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In vitro high frequency axillary shoot regeneration of Roundleaf toothcup-*Rotala rotundifolia* [(BuchHam. ex Roxb) Koehne]

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Abstract

Roundleaf toothcup-*Rotala rotundifolia* [(BuchHam. ex Roxb) Koehne] is an amphibious aquatic plant of Southeast Asia used as medicinal and ornamental purpose. The shoot tip explant of *R. rotundifolia* was aseptically excised and cultured on MS (Murashige and Skoog) medium enriched with 0.05-1.60 mg/l Thidiazuron (TDZ) that resulted in callus induction without any shoot induction after 8 weeks of inoculation. Thereafter, the explants were transferred to MSO for further 4 weeks that resulted in 100% shoot regeneration frequency, 29.33-59.67 shoots per explant alongwith mean shoot length ranged 0.20-1.87 cm. Maximum number of 59.67 shoot alongwith mean longer shoots (1.87 cm) were achieved on MS medium with 0.05 mg/l TDZ. Further increase of TDZ concentration in the culture medium inhibited the both shoots per explant and shoot length. Soots with ≈1.0 cm length were successfully rooted on medium enriched with 0.25 mg/l Indole-3-butyric Acid (IBA) that was followed by successfiul adaptation of plantlets in the aquariums filled with tap water. The protocol developed can be employed for future biotechnological studies like genetic transformation.

Keywords: Axillary shoots, In vitro, Shoot tip, Thidiazuron

INTRODUCTION

Roundleaf toothcup [*Rotala rotundifolia* (BuchHam. ex Roxb) Koehne] is submerged aquatic and amphibious plant of tropical and sub-tropical regions. The plant mainly belongs to Southeast Asia that extends from Eastern India to Japan [1-3]. It can be grwon in shallow water as obligate aquatics or in marshy lands as semi-aquatics or terrestrial plant. The stem of plant is succulent and abundantly branched with pinkish to purplish colour. Leaves are either sessile or with shorter petioles. Flowers are rose colored and bloom in spring to early summer time [4].

Roundleaf toothcup is an an important aquarium plant due to its relatively attractive leaves with reddish appearnace which mainly depends on the availibility of light. It is one of the important aquatic plant in Turkey used in aquarium industry as an ornamental plant [5]. The plant is considered as an important medicinal plant for curing diseases and disorders like piles, menstrual cramps, gonorrhea frheumatism, cirrhosis ascetic fluids uruncle, carbuncle and arthralgia [2,5-6]. It also possesses anti pyretic, antiswelling, detoxication, diuresis properties and antioxidant activity [7]. A study on *R. rotundifolia* revealed the suppression of HBV surface antigen (HBsAg) production in HepA2 cells [8].

Although, Roundleaf toothcup is well established ornamental and medicinal plant, its propagation is mainly through vegetaive propagation. The plant has vast potential and there is need to propagate through plant tissue culture techniques. In recent years, this plant has been propagated under in vitro condiitons using different explants and growth variants [5, 9]. There is still need to focus more on developing more reliable and repaeatble protocols for mass propagation of *R. rotundifolia* for further studies like application of biotechnological tools like genetic transformation, secondary metabolites production and pharmaceutical studies. Keeping in view, the study preseneted was designed to analyze the potential of shoot tip explant of *R. rotundifolia* on in vitro shoot regenration using TDZ.

MATERIAL AND METHODS

In vitro rooted plantlets of *R. rotundifolia* (Karataş et al 2014) were used for achieving required number of explants. The apical/shoot tip explants were excised and cultured directly on MS [10] medium enriched with sucrose (30 g) and phytagel (0.25%) was used for medium solidification. The MS medium was also supplemented with TDZ (0.05, 0.10, 0.20, 0.40, 0.80 and 1.60 mg/L) concentrations. The pH of the medium was calibrated to ≈ 5.8 after adding TDZ and sterilization of the medium was done in autoclave adjusted at 120°C for 21 min with 118 kPa atmospheric pressure.

The experiment was replicated thrice with eqaul number of shoot tip explants per replicate and cultured under 16 h light photoperiod by using cool flourescent lamps. After 8 weeks of initial culture, all explants were shifted to MSO medium (no TDZ or any other growth variant) for further 4 weeks. The data regarding shoot regeneration frequency (%), number of shoots per explant and average shoot length were recorded after total of 12 weeks of culture. Regenerated shoots after 12 weeks of culture were transferred to rooting medium (Magenta GA7 vessels) containing 0.25 mg/l IBA. After 2 weeks of culture, adhering gel was removed and plantlets were washed under tap water followed by acclimatization in aquariums filled with tap water. Statistical

analysis of data taken was performed using SPSS17 for Windows (One Way ANOVA). Post hoc tests performed using DMRT test was used for significance level. Arcsine transformation was used for given data (percentages) prior to statistical analysis [11].

RESULTS AND DISCUSSION

Shoot tip explant of *R. rotundifolia* was used for multiple shoot induction under in vitro conditions. Shoot tip or apical meristem explant is very potent for multiple shoot induction of many aquatic plants irrespective of growth variants used [12-19]. On the other hand, TDZ is also very useful, powerful and potent cytokinin type hormone for multiple shoot induction and successfully reported for other aquatic plants [8,12, 20, 22-25].

The shoot tip explants inoculated on MS medium enriched with varable TDZ concentrations resulted in callus induction with multiple shoot buds but with no shoot induction upto 8 weeks of culture. It is well established that exposure of explants to TDZ for longer time may lead to callus induction or low shoot induction with stunted grwoth. [20-21, 24-25] due to carry over effects of TDZ [26]. In another study on *R. rotundifolia*, leaf explants cultured on 6-Benzylaminopurine (BA) generated large number of shoots buds but with no shoot induction and obtained multiple shoots after transfer to mew medium containg BA- GA_3 (Gibberellic acid) [5]. Similarly, transfer of TDZ-exposed explants to MS medium without any growth variants exert positive effects and tend to shoot induction in other plants [5, 27, 28].

In this study, transfer of calli from all culture media to phytagel solidified MSO medium without any growth variants showed positive effects and resulted in well developed shoot buds followed by multiple shoot induction with the passage of time. Similarly, Karataş et al. [5] gained multiple shoots after transferring explants to medium containing BA-GA_3. Contraraily, Doğan [9] achieved direct regeneration from shoot tip and nodal explants culturing on medium containing Kinetin-GA_3. The possible reason might be the provision of GA_3 from the start of the experiment which helped to induce multiple shoots directly. Similarly, positive bearings of GA_3 or transfer to new medium on shoot induction has also been reported by Hoque et al. [29] and Purkayastha et al. [30].

After 4 weeks of further culture on MSO, shoot regenration frequency, shoots per explant and avearge shoot length (cm) were recorded. As shoot regenration frequency was recorded 100%, therefore data taken was not subjected to statistical analysis. Higher shoot regeneration frequency using shoot tip and nodal explants [9] and leaf [5] has been reported for *R. rotundifolia*. On the other hand, data pertaining to shoots per explant ($p < 0.01$) and shoot length ($p < 0.01$) subjected to statistical analysis was significant (Table 1) and this siginificance level was determined by Duncan test (Table 2).

Data pertaining to number of shoots per explants significantly showed the impact of TDZ concentration on number of shoots per explants and mean shoot length in similar way. Shoots per explants ranged between 29.33-59.67 with mean shoot length range of 0.20-1.87 cm. It was also noteworthy that shoot tip explants required very low concentration of TDZ (0.05 mg/l) for maximum number of shoots and longer shoots which were recorded 59.67 and 1.87 cm respectively. Successful application of TDZ with variable effects on number of shoot per explants has been reported for other aquatic plants like *Ipomoea aquatica* [31] ,[*Trapa japonica* Flerov [29], *Spartina alterniflora* [32], Lemna gibba var. Hurfeish and *Spirodela punctata* [33], *Ludwigia repens* [Öztürk et al 2004], *Bacopa monnieri* [21, 34] and *Pistia stratiotes* [35].

Results further revealed that increased TDZ concentration was detrimental for both number of shoots and shoot length [21]. Almost half number of shoots were recorded at higher TDZ concentration (0.80-1.60 mg/l) comapred to 0.05 mg/l TDZ (Table 2). However, shoot length was affected more due to exposure of explants to higher concentrations of TDZ (0.40-1.60 mg/l) which resulted in 3-8 fold stunted shoots comapred to shoots achieved from medium having 0.05 mg/l TDZ. Contrarily, positive bearings of increased TDZ concentration on shoot length has been reported by Vijaykumar et al. [36].

There was no direct rooting on in vitro regenerated shoots and therefore subjected to rooting experiment using 0.25 mg/l IBA. Contrarily, Karataş et al. [5] reported direct in vitro regeneration of plantlets of *R. rotundifolia* using BA-GA_3 in the experiment. Stunted shoots (≤ 1.0 cm) failed to induce rooting on rooting medium. However, shoots taken above 1.0 cm were rooted successfully on MS medium containing 0.25 mg/l IBA [9]. Rooted plantlets were acclimatized successfully in the aquariums containing tap water. Earlier, Karataş et al. [5] reported the acclimatization fo *R. rotundifolia* plantlets in the water with pH range of 5-9.

Table 1. Analysis of variance for shoot tip explants of R. rotundifolia cultured on MS medium containing different concentrations of TDZ

Source of Variance	df	Number of Shoots per Explant		Shoot length (cm)	
		Mean Square	F value	Mean Square	F value
Medium	4	368.86	21.21**	1.82	363.91**
Error	10	17.39	-	0.005	-
General Total	14	-	-	-	-

**Significant at $p < 0.01$ level

Table 2: Effect of different Concnetrations of TDZ on multiple shoot regeneration from shoot tip explants of R. rotundifolia

TDZ (mg/l)	Shoot Regeneration Frequency (%)	Number of Shoots per Explant	Shoot length (cm)
0.05	100ns	59.67a	1.87a
0.10	100	36.67bc	1.67b
0.20	100	44.00b	1.60b
0.40	100	40.00b	0.50c
0.80	100	29.33c	0.23d
1.60	100	30.67c	0.20d

*Values followed by different small letters in the same column differ significantly at $p < 0.01$

ns: nonsignificant

The study presents the successful use of shoot tip explant for multiple axillary shoot induction of *R. rotundifolia* under in vitro conditions using TDZ. It is concluded that lower TDZ concentration is excellent for multiple shoot induction but longer exposure to lower TDZ concnetration is also detrimental for shoot induction and shoot length. This protocol can be used for the application of other biotechnological tools for its improvement.

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