and nodal explants of *R. rotundifolia* cultured on MS nutrient medium containing 0.05-1.25 mg/L KIN and 0.25 mg/L GA_3 combinations. This study may help to use protocol for isolation of pharmacologically useful components from the plant and may offer an alternative method for the mass production of *R. rotundifolia* in the aquarium trade industry.

2. Materials and Method

The plants of *R. rotundifolia* were obtained from the local aquarium of Konya province of Turkey. After taxonomic studies, 3-5 cm long twigs were washed under tap water for 10 minutes. Surface sterilization was performed with 20% hydrogen peroxide (H_2O_2) for 10 min followed by rinsed thrice with sterilized distilled water by continuous stirring for 5 min each. After sterilization, shoot tip and nodal explants were isolated under sterile conditions and cultured on Murashige and Skoog (1962) medium (MS) devoid of growth variants (Table 1) for 2 weeks to obtain contamination free explants. Thereafter, the explants were cultured on MS medium supplemented with 3% sucrose, different concentrations (0.05, 0.25, 0.50, 0.75, 1.00 and 1.25 mg/L) of Kinetin (KIN) and 0.25 mg/L Gibberellic Acid (GA_3) in Magenta GA^7 vessels (Table 3).

Table 1. The content of Murashige and Skoog (1962) basic nutrient medium

Components		Concentrations (mg/L)
Macroelements	NH_4NO_3	1650.00
	KNO_3	1900.00
	$CaCI_2.2H_2O$	440.00
	$MgSO_4.7H_2O$	370.00
	KH_2PO_4	170.00
Microelements	KI	0.83
	H_3BO_3	6.20
	$MnSO_4.4H_2O$	22.30
	$ZnSO_4.7H_2O$	8.60
	$Na_2MoO_4.2H_2O$	0.25
	$FeSO_4.7H_2O$	27.85
	$CoCl_2.6H_2O$	0.025
	$CuSO_4.5H_2O$	0.025
	$Na_2EDTA.2H_2O$	37.25
Vitamins	Myo-Inositol	100.00
	Nicotinic Acid	0.50
	Pyrotinic Acid	0.50
	Thiamine-HCI	0.10
	Glycine	2.00

The pH of the culture media was adjusted to 5.7±1 before the autoclaving (1.2 atmospheric pressure, 120°C for 20 min). All cultures were incubated under 16 h light photoperiod. After 8 weeks of culture, the experiment was terminated and the data were recorded for shoot regeneration and analyzed.

The regenerated shoots were rooted on agar-solidified MS rooting medium containing 0.25 mg/L indole-3-butyric acid (IBA) in Magenta GA^7 vessels. After 4 weeks of culture, the agar medium was removed carefully from the rooted plantlets without damaging the roots by washing under running tap water. Thereafter, the plants were transferred to an aquarium containing tap water and sand for acclimatization (23°C with 16 h light photoperiod).

The experiment was replicated 6 times. Statistical analysis was performed as One Way ANOVA using SPSS 16 for Windows and post hoc tests were performed using Duncan. Data given in percentages were subjected to arcsine transformation (Snedecor and Cocharan, 1967) before statistical analysis

3. Results and Discussion

For *in vitro* shoot regeneration, shoot and nodal explants of *R. rotundifolia* were cultured on MS medium containing combinations of 0.05-1.25 mg/L KIN and 0.25 mg/L GA_3. At the end of two weeks, the first shoot formations were started to be observed. In the fifth week, red colorations were observed on the ends and leaves of some regenerated shoots. After eight weeks, *in vitro* shoot regeneration from shoot tip (Figure 1a) and nodal (Figure 1b) explants of *R. rotundifolia* was successfully achieved and then variance analysis was performed for shoot regeneration frequency, the mean number of shoots per explant, shoot length, and root regeneration frequency (Table 2). Similarly, the use of shoot tip or nodal explants for multiple shoot regeneration has been reported for some aquatic plants such as *Mentha viridis* L. (Raja and Arockiasamy, 2008), *Veronica anagallis-aquatica* L. (Shahzada et. al., 2011), *Ludwigia palustris* (L.) Ell. (Fontanili et al., 2015) *Ceratophyllum demersum* L. (Karatas et al., 2015; Emsen et al., 2016; Dogan et al., 2017) and *Pogostemon erectus* (Dalzell) Kuntze (Dogan et al., 2016).

As seen in Table 2, while there was no statistically significant difference in terms of root regeneration frequency, there was a statistically significant difference in shoot regeneration frequency, the mean number of shoots per explant and shoot length for shoot tip explants ($p<0.05$). In the analysis of variance of the nodal explants, a statistically significant difference was not detected for root regeneration frequency and shoot regeneration frequency, but a statistically significant difference was found in the 99% confidence interval for mean number of shoots per explant and shoot length ($p<0.01$). Duncan test was performed to determine the significance level of these differences (Table 3).

The shoot regeneration frequency was recorded between 83.33-100.00% for both explants (Table 3). In the shoot tip explants, 100% shoot regeneration was observed in MS medium containing 0.05-1.00 mg/L KIN + 0.25 mg/L GA_3. The highest shoot regeneration frequency (100%) in the nodal explant was obtained in the MS medium containing 0.75, 1.00 and 1.25 mg/L KIN + 0.25 mg/L GA_3. Similarly, Manik et al. (2012) reported high shoot regeneration frequency (78-92%) from shoot tip explants of *Mentha piperata* cultured on MS medium containing 0.75-2.0 mg/L KIN.

Mean number of shoots per explants of shoot tip and nodal explants was recorded 13.17-38.66 and 13.44-30.77, respectively (Table 3). The maximum number of 38.66 and 30.77 shoots per explant were obtained from shoot tip and nodal explants on MS medium supplemented with

Figure 1. *In vitro* shoot regeneration of *R. rotundifolia*. Multiple shoot regeneration from shoot tip (a) and nodal (b) explants on MS medium containing 0.25 mg/L KIN + 0.25 mg/L GA_3 after 8 weeks of culture

Table 2. Analysis of variance of shoot tip and nodal explants of *R. rotundifolia* in MS medium containing different KIN and GA_3

Source of variance	Degree of freedom	Shoot regeneration frequency (%)		Mean number of shoots per explant		Shoot length (cm)		Root regeneration frequency (%)	
		Mean square	*F* value	Mean Square	*F* value	Mean Square	*F* value	Mean Square	*F* value
Shoot tip									
Medium	5	138.89	4.00*	220.26	3.19*	0.26	4.57 *	145.83	2.10 ^{is}
Error	12	34.72	-	69.13	-	0.06	-	69.44	-
General Total	17	-	-	-	-	-	-	-	-
* Significant at p <0.05 level; is: Insignificant									
Nodal									
Medium	5	138.89	1.333 ^{is}	121.34	7.44**	0.20	5.42**	55.56	0.80 ^{is}
Error	12	104.17	-	16.30	-	0.04	-	69.44	-
General Total	17	-	-	-	-	-	-	-	-
**Significant at p <0.01 level; is: Insignificant									

Table 3. Effect of different combinations of KIN and GA_3 on multiple shoot regeneration from shoot tip and nodal explants of *R. rotundifolia* after eight weeks of culture

Plant growth regulators (mg/L)		Shoot regeneration frequency (%)		Mean number of shoots per explant		Shoot length (cm)		Root regeneration frequency (%)	
KIN	**GA_3**	**Shoot tip***	**Nodal ^{is}**	**Shoot tip***	**Nodal****	**Shoot tip***	**Nodal****	**Shoot tip***	**Nodal ^{is}**
0.05	**0.25**	100.00^{a}	83.33	18.83^{b}	16.55^{bc}	1.16^{b}	1.28^{abc}	100.00^{a}	100.00
0.25	**0.25**	100.00^{a}	91.67	38.66^{a}	30.77^{a}	1.31^{b}	1.39^{abc}	100.00^{a}	100.00
0.50	**0.25**	100.00^{a}	91.67	24.33^{ab}	25.92^{ab}	1.46^{ab}	1.79^{a}	100.00^{a}	100.00
0.75	**0.25**	100.00^{a}	100.00	23.69^{ab}	21.25^{abc}	1.87^{a}	1.67^{ab}	100.00^{a}	100.00
1.00	**0.25**	100.00^{a}	100.00	20.61^{b}	18.64^{bc}	1.49^{ab}	1.24^{bc}	91.67^{ab}	91.67
1.25	**0.25**	83.33^{b}	100.00	13.17^{b}	13.44^{c}	1.03^{b}	1.13^{c}	83.33^{b}	91.67

*Values followed by different small letters in the same column differ significantly at p < 0.01
**Values followed by different small letters in the same column differ significantly at p < 0.05
is: Insignificant

0.25 mg/L KIN + 0.25 mg/L GA_3, respectively. On the other hand, minimum number of shoots per explants for both explant types was determined on MS medium with 1.25 mg/L KIN + 0.25 mg/L GA_3. The results revealed that the increase in the KIN + GA_3 combination in the MS medium had a negative effect on the number of shoots per explant. These results are in line with Bhattacharyya and Bhattacharya (2001) who cultured the shoot tip explants of *Phyllanthus amarus* Schum. &Thom. on MS medium containing 0.05-5.0 mg/L KIN and reported a decrease in the number of shoots per explant with an increase in KIN ratio. Banerjee and Shrivastava, (2008) obtained the minimum number of 8 ± 1.86 shoots per explant of *B. monnieri* cultured on MS medium with 2.0 mg/L KIN.

Shoot lengths ranged from 1.03 to 1.87 cm for the shoot tip explant and from 1.13 to 1.79 cm for the nodal explant (Table 3). The highest shoot length of shoot tip (1.87 cm) was obtained on MS medium containing 0.75 mg/L KIN + 0.25 mg/L GA_3, whereas the highest shoot length of nodal explant (1.79 cm) was obtained on MS medium supplemented with 0.50 mg/L KIN + 0.25 mg/L GA_3. In both explant types, the shortest shoots were determined in MS medium containing 1.25 mg/L KIN + 0.25 mg/L GA_3. Kaviani et al. (2013) reported that the longest shoots (1.20 cm) were obtained from the shoot tip explants of Matthiola incana on MS medium supplemented with 2 mg/L KIN.

Root occurrences with KIN effect were recorded on *in vitro* propagation medium. For both types of explants, 100% root formation was obtained on MS medium containing 0.05-0.75 mg/L KIN + 0.25 mg/L GA_3. It has been determined that high KIN doses have an adverse effect on root formation.

In spite of root formation on the propagation medium, *in vitro* rooting studies of regenerated shoots were carried out in MS medium containing 0.25 mg/L IBA for four weeks. *In vitro* rooted plantlets were successfully acclimatized to external conditions in the aquarium environment. Similarly, successful acclimatization of *in vitro* regenerated aquatic plants had been reported for *Cryptocoryne wendtii* and *Cryptocoryne beckettii* (Stanly et al., 2011), *A. sessilis* (Gnanaraj et al., 2011), and *C. demersum* (Dogan et al., 2015).

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