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IN VITRO PLANT REGENERATION FROM NODAL EXPLANTS OF *RUELLIA TUBEROSA* L. -AN IMPORTANT MEDICINAL HERB

Dr. M. M. Rahman[1], Md. Tajul Islam and Dr. Animesh Biswas[1]*

Department of Botany, University of Chittagong 4331, Bangladesh.

Corresponding Author: Dr. Animesh Biswas
Department of Botany, University of Chittagong 4331, Bangladesh.

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ABSTRACT
An *in vitro* mass propagation protocol of *Ruellia tuberosa* L. was developed. Seeds were germinated *in vitro* for the supply of aseptic explants. Quick germination of seeds was occurred in the medium contained 1.0mg/l Kn + 0.5mg/l IAA. Nodal segments, obtained from aseptically grown seedlings, were used as explants. Multiple shoots were developed onto MS medium (2.0 mg /l BAP + 1.0 mg/l NAA) within 20-25 days of culture. In an average four shoots were produced from a single explant after two successive subcultures at 14 days interval. Microshoots were elongated onto MS medium fortified with 2.0 mg/l Kn + 0.5 mg/l IAA. The shoots were rooted best on half strength MS medium supplemented with 1.0 mg/l IBA. Plantlets with profuse root systems were successfully established in natural environment through gradual acclimatization. The survival rate of *ex vitro* plants was 80% without any visual deformities.

KEY WORDS: *R. tuberosa*, important medicinal plants, nodal segments, direct organogenesis.

1. INTRODCTION

Ruellia tuberosa L. (Acantheceae) is a perennial herb with thick fusiform tuberous roots. It is commonly known as "*Chatpoty*" in Bangali. This plant is native to tropical America and introduced in Southeast Asia. In Bangladesh this plant can be found in roadsides, village thickets and surrounding of homesteads.

The plant contains a wide range of bioactive compounds[1]. It has been reported that this plant possesses antioxidant, antimicrobial, anticancer, gastroprotective, antinociceptive, and anti-inflammatory activity[2, 3]. The flavonoid isolated from this plant has shown to be antiproliferative against HepG2 and KB cancer cell lines[3]. The root extracts of this plant are emetic and purgative, used in reducing the cholesterol level and as an antioxidant in hypercholesterolomic rats and was found scavenging effects on free radicals[4, 5, 6]. In folk and Ayurvedic medicine it has been used as an antidiabetic, antipyretic, analgesic, anti-hypersensitive, gestroprotective and in treating gonorrhea. Different parts of this plant are used as remedy for gonorrhea, ear diseases, stomach cancer, sore eye and promoting abortions[5]. The indigenous communities of Chittagong Hill Tracts use the tubers of this plant as stimulants[7]. The species is facing rarity due to indiscriminate collection by local herbalists and depletion of natural habitats by human interference. The present study was under taken with a view to develop an easy micropropagation protocol for massive multiplication followed by *ex vitro* establishment of plantlets of this medicinally important plant.

Plant tissue culture is a potential technique for exploitation of plant resources in multifarious way. These include production of huge plantlets in a short time without any climatic interference, genetic transformation and plant cell culture and secondary metabolites isolation. This protocol will help to produce identical clones of this species and future research is aimed at to screening and isolation of active bio-compounds from callus and cell culture.

2. MATERIALS AND METHODS

Mature seeds of *R. tuberosa* were collected from the road side of Khulna Sathkira Highway in January 2013. Plants were grown from seeds in earthen pots housed in the Department of Botany, University of Chittagong for future utilization. Nodal segments (0.5-1.0cm) of young healthy sprouts of two months old were used for *in vitro* culture.

In order to standardize *in vitro* culture experiments in *R. tuberosa* nodal segments of pot grown plants were first collected and cultured on MS medium following standard procedure of sterilization and supplemented with different concentrations and combinations of various PGRs (Plant Growth Regulators). These explants did give little response due to high contamination rate. It was then decided to grow *in vitro* aseptic seedlings of

this species from mature seeds. Nodal segments (0.5-1.0) of aseptically grown one month old seedlings were further used in *in vitro* culture for direct organogenesis.

Seeds were first thoroughly washed under running tap water for 10 minutes and then treated with 1% savlon plus few drops of Lifebouy (special liquid soap) for 5-10 mins with constant shaking. The seeds were then washed 3-4 times with distilled water and taken under running laminar airflow cabinet and transferred to 250ml beakers. In the laminar airflow cabinet, the explants were washed 2-3 times by doubled distilled water (DDW) and materials were immersed carefully on 0.1% $HgCl_2$ solution for different duration of time. After these, seeds were washed with sterile distilled water for 3-4 times to remove the every trace of sterilant. The material then rinsed with 70% ethanol solution for less than 60 seconds and were washed with sterile distilled water for 3-4 times. The sterilized seeds were inoculated on 0.8% (w/v) ager solidified MS[8] medium supplemented with different concentrations and combinations of PGRs (BAP: 1.0-3.0 mg/l; IAA and NAA: 0.5-1.0 mg/l).

The procedure of media preparation and incubation for *in vitro* seed germination and explants culture was same. In both of the cases, pH of the media was adjusted to 5.8 before adding ager and was autoclaved at 121^0C and 1.5 kg/cm^2 pressure for 30 minutes. The culture vessels were kept in the culture room under a regular cycle of 14/10h photoperiod at 2000-4000 Lux light intensity with a temperature 25 ± 2^0C.

Seeds were not germinated on MS without any PGR. MS with all combinations of PGRs were responded and differential results were recorded. The best result was obtained in MS+1.0mg/l Kn + 0.5mg/l IAA. In this combination 100% seeds were germinated within 10 days of culture. Scanty callus formation was noticed in some of the combinations. *In vitro* grown seedlings of one month old were used as explants in further experiments.

Nodal explants were cultured on MS fortified with various growth regulators (BAP: 0.5-3.0mg/l, Kn:0.5-3.0mg/l, NAA:0.5-2.0mg/l and IAA:0.5-2.0mg/l) at different concentrations and combinations. Initially the explants were cultured individually for 21 days and then subcultured at a regular interval of 14 days. For the rapid elongation of microshoots, various concentrations and combinations of growth regulators were used singly or in combination with BAP:1.0-3.0mg/l, Kn: 0.5-2.0mg/l, NAA: 0.5-1.0mg/l, 2, 4-D: 0.5-1.0mg/l, IAA: 0.5-1.0mg/l. Two subcultures were required for getting the appropriate length of microshoots for rooting experiments. When shoots were attained 4-5cm long they were then cultured for rooting in ager solidified MS, $^1/_2$MS and $^1/_4$MS media fortified with different concentrations of auxins (IBA:0.5-2.0mg/l, NAA:0.5-1.0mg/l, IAA:0.5-1.0mg/l) along with 0.3% sucrose. All cultures were compared to control. Each experiment was repeated thrice using 15 explants. Vessels containing well rooted plantlets were isolated from culture room and kept in the normal laboratory temperature for 3-4 days. Plantlets were then removed from culture tubes and washed thoroughly under running tap water to remove all trace of medium attached to the roots. These were then transplanted in the plastic pot containing 2:1 garden soil and compost. The soil was treated with 0.1% Agrosan (Fungicide) solution. Pots were then covered with transparent polythene bags and placed outside the laboratory. Plantlets were successfully established in field condition after 2 week of hardening.

3. RESULTS AND DISCUSSION

This study was aimed at to develop an *in vitro* propagation technique and regeneration protocol for an important medicinal plant of *R. tuberosa*. Explants collected from pot grown donors were very infection prone. So we had to establish *in vitro* seeds germination protocol for continuous supply of explants. Accordingly seeds were grown aseptically in sterile medium to raise contamination free *in vitro* seedlings.

The highest germination rate was recorded when seeds were cultured on MS medium fortified with 1.0mg/l Kn + 0.5 mg/l IAA (Table -1, Figure 1-2). One month old seedlings were used as explants source. Nodal segments obtained from those seedlings were then used as explants. Explants were responded in almost all of the media combinations used but multiplication rate of the cultured explants varied depending upon concentrations and combinations of the growth regulators. When BAP and Kn applied singly, proliferation response was found to be poor with only 2-3 shoot per culture. But shoots multiplication enhanced remarkably when low concentration of auxins was used in various combinations with BAP. It was observed that combinations of BAP + NAA were very effective for multiple shoot induction from nodal explants of *R. tuberosa*. The synergistic effect of BAP in combination with auxins has been demonstrated in many medicinal plants[9, 10, 11, 12, 13]. The highest frequency (100%) of multiple shoot induction was recorded from nodal segment in the medium containing 2.0 mg/l BAP + 1.0 mg/l NAA. The average number of shoots per culture were also highest in this combination and this was 4.0 ± 0.21 (Table- 2, Figure 3-4). For shoot bud elongation microshoots were cultured on different concentrations and combinations of growth regulator (BAP; BAP + NAA; BAP + 2,4-D; Kn; Kn+IAA; Kn+ NAA). Maximum elongation (5.0 ± 0.63 cm) was recorded in 2.0 mg/l Kn + 0.5 mg/l IAA (Table-3) (Figure 5).

For root induction, shoot were culture on MS, $^1/_2$MS, $^1/_4$MS basal media with 3% sucrose supplemented with different concentrations of IAA, NAA, IBA (Table 4). Root induction was observed on to $^1/_2$MS medium with different rooting percentages but the best response was found in $^1/_2$MS +1.0 mg/l IBA. Hundred per cent microshoots induced root within 30 days of culture with

an average 4.4 ± 1.54 roots per culture and 3.4 ± 0.98 mean of length of root per culture (Figure 1f). Reduced strength of basal medium is often used for rooting[14]. The efficiency of IBA at lower concentration in *in vitro* rooting has been reported in many medicinal plants[15-21]. On the other hand, full strength MS medium with different concentrations of auxins failed to induce any roots from the *in vitro* grown shoots.

In vitro raised shoots with well developed root systems were planted in small pots containing normal garden soil and compost (1:1). Plantlets were hardened in normal room temperature for another one week. Covering the pots, containing plant lets with transparent polythene bags facilitated to increase the survival percentage. Eighty per cent plantlets were survived in outside environment without any abnormal morphological differences compared to the donor plants (Figure 6).

Table - 1: Effects of different concentrations and combinations of PGRs on seed germination of *R. tuberosa.* In all cased MS basal medium was used.

Plant growth regulators (mg/l)	Total no. of seed cultured	Number and % of germinated seeds		Time / d required for seed germin-ation	Callus induction	Remarks
		No.	%			
MS	25	-	-	-	-	No visible changes had occurred.
BAP						
0.5	15	2	13.33	24-30	-	Green seedlings were produced.
1.0	15	6	40.00	20-25	-	
2.0	15	3	20.00	20-25	-	
3.0	15	2	13.33	24-26	-	
BAP + IAA						
1.0 + 0.5	10	8	80.00	8-12	+ +	Quickly germinated & green seedlings were produced.
1.0 + 1.0	10	7	70.00	8-12	+ + +	
BAP+NAA						
1.0 + 0.5	15	7	46.66	18-25	-	Green seedlings were produced.
1.0 + 1.0	12	3	25.00	18-25	-	
Kn						
0.5	15	7	46.66	18-22	-	Light green seedlings were produced.
1.0	15	9	60.00	18-20	+	
2.0	20	18	90.00	12-15	-	
3.0	20	15	75.00	12-15	-	
Kn + IAA						
1.0 + 0.5	15	15	**100.0**	**8-10**	-	Quickly germinated & produced more vigorous seedlings.
1.0 + 1.0	15	12	80.00	8-10	-	
Kn + NAA						
1.0 + 0.5	12	4	33.33	20-25	-	Green seedlings were produced.
2.0 + 0.5	12	7	58.33	20-25	-	

d = days.
- = no response.
+ = positive response to callus formation.
+ + = more response to callus formation.
+ + + = better response to callus formation

Table - 2: Effect of BAP and Kn in combination with IAA or NAA in MS medium for induction of MSBs in nodal explants of *Ruellia tuberosa.*

Plant growth mg/l regulators	No. of explants cultured	No. of explants responded	% of explants responded	Time /d required for induction of MSBs	Average* no. of MSBs/explants (mean ± SE)
BAP					
0.5	15	3	20.00	20-25	1.2 ± 0.20
1.0	15	5	33.33	20-22	1.6 ± 0.24
1.5	12	5	41.67	25-30	2.2 ± 0.20
2.0	15	10	66.67	18-20	2.8 ± 0.58
3.0	15	11	73.33	15-20	3.0 ± 0.80

BAP+NAA					
1.5 + 0.5	10	6	60.00	15-20	3.4 ± 0.60
2.0 + 0.5	15	7	46.67	15-20	3.2 ± 0.80
3.0 + 0.5	10	4	40.00	25-30	2.0 ± 0.63
1.5 + 1.0	10	6	60.00	15-20	3.4 ± 0.60
2.0 + 1.0	**10**	**10**	**100.00**	**20-25**	**4.10 ± 0.21**
3.0 + 1.0	10	2	20.00	20-24	0.8 ± 0.20
BAP+IAA					
1.5 + 0.5	14	2	14.29	14.29	18-22
2.0 + 0.5	14	5	35.72	35.72	25-30
3.0 + 0.5	12	3	25.00	25.00	26-28
1.5 + 1.0	14	3	21.43	21.43	25-30
2.0 + 1.0	15	6	40.00	40.00	25-30
3.0 + 1.0	14	2	26.67	14.29	18-22
Kn					
0.5	10	4	26.67	25-27	2.9 ± 0.12
1.0	15	4	33.33	20-22	3.1 ± 0.18
1.5	14	2	14.29	25-27	2.2 ± 0.20
2.0	12	4	33.33	18-20	3.0 ± 0.19
3.0	10	2	20.00	20-24	0.8 ± 0.20
Kn+IAA					
1.5 + 0.5	10	3	30.00	18-20	3.10 ± 0.18
2.0 + 0.5	16	13	81.25	18-22	3.5 ± 0.26
3.0 + 0.5	10	5	50.00	25-27	3.3 ± 0.23
1.5 + 1.0	10	2	20.00	20-24	2.4 ± 0.20
2.0 + 1.0	10	3	30.00	18-20	3.10 ± 0.18
3.0 + 1.0	10	3	30.00	18-20	3.10 ± 0.18
Kn+NAA					
1.5 +0.5	14	4	28.57	18-22	3.0 ± 0.17
2.0 +0.5	14	5	35.72	25-28	3.1 ± 0.19
3.0 +0.5	12	3	25.00	26-28	2.9 ± 0.12
1.5+ 1.0	15	3	20.00	20-26	2.8 ± 0.13
2.0+ 1.0	14	4	28.57	18-22	3.0 ± 0.17
3.0+ 1.0	14	5	35.72	25-28	3.1 ± 0.19

d = days.
MSBs = multiple shoot buds.
*Values are the means of three replicate with 15 explants

Table - 3: Effect of different concentrations and combinations of PGRs on elongation of multiple shoot buds of *Ruellia tuberosa.*

Plant growth regulators	**mg/l**	**Average* initial length /cm of individual shoot bud (Mean ± SE)**	**Average *length /cm of multiple shoot bud after 30d of culture (Mean ± SE)**
BAP	1.0	1.6 ± 0.40	2.8 ± 0.73
	2.0	1.8 ± 0.37	3.0 ± 0.55
	3.0	1.4 ± 0.24	2.0 ± 0.77
BAP + NAA	1.0 + 0.5	0.8 ± 0.20	1.8 ± 0.37
	1.0 + 1.0	1.6 ± 0.25	2.6 ± 0.81
	2.0 + 0.5	0.8 ± 0.20	1.4 ± 0.50
BAP + 2,4-D	1.0 + 1.0	2.8 ± 0.58	3.8 ± 0.97
	2.0 + 0.5	3.0 ± 1.26	4.0 ± 1.00
	2.0 + 1.0	3.2 ± 0.80	4.2 ± 1.07
Kn	0.5	1.2 ± 0.20	2.2 ± 0.73
	1.0	0.4 ± 0.24	1.4 ± 0.40
	2.0	0.6 ± 0.24	1.2 ± 0.20
Kn + IAA	0.5 + 0.5	3.4 ± 0.60	4.6 ± 1.69
	1.0 + 1.0	3.6 ± 0.87	4.8 ± 0.73
	2.0 + 0.5	**3.8 ± 1.53**	**5.0 ± 0.63**

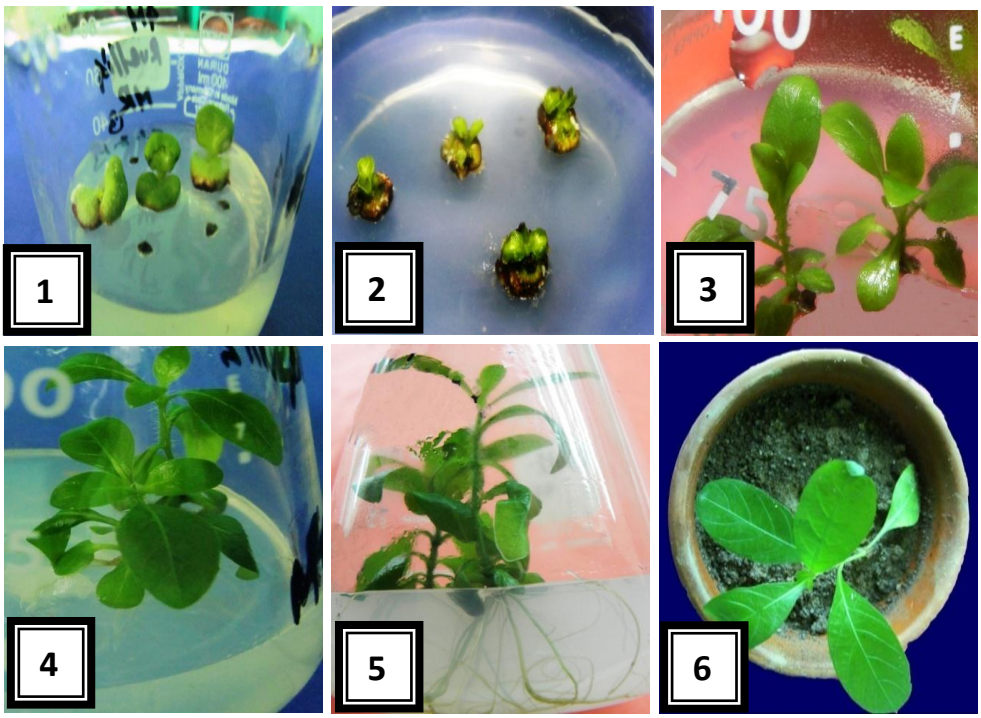
Kn + NAA	0.5 + 1.0 1.0 + 1.0 2.0 + 0.5	1.2 ± 0.68 2.4 ± 0.60 2.2 ± 0.20	3.6 ±1.40 3.4 ± 0.60 3.2 ± 0.66

d = days.
*values are the means of five replicates each with 15 explants.

Table - 4: Effect of different concentrations and combinations of PGRs on induction of roots of *Ruellia tuberosa.*

Plant growth Regulators	mg/l	% of micro shoot produced roots	Average* Number of roots/micro shoot (Mean ± SE)	Average*length/ cm of roots after 30d of culture (Mean ± SE)
Full MS + IBA	0.5 1.0	15.00 12.00	1.8 ± 0.49 1.6 ± 0.40	1.2 ± 0.20 0.8 ± 0.20
$^1/_2$MS	-	48.00	2.6 ± 0.68	1.8 ± 0.37
$^1/_2$MS + BAP	0.5 1.0	72.00 87.00	3.2 ± 0.20 3.6 ± 0.60	2.4 ± 0.68 2.8 ± 1.32
$^1/_2$MS + IAA	0.5 1.0	75.00 55.00	3.4 ± 0.60 2.8 ± 0.66	2.6 ± 0.64 2.0 ± 0.63
$^1/_2$MS + NAA	0.5 1.0	35.00 23.00	2.2 ± 0.73 2.0 ± 0.45	1.6 ± 0.40 1.4 ± 0.40
$^1/_2$MS + IBA	0.5 **1.0** 2.0	95.00 **100.00** 90.00	4.0 ± 0.77 **4.4 ± 1.54** 3.8 ± 1.35	3.2 ± 0.73 **3.4 ± 0.98** 3.0 ± 0.84
$^1/_4$MS	-	69.00	3.0 ± 1.05	2.2 ± 0.58

d = days.
- = without PGRs.
*values are the means of five replicates each with 15 explants.



1. In *vitro* germination of seeds of *Ruellia tuberosa.*
2. Induction of multiple shoot buds in nodal segments of *Ruellia tuberosa* on 2.0 BAP + 1.0 mg/l NAA.
3-4. Elongation of *in vitro* raised multiple shoot buds from nodal segments of *R. tuberosa.*
5. Induction and proliferation of root in $^1/_2$MS medium + 1.0 mg/l IBA.
6. *In vitro* developed plant after transplantation in small earthen pot.

4. CONCLUSION

This research article presents a reproducible protocol for *in vitro* regeneration in *R. tuberosa*. This protocol can be used for mass production and *ex-situ* conservation and this effort can help to reduce pressure on wild population and contribute to the conservation of this valuable medicinal plant. This protocol can be reliably used in mass propagation of this species. In addition, it will help in screening and isolation of active bio-compounds from this medicinal plant without destroying its natural vegetation.

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