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IN VITRO PROPAGATION OF THE AQUARIUM PLANT *Rotala rotundifolia (ROXB.) KOEHNE*

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ABSTRACT

An efficient cost effective micropropagation protocol using liquid medium was developed for *Rotala rotundifolia,* a popular aquarium plant. Comparative analysis of shoot growth and proliferation in liquid Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinin, 6-Benzyladenine (BA) and auxin Indole - 3- acetic acid (IAA) was conducted. Better response in terms of shoot proliferation and number of branches/ shoot was observed in the liquid medium supplemented with 1.0 µM of BA and 1.0 µM of IAA. Micropropagation was best achieved from nodal explants in this medium. Regenerated shoots spontaneously developed roots within 6 weeks on the same medium. Standardization of the medium further lays the foundation for the shifting of plant production from small-scale to commercial scale.

KEYWORDS: *Rotala rotundifolia*, Micropropagation, Benzyl Adenine, Indole - 3 Acetic Acid

Aquarium plants, the water purifiers and aesthetisers of aquaria are a valuable commodity in the aquarium trade. It has opened a new era of aquascaping in various styles termed Japanese, dutch and nature styles. These styles are a copy of submersed or emersed states of nature. A large number of aquatic plants are made use of in aquascaping. The genus *Rotala*, a member of the family Lythraceae has more than 46 species around the world, of which *Rotala indica* and *Rotala rotundifolia* are seen in India (Vardhana, 2006). *Rotala rotundifolia* is an annual or perennial amphibious herb often seen in a semi-emersed state in marsh lands, paddy fields and stream sides of China, Bangladesh, Bhutan, India, Japan, Laos, Myanmar, Nepal, Thailand and Vietnam (Haining *et al*., 2007). This is a popular aquarium plant on account of its ease of cultivation and beautiful growth pattern. Decussate, elliptic leaves form at two per node which attains a wine red color when exposed to high light intensity. The plants form a good bunch in the aquarium with lots of side shoots and often featured in the dutch and nature – style aquascapes. The preferred temperature range of 20 – 28 °C and a pH of 5 – 8 is optimal for growth. Bright rose petalled flowers bloom on the emergent spike like inflorescences. This fast growing plant is, however, sensitive to unfavourable conditions, when it will react by producing smaller leaves (Jacobsen, 1979).

Most of the *Rotala* species are collected from natural populations and marketed in an improper manner which leads to the wilting of plants in the aquarium post setting up. These plants show seasonal abundance in their natural habitats. Unreliable supply and loss of plants due to poor marketing strategies leads to decreased availability of quality aquarium plants. Vegetative propagation has the limitation of producing lesser number of individuals from a single plant. Propagation through stem cutting requires enough stocks of stem and high labour inputs (Carneiro *et al*., 1997). Application of micropropagation technology for commercial production of aquarium plants could alleviate both, problems of supply and quality in aquarium trade, and provide a means of conservation of endangered species (Kane *et al*., 1999). Micropropagation efforts have been very few in the case of aquarium plants and are reported for *Anubias barteri, Aponogeton madagascariensis*, *Bacopa monnieri, Cryptocoryne wendtii, Myriophyllum aquaticum* and *Ludwigia repens* (Kukulezanga *et al*., 1980; Huang *et al*., 1994; Kane *et al*., 1999; Ozturk *et al*., 2004; Smitha *et al*., 2007; Banerjee and Shrivastava, 2008; Carter and Gunawardena, 2011).

The objectives of the present investigation were to determine whether *R. rotundifolia* could be grown under *in vitro* culture conditions, and to determine growth responses to two plant growth regulators with a view to standardize the medium for optimum growth of the plant.

MATERIALS AND METHODS

The experimental procedures followed the descriptive stages employed for the *in vitro* propagation of terrestrial plants (Pierik, 1987). Stage I describes the collection and surface sterilization of plant tissues and the initial growth of the explants on a starting medium. Stage II describes experiments designed to optimize growth and

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propagation on a sucrose based medium. Stage III (if required) describes medium development to enhance rooting, and Stage IV describes acclimation of plants outside of their sterile *in vitro* environment. In the present study, the plants were taken from Stage II directly to Stage IV as Stage III was not found necessary (since rooting was established in the Stage II itself). The different stages followed in the present study are described.

Stage I: Plant collection, surface sterilization and culture initiation

Healthy plants of *Rotala rotundifolia* were collected from aquarium plant vendors (Sree Padma Aquariums, Cochin) in February, 2015 as the donor plants for the experiments. The plant stocks were maintained under environmentally controlled fresh water fibre glass tanks at 30 ± 2°C with 12 h photoperiod. Nodal segments (length, 60.0 to 70.0 mm with 3.0 to 4.0 internodes; diameter, 1.0 to 3.0 mm) excised from the actively growing shoots were used for inoculation. Surface sterilization was carried out with 5.25 % (v/v) sodium hypochlorite solution containing a drop of 0.1 % Tween – 20 (w/v) by vigorous shaking for 35 minutes followed by rinsing with sterile distilled water until frothing disappears. Sterilized nodal explants were further trimmed to 40- 50 mm pieces, enclosing 2- 3 nodes before transferring singly into culture tubes.

Murashige and Skoog (1969) basal salts enriched with 3 % (w/v) sucrose and 0.56 mM myo – inositol were used as the basal media for all the experiments. The cultures were then grown in a Plant Growth Chamber at a temperature of 25 ± 2 °C and a (light fluence) or (lux) of 20 ± 5 µmol $m^{-2}s^{-1}$ irradiance provided by cool white fluorescent tubes (Philips, India). The photoperiod was 12 L: 12 D.

Stage II: *In vitro* propagation

Experimental cultures were grown for a period of 4 weeks. Each experiment had 10 replicates. Explants for the experimental cultures came from contaminant – free stock cultures. Cultures were evaluated for microbial contamination by examining the cultures Lack of any visible contamination indicated that these cultures could be considered operationally axenic.

In order to optimize the concentration of cytokinin and auxin combination for growth and shoot proliferation, the explants were initiated on MS media supplemented with different concentrations of cytokinin, 6-benzyl adenine, BA (0.1, 0.5, 1.0, 1.5 and 2.0 µM) and auxin, Indole -3 Acetic Acid, IAA (0.5 and 1.0 µM) were tested. All the 10 treatments had 10 replicates per treatment. A control was also set with plant growth regulator free medium. Each replicate had single explants per tube. The explants submerged in the liquid MS medium were placed on growth racks with fluorescent tubes per shelf. The cultures were incubated at 25 ± 2 °C under a 12 h photoperiod of 20 ± 5 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India). The number of explants producing shoots and the number of shoots per explant were scored after four weeks of incubation. The induced shoots were excised and sub cultured in basal media containing the most effective plant growth regulator in terms of growth for multiplication and rooting in one single step.

Stage IV: Establishment of plantlets in soil

Stage II cultures were moved directly into a Stage IV acclimation trial. Rooted plantlets were removed from the culture tubes and washed under running tap water to make it free of all the nutrient traces and placed into plastic cup trays filled with fine sand. The trays were kept immersed in the aquaria under 12 h illumination of 20 ± 5 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes for two weeks before they were transferred to outside under field conditions. The created environment of the aquarium was non-axenic, and simulated *R.rotundifolia*'s natural environment, giving the plants the opportunity to adjust to life outside of axenic culture conditions.

Acclimation

Plants that were cultured in Stage II medium produced roots that were proximate to each erect shoot. The production of roots in Stage II meant that the development of a Stage III root induction medium would not be necessary. Thus, the plants were moved directly from Stage II (*in vitro* propagation) to Stage IV (acclimation). When the plants were removed from the Stage II cultures and transferred to an aquarium, they continued to produce new erect shoots and roots. Shoot proliferation started within two weeks of transfer. Acclimation was successful.

Experimental Design and Analysis of Data

All experiments were statistically designed and used the completely randomized design. The experiments

were repeated at least twice to confirm the conditions for optimum growth. Data presented are mean ± standard error.

RESULTS

Plant Growth Regulator Effects

The cytokinin and auxin combination stimulated growth as measured by the production of erect green shoots and branches (Figure 1). The number of new erect shoots ranged from 12 to 45 in media containing cytokinins and auxins, while the number of erect shoots was only 10 in the control media without plant growth regulators (Table 1). Use of auxin – cytokinin combination at all tested concentrations led to the production of erect shoots and branches with multiple leaves and roots. Growth of *R. rotundifolia* increased as a function of BA concentration. Production of erect shoots increased up to a BA concentration of 1.5 µM, after which they started producing stunted shoots indicating an upper limit on the BA concentration (Figure 2). In all the media individual shoot development initiated within 7 to 14 days. The highest number of shoots per explant and the tallest plants were obtained on 1.5 µM BA and 1.0 µM IAA, T_9 (45.9 ± 0.081 nos. and 1.63 ± 0.710 cm, respectively), while the number of shoots was only 12.6 ± 0.358 in 0.1 µM BA and 1.0 µM IAA (Table 1).

Table 1: Effect of auxin – cytokinin combination on the shoot proliferation from nodal segments of *R. rotundifolia* after four weeks culture on MS medium

Different concentration of cytokinin and auxin (µM)	No. of shoots induced per explant (Mean ± SD)	Average length of shoot (cm) (Mean ± SD)
MS + PGR free media (Control)	10.1 ± 0.051	0.76 ± 0.002
MS + 0.1 BA + 0.5 IAA (T_1)	15.3 ± 0.065	0.85 ± 0.915
MS + 0.5 BA + 0.5 IAA (T_2)	21.7 ± 0.269	0. 92 ± 0.006
MS + 1.0 BA + 0.5 IAA (T_3)	28.9 ± 0.551	1.31 ± 0.054
MS + 1.5 BA + 0.5 IAA (T_4)	38.7 ± 0.073	1.58 ± 0.462
MS + 2.0 BA + 0.5 IAA (T_5)	33.8 ± 0.081	0.64 ± 0.650
MS + 0.1 BA + 1.0 IAA (T_6)	12.6 ± 0.358	0.71 ± 0.007
MS + 0.5 BA + 1.0 IAA (T_7)	23.9 ± 0.625	0.78 ± 0.221
MS + 1.0 BA + 1.0 IAA (T_8)	30.6 ± 0.004	1.27 ± 0.035
MS + 1.5 BA + 1.0 IAA (T_9)	45.9 ± 0.081	1.63 ± 0.710
MS + 2.0 BA + 1.0 IAA (T_{10})	27.4 ± 0.183	0.85 ± 0.008

Figure 1: Micropropagation stages of *Rotala rotundifolia* a) shoot induction after 7 days b) Shoot induction after 14 days c) shoot multiplication d) *in vitro* plant at 1.5 mg l^{-1} BA e) *in vitro* rooting.

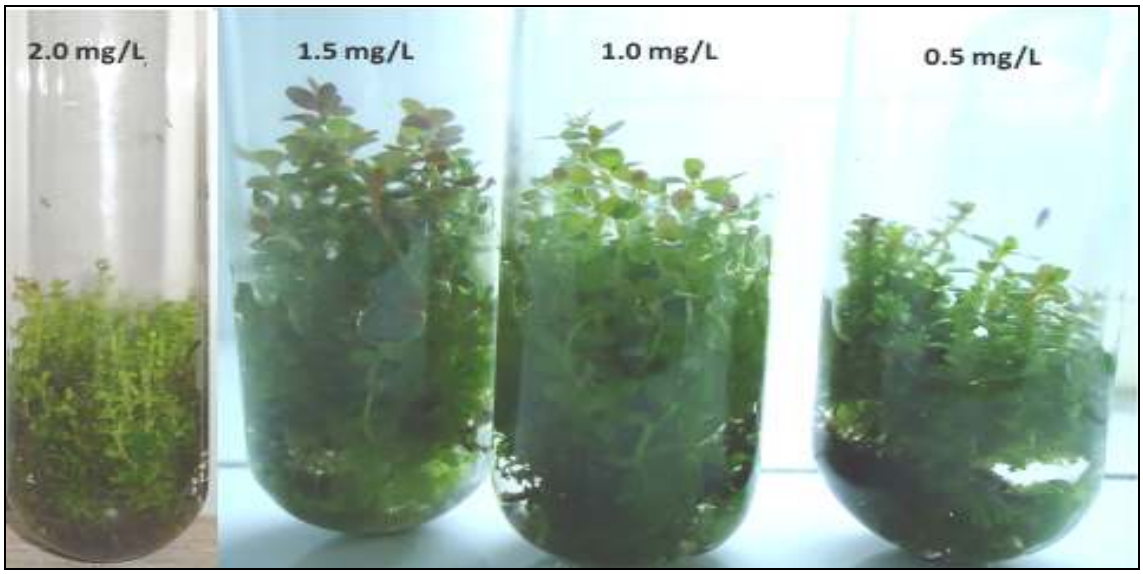

Figure 2: Effect of varying levels of BA on shoot number and length in *R. rotundifolia* four weeks after inoculation

DISCUSSION

Multiple shoots were raised using nodal explants in a liquid culture system using an auxin – cytokinin combination. Most of the reports of aquatic plant tissue culture refer to a culture in semisolid media using 0.7% agar (Kane and Gilman, 1991; Kane *et al.,* 1999; Ozturk *et al.,* 2004; Sharma *et al.,* 2010). Jenks *et al.,* (2000) opines that a liquid basal media is good for the establishment of stock cultures of *Nymphoides indica*. Liquid medium has several advantages over solid medium like the elimination of agar costs and enhanced retention period in the liquid medium (6 weeks) compared to 4 weeks in solid medium as reported by Pati *et al*., (2011) in his works on *Catharanthus roseus*.

The present report is the first to suggest the effectiveness of BA and IAA in shoot proliferation of *R. rotundifolia*. A combination of BA (1.5 µM) and IAA (1.0 µM) was effective in inducing multiple shoots. George (1993) has stated that a combination of cytokinins and auxins stimulate the *in vitro* multiplication and the growth of shoots of several plant species. The results of the present work are consistent with the findings of Kane *et al.,* (1991) in *Myriophyllum heterophyllum* and Jenks *et al.,* (2000) in *Nymphoides indica* where shoot organogenesis was completely inhibited with cytokinins alone. A combination of BA and NAA was effective in *in vitro* multiplication of Lotus (*Nelumbo nucifera*) where BA alone was effective for shoot formation but shoot length was disappointing which was compensated by the addition of NAA (Shou *et al.,* 2008). This experiment showed that the presence of cytokinin on the culture media positively influenced the micropropagation of *R. rotundifolia.* The number of shoots produced increased as the BA concentration increased from 0 to 1.5 µM at both levels of IAA (0.5, 1.0 µM). The high number of adventitious shoots produced in presence of BA, is consistent with the general knowledge that cytokinins overcome apical dominance, release lateral buds from dormancy, and promote shoot formation (George, 1993). However, the explants on 2.0 µM BA produced stunted shoots indicating an upper limit of BA. Similarly, Fracaro and Echeverrrigaray (2001) found that the number of shoots in *Cunila galioides* increased but their elongation ceased with higher levels of BA.

The liquid medium was found to be most suitable for *in vitro* root induction too. Presence of auxin, IAA (1.0 µM) helps in induction of roots. Earlier reports by Pati *et al*., (2011) demonstrated that liquid medium is most suitable for root induction compared to agar medium due to the difference in osmotic potential of the two media. Rooted plantlets were removed from the medium and transferred to aquaria for acclimation with 100 % success.

CONCLUSION

The present study reveals that aquarium plants are easier to micropropagate compared to terrestrial plants. The protocol described here provides a rapid micropropagation

system that may also be applicable to other species belonging to *Rotala* genus with minor modifications. *R. rotundifolia* shoots regenerated *in vitro* proved to be easily adaptable to *ex vitro* plant establishment since it is an easy to root plant.

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