

## ADVENTITIOUS SHOOT REGENERATION OF ROUNDEAF TOOTH CUP-*ROOTALA ROTUNDIFOLIA* [(BUCH-HAM. EX ROXB) KOEHNE]

M. Karatas, M. Aasim and M. Çiftçio lu

Department of Biology, Kamil Ozdag Faculty of Science, Karamanoglu Mehmetbey University, Yunus Emre Campus, 70200, Karaman, Turkey.  
Corresponding author: mshazim@gmail.com

### ABSTRACT

Roundleaf toothcup - *Rotala rotundifolia* [(Buch-Ham. ex Roxb) Koehne] is an important aquatic, ornamental and medicinal plant of family Lythraceae. Present study was designed to propagate adventitious shoots regeneration from leaf explants cultured on MS medium supplemented with 5 levels of 0.25-2.00 mg/L BA. Maximum shoot regeneration frequency (53.33 percent) and mean number of 17.06 shoots per explant was recorded on MS medium supplemented with 1.0 mg/L BA. Multiple shoots buds were also induced on all culture mediums most of which were either slow to grow or failed to convert in to shoots. Transfer of explants to 0.20 mg/L GA<sub>3</sub> promoted the shoot regeneration frequency to 100 percent on MS medium containing 1.00 mg/L BA and increased mean number of shoots (24.0) on MS medium supplemented with 0.25 mg/L BA-0.20 mg/L GA<sub>3</sub>. The developing shoots rooted in the shoot elongation medium showed positive effects of BA- 0.20 mg/L GA<sub>3</sub> combinations. Thereafter, the developing plantlets were acclimatised in water using pH level of 4.0-10 (7 levels). All plants at pH 4.0 developed necrosis and died; whereas, rest of the plants showed variable growth at other pH levels. A comparison among the pH treatments suggested slightly acidic to alkaline medium (pH 6-9) was more appropriate for the acclimatisation of *in vitro* regenerated plants without having negative effects on plant growth and development.

**Key words:** Acclimatisation, Adventitious, Aquatic, *In vitro*, Shoots, Regeneration.

### INTRODUCTION

Roundleaf toothcup-*Rotala rotundifolia* [(Buch-Ham. ex Roxb) Koehne] is an aquatic, amphibious, tropical and sub-tropical plant that has considerable phenotypic plasticity and grow as obligate aquatics in shallow water, and semi-aquatics or terrestrial habitats like marshy lands. It is native to south and southeast Asia extending from Eastern India to Japan (Cook, 1996).

*Rotala* has soft, succulent, dark pink to purplish stems that branch abundantly, with prostrate, creeping growth form. Aerial leaves are round to broadly ovoid and are either sessile or with short petioles. Whereas, submersed leaves are more linear to elongate-elliptical in outline, and appear distinctly four-ranked along submersed stems. It produces abundant rose colored flowers at the tips of aerial stems in dense racemose spikes that bloom during spring into early summer followed by development of small capsuled fruits that split to release seeds (Cook 1979).

Roundleaf toothcup is used as an aquarium plant that can grow in medium light but needs relatively high light to show its true colors. The plant has also medicinal properties. The ethanol extract of *R. rotundifolia* also showed the promising effect in suppression of HBV surface antigen (HBsAg) production in HepA2 cells (Zhang *et al.* 2011). It is well known as anti pyretic, detoxication, anti swelling and diuresis properties and also

useful in treatments of cirrhosis ascetic fluids, gonorrhoea, menstrual cramps and piles in the south of China (Anonymous, 2004). Roundleaf toothcup is not native plant of Turkey. It is a popular plant that is imported for aquarium trade only. It is difficult to come across reliable and comprehensive statistics about aquarium plants sector in Turkey. Regarding the supply issue, all aquarium plants including roundleaf toothcup are imported. Annual imports in the Turkey, associated with the ornamental fish trade, sale of water tanks, live fish and ornamental plants, is estimated to be about 137.1 million Euro (Atar, 2012).

Besides its medicinal properties, the plant is mainly used as aquarium plant in Turkey. The main objective of this study is to develop a reliable and repeatable *in vitro* regeneration protocol followed by acclimatization locally to spread its use as aquarium plant in Turkey, so as to prohibit introduction of exotic microflora and fauna with its import in Turkish waters and unrestricted safe availability of the plant for future pharmaceutical studies

### MATERIALS AND METHODS

*R. rotundifolia* plants were obtained from local traders of aquatic plants at Karaman province of Turkey. Many plant twigs with 4-5 nodes with attached leaves were washed under tap water for 5 minutes. Thereafter,

these were surface sterilized with 40 percent diluted H<sub>2</sub>O<sub>2</sub> (v/v) for 12 min. followed by 3x5 min rinsing with bidistilled sterilized water. The leaf explants were detached from twigs under aseptic conditions and cultured on MS (Murashige and Skoog, 1962) medium supplemented with 3.0 percent sucrose, solidified with 0.65 percent agar for two weeks to evaluate the extent of sterilisation.

All leaves that did not show sign of any fungal or bacterial contaminations were cultured on MS medium containing 0.25, 0.50, 1.0, 1.50 and 2.0 mg/L BA supplemented with 3 percent sucrose solidified with 0.65 percent agar in Magenta GA7 vessels for regeneration. The pH of all media was adjusted to 5.8±0.1 before autoclaving (118 kPa atmospheric pressure, 120°C for 21 min). All cultures were incubated under 16 h light photoperiod (6000 lux). After 8 weeks of culture, *in vitro* regenerated shoots were isolated and the data were scored. Thereafter, explants with developing shoots were cultured on MS shoot elongation and maturation medium containing 0.25, 0.50, 1.0, 1.50, 2.0 mg/L BA - 0.20 mg/L GA<sub>3</sub>. After 6 weeks of culture, data regarding frequency of shoot regeneration, number of shoots per explant and shoot length were scored again and *in vitro* regenerated shoots were transferred to glass jars for acclimatisation at differential (4, 5, 6, 7, 8,9,10) pH levels.

Each treatment contained 8 explants, replicated 6 times (8 x 6 = 48 explants) and repeated twice. Statistical analysis was done with the help of One Way ANOVA using SPSS17 for Windows and the post hoc tests were performed using Duncans Multiple Range Test. Care was taken to arcsine transform all data given in percentages before statistical analysis (Snedecor and Cochran, 1967) before statistical analysis.

## RESULTS

Callus induction from leaf explants started within 2 weeks of culture followed by shoot buds initiation within four weeks on all explants irrespective of BA concentration (Figure 1a). It was noted that all explants had high regeneration potential and continued to develop multiple number of shoot buds (Figure 1b). These shoot buds started to sprout and after 6 weeks of culture, multiple shoots were recorded and were further allowed to regenerate for four more weeks but regenerated shoots did not gain length properly (Figure 1c). Therefore, all of the regenerated explants were cultured on differential concentrations of 0.25 to 2.00 mg/L BA -0.20 mg/l GA<sub>3</sub> (5 combinations) for six weeks (Figure 1d) for shoot initiation and elongation. Data regarding frequency (percent) of shoot regeneration, mean number of shoots per explant and mean shoot length were recorded before (after 10 weeks) and after subculture to BA-GA<sub>3</sub> mediums (after 16 weeks).

The results showed that frequency (percent) of shoot regeneration was in range of 26.67-53.33 percent and had statistically insignificant differences among them (Table 1). Maximum shoot regeneration of 53.33 percent was noted on MS medium containing 1.00 mg/L BA. Subculturing of explants to their respective cultures containing 0.20 mg/L GA<sub>3</sub> exerted positive effects on shoot regeneration frequency that ranged 73.33-100.0 (Table 1). Maximum shoot regeneration frequency (100 percent) was recorded on MS medium containing 1.00 mg/L BA.

Differences among mean number of shoots per explant were statistically significant after 10 weeks of culture and ranged 6.73-17.06 (Table 2). Maximum number of shoots per explant (17.06) was recorded on MS medium supplemented with 1.00 mg/L BA. Whereas, minimum number of 6.73 shoots per explant was recorded on MS medium containing 0.25 mg/L BA. After transfer of explants to BA-GA<sub>3</sub> mediums, 8.17-24.00 more shoots per explant were obtained on leaf explants after transfer to GA<sub>3</sub> containing medium. Minimum number of shoots per explants were recorded on MS medium supplemented with 1.00 mg/L BA-0.20 after transfer to GA<sub>3</sub> containing medium. Whereas, maximum number of 18.67 shoots per explants were obtained from MS medium containing 2.00 mg/L BA-0.20 mg/L GA<sub>3</sub>. Overall, 1.5-4 fold more shoots per explants were obtained after transfer to 0.25-2.00 mg/L BA+0.20 mg/L GA<sub>3</sub> culture mediums. (Table 2). However, total number of shoots per explant was found insignificant that ranged 25.23-30.73.

Data regarding mean shoot length taken before subculture to BA-GA<sub>3</sub> mediums ranged 0.59-1.37 cm with maximum shoot length (1.37 cm) was recorded on MS medium containing 0.25 mg/L BA (Table 3). An increase in BA concentration resulted in decreased shoot length and minimum shoot length was recorded on MS medium supplemented with 2.00 mg/l BA. Transfer of explants to mediums containing GA<sub>3</sub> resulted in enhanced shoot length and ranged 2.69-4.01 cm. Maximum longer shoots were obtained on MS medium with 0.25 mg/l BA-0.20 mg/L GA<sub>3</sub> followed by decreased with increased BA concentration. (Table 3).

Regenerated shoots rooted directly (Figure 2a) in the culture medium and no experiment for rooting was carried out separately. For acclimatisation, 5 plants with average shoot length of 5 cm were directly transferred to glass jars containing water at pH level of 4.0-10.0 (7 levels) in growth room at 23±1°C with 16h light (6000 lux) photoperiod for 30 days. All plants at pH 4.0 turned brown due to necrosis within 1 week and died in 2 weeks time. Whereas, plants at pH 5.0 slowed down the growth. The plants at pH 6.0 and 7.0 gained average length of 7.2 cm within one month and their leaves did not show any signs of chlorosis or necrosis. Whereas, at pH 8.0 and 9.0, plants responded well and gained maximum length

achieving mean shoot length of 9.0 cm (Figure 2b). Again pH 10.0, was unsuitable with partial development of chlorosis and stunted shoot length of 5.8 cm.

**Table 1. Frequency (percent) of shoot regeneration from leaf explant of Roundleaf toothcup at different BA concentrations.**

BA (mg/L)	Shoot Regeneration Frequency (percent)	BA-GA <sub>3</sub> (mg/L)	Shoot Regeneration Frequency (percent)
0.25	26,67 <sup>ns</sup>	0.25-0.20	73.33 <sup>b</sup>
0.50	33,33	0.50-0.20	73.33 <sup>b</sup>
1.00	53,33	1.00-0.20	100.00 <sup>a</sup>
1.50	46,67	1.50-0.20	73.33 <sup>b</sup>
2.00	33,33	2.00-0.20	73.33 <sup>b</sup>

Means followed by different small letters within columns are significantly different using Duncan test at P<0.001

**Table 2. Mean number of shoots per explant from leaf explant of Roundleaf toothcup at different BA concentrations**

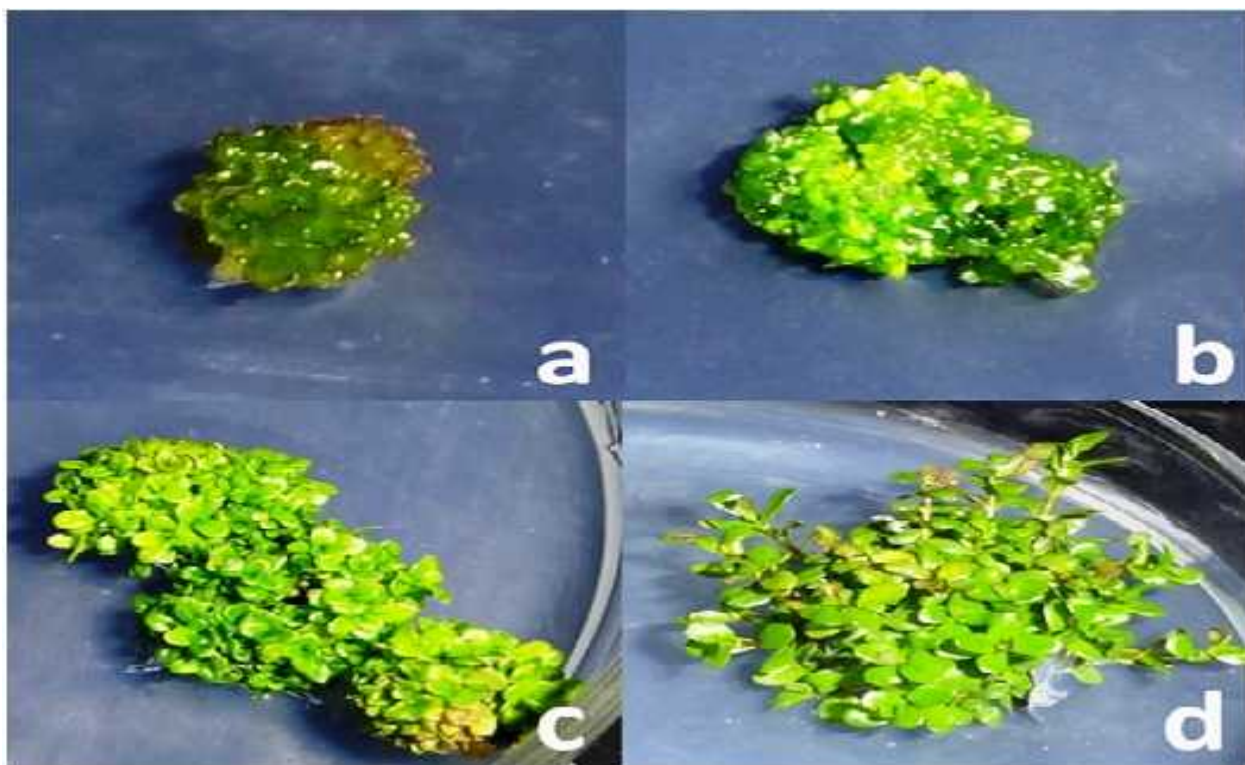
BA (mg/L)	Shoots per explant	BA-GA <sub>3</sub> (mg/L)	Shoots per explant	Total no. of shoots
0.25	6.73 <sup>ns</sup>	0.25-0.20	24.00 <sup>a</sup>	30.73 <sup>ns</sup>
0.50	10.20	0.50-0.20	16.00 <sup>b</sup>	26.20
1.00	17.06	1.00-0.20	8.17 <sup>c</sup>	25.23
1.50	14.73	1.50-0.20	11.67 <sup>c</sup>	26.40
2.00	7.80	2.00-0.20	18.67 <sup>b</sup>	26.47

Means followed by different small letters within columns are significantly different using Duncan test at P<0.001

**Table 3. Mean shoot length from leaf explant of Roundleaf toothcup at different BA concentrations**

BA (mg/L)	Shoot length (cm)	BA-GA <sub>3</sub> (mg/L)	Shoot length (cm)
0.25	1,37 <sup>a</sup>	0.25-0.20	4.01 <sup>a</sup>
0.50	1,32 <sup>ab</sup>	0.50-0.20	3.77 <sup>a</sup>
1.00	1,06 <sup>bc</sup>	1.00-0.20	2.93 <sup>b</sup>
1.50	0,89 <sup>c</sup>	1.50-0.20	2.69 <sup>b</sup>
2.00	0,59 <sup>d</sup>	2.00-0.20	3.03 <sup>b</sup>

Means followed by different small letters within columns are significantly different using Duncan test at P<0.001.



**Figure 1. Adventitious shoot regeneration on leaf explant of *R. rotundifolia* (a) shoot bud inductions (b) shoot buds and shoot induction (c, d) shoot induction on GA<sub>3</sub> containing medium**



**Figure 2. Rooting and acclimatisation of *R. rotundifolia* (a) direct rooting of regenerated plantlets (b) developed plants at pH 8.0 after one month of acclimatisation.**

## DISCUSSION

The study presents efficient adventitious shoot regeneration from leaf explant of Roundleaf toothcup, an important aquatic and medicinal plant. Leaf explant has been used for shoot regeneration in other aquatic plants like *Nymphaea* (Jenks *et al.* 1990), *Hygrophila auriculata* (Panigrahi *et al.* 2006), *Rotala macaranda* (Umlu, 2009), *Bacopa monnieri* (Karatas *et al.* 2013a) and *Hygrophylla polysperma* (Karatas *et al.* 2013b). Leaf explants responded well to growth variants and callus induction followed by shoot buds initiation. Callus induction from the leaf explant has been reported in other aquatic plants like water lettuce (Yong *et al.* 2008) and *B. monnieri* (Karatas *et al.* 2013). It was also noted that growth variants in the culture medium were more supportive for shoot buds induction but failed to develop properly in the shoots.

Results further showed that BA concentrations exerted variable effects on shoot induction. Multiple shoot buds were observed on all culture media but their conversion into shoots was very low that might be due to carry over effects of BA. The results are in partial agreement with Vijaykumar *et al.* (2010), who reported 30.0-95.0 percent shoot regeneration frequency of *B. monnieri* cultured on BA. Whereas, Karatas *et al.* (2013) reported 100.0 percent shoot regeneration on leaf explant of *B. monnieri* using various concentrations of BA-NAA. Similarly, Xu *et al.* (2009) reported 70.83- 90.48 percent shoot regeneration frequency at 0.5 mg/L BA containing medium on different explants of mat rush varieties. To enhance shoot regeneration frequency, the explants were cultured to their respective culture medium with extra of 0.20 mg/l GA<sub>3</sub> that ultimately released dormancy of shoot buds and resulted in shoot initiation and proliferation from shoot buds in variable frequency.

Results on mean number of shoots showed the carry over effects of BA used singly on shoot initiation as to shoots properly. Results suggested that explants needed subculture and addition of 0.20 mg/l GA<sub>3</sub> in the respective regeneration medium resulted in shoot proliferation. Similarly, Tiwari *et al.* (2001) also reported positive effects of subculturing on number of shoots per explants in *B. monnieri*. Bhagwat *et al.* (1996) exposed nodal segments of Cassava to 0.11-0.22 μM TDZ for 6-8 days followed by culturing of explants to medium containing 2.2 μM TDZ and 1.6 μM GA<sub>3</sub> and recorded continuous growth during culture with BA and GA<sub>3</sub>.

Results on mean shoot length showed clear negative effects of BA concentration as it decreased with increase in BA concentration. This negative phenomenon of increased concentration of BA and other cytokinins on shoot length has been reported in other plants like Hungarian vetch (Sahin-Demirbag *et al.* 2008), narbon vetch (Kendir *et al.* 2009) and Chickpea (Aasim *et al.* 2011, 2013). Contrarily, Vijaykumar *et al.* (2010) reported increase in shoot length with increase in BA concentration in the culture medium using leaf explant of *B. monnieri*. On the other hand, culture to GA<sub>3</sub> containing medium exerted positive effects on mean shoot length. Hoque *et al.* (2006) reported stimulation of the axillary shoot elongation of water chestnut by the addition of 0.5 mg/L GA<sub>3</sub> with 2,4-D and BA combinations.

Acclimatisation of aquatic plants is an important step for *in vitro* regenerated plants and has been reported in other aquatic plants like *Ludwigia repens* (Öztürk *et al.* 2004) and *B. monnieri* (Karatas *et al.* 2013). Regenerated plantlets tested for acclimatisation under *in vitro* conditions at various pH levels. The results suggested that pH 4.0 was detrimental for plant growth and all other plants died within 2 weeks. Whereas, visible effects of nutrient deficiency, chlorosis and necrosis at leaf margins at pH 5.0 also indicated unsuitability of this acidic pH. This suggested likely deficiency of nitrogen and other micro and macro nutrients elements due to acidic pH and these plantlets did not gain length. Results further showed that plants required slight acidic (pH 6), neutral (pH 7) to slight alkaline (pH 8 and 9) medium for better acclimatisation and development of *Rotala* plants. Contrarily, Karatas *et al.* (2013) reported no negative effects of pH levels on acclimatisation of aquatic plant *Hygrophila polysperma* at pH 4.0-10.0.

The establishment of a successful regeneration and acclimatization protocol of Roundleaf toothcup provides an opportunity for the application of biotechnological tools to multiply the plant for multiple uses as medicinal and an ornamental plant. The protocol also provides a base for the extraction of medicinally important compounds from this important aquatic plant.

**Acknowledgment:** The Authors acknowledged the Financial assistance by the Karamano lu Mehmetbey

University through the Scientific Research Project commission (BAP) for funding project number 32-M-12.

## REFERENCES

- Aasim, M., S. Day, F. Rezai, M. Hajyzadeh, S.T. Mahmud and S. Ozcan (2011). *In vitro* shoot regeneration from preconditioned explants of chickpea (*Cicer arietinum* L.) cv. Gokce. Afr J. Biotechnol. 10, 2020-2023
- Aasim, M., S. Day, F. Rezai, and M. Hajyzadeh (2013). Multiple shoot regeneration of plumular apices of chickpea. Turk J Agric. For. 37, 33-39
- Anonymous. (2004). Dictionary of Chinese Materia Medica. Shanghai Scientific and Technical Publishers, Shanghai, pp. 531.
- Atar, H.H (2012). Current Status of Marketing and Seafood Trade in Turkey. (<http://www.eurofish.dk/images/stories/files/Turkey/Cesme/3-HA.pdf>)
- Bhagwat, B.B., L.G.F. Vieiral and L.R. Erickson (1996). Stimulation of *in vitro* shoot proliferation from nodal explants of cassava by thidiazuron, benzyladenine and gibberellic acid. Plant Cell Tiss. Org. Cult. 46, 1-7
- Cook, C.D.K. (1979). A revision of the genus *Rotala* (Lythraceae). *Boissiera* 29: 1-155.
- Cook, C.D.K., (1996). Aquatic Plant Book. SPB Academic Publishing, Amsterdam/New York. 60 p
- Hoque A., A. Nahar, M.A. Razvy, M.K. Biswas, and A.H. Kabir (2006). Micropropagation of water chestnut (*Trapa* sp.) through local varieties of Rajshahi Division. Asian J. Plant Sci. 5, 409-413
- Jenks, M., M. Kane, F. Marasca, D. Mcconnell, and T. Sheeran (1990). *In vitro* establishment and epiphyllum plantlets re-generation of *Nymphaea* "Daubeniana". Hortsci. 25, 1664-1665
- Karatas, M., M. Aasim, M. Dogan, and K.M. Khawar (2013). Adventitious shoot regeneration of the medicinal aquatic plant water hyssop (*Bacopa monnieri* L. PENNELL) using different internodes. Arch. Biol. Sci. Belgrade, 65 (1), 297-303
- Karatas, M., M. Aasim, A. Çınar, M. Dogan (2013). Adventitious shoot regeneration from leaf explant of dwarf hygro (*Hygrophila polysperma* (Roxb.) T. Anderson). Scientific World J. <http://dx.doi.org/10.1155/2013/680425>
- Kendir, H., N. Sahin-Demirbag, M. Aasim, and K.M. Khawar (2009). *In vitro* plant regeneration from Turkish narbon vetch (*Vicia narbonensis* L. VAR. *Narbonensis* L.). Afr J. Biotechnol. 8 (4), 614-618
- Murashige, T. ve Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15(3), 473-497.
- Öztürk, M., K.M. Khawar, H.H. Atar, C. Sancak, and S. Özcan (2004). *In vitro* micropropagation of the aquarium plant *Ludwigia repens*. Asia Pac J. Mol. Bio. Biotechnol. 12, 21-25.
- Panigrahi, J., R.R. Mishra, and M. Behera (2006). *In vitro* multiplication of *Asteracantha longifolia* (L.) Nees-a medicinal herb. Indian J. Biotech. 5 (4), 562-564
- Sahin-Demirbag, N., H. Kendir, K.M. Khawar, and M. Aasim (2008). *In vitro* plant regeneration from Hungarian vetch (*Vicia pannonica*) using cotyledonary node explants. Biotechnol. & Biotechnol. Eq. 22, 929-932
- Snedecor, G.W., W.G. ve Cochran (1967). Statistical Methods. The Iowa State University Press, Iowa, USA.
- Umlu, ., K.M. Khawar, M. Öztürk, and H.H. Atar, (2009). Akvaryum bitkisi *Rotala macranda*'nın *in vitro* ko ullarda hızlı ço altımı ve gen aktarımı. PhD Thesis (Unpublished). Deptt. of Field Crops, Ankara Univ. (In Turkish).
- Tiwari, V., K.N. Tiwari, and B.D. Singh (2001). Comparative studies of cytokinins on *in vitro* propagation of *Bacopa monniera*. Plant Cell Tiss. Org. Cult. 66, 9-16
- Vijayakumar, M., R. Vijayakumar, and R. Stephen (2010). *In vitro* propagation of *Bacopa italicsmonnieri* L.-a multipurpose plant. Ind. J. Sci. Tech. 3, 781-786
- Xu, L., U. Najeeb, R. Raziuddin, W.Q. Shen, J.Y. Shou, G.X. Tang, and W.J. Zhou (2009). Development of an efficient tissue culture protocol for callus formation and plant regeneration of wetland species *Juncus effusus* L. In Vitro Cell. Dev. Biol. Plant. 45, 610-618
- Yong, Z., W. Yao, Y. Baoyu, and C. Shiyun (2008). *In vitro* Regeneration and Propagation of *Pistia stratiotes*: An Ideal Aquatic Plant for Biomanufacturing and Bioremediation. Chinese J. App. Environ. Biol. 14, 445-449
- Zhang, L.J., S.F. Yeh, Y.T. Yu, L.M.Y. Kuo, and Y.H. Kuo (2011). Antioxidative Flavonol Glucuronides and AntiHBsAg Flavonol from *Rotala rotundifolia*. J. Trad. Comp. Med. 1, 57-6.