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# MICROPROPAGATION OF PHARMACEUTICAL CROP *RUBIA CORDIFOLIA*

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## ABSTRACT

Plants are cherished as the chief resource for healthcare products attributed to synthesizing diverse array of bio-molecules through various metabolic alterations. Over exploitation of such resources can intimidate its existence. Developing medicinal plants into crops and the use of micropropagation as technique to mass produce high-demand biomass can make a continuous supply of natural therapeutic products. *Rubia cordifolia* is one such plant, so an improved, efficient, reproducible and reliable *in vitro* clonal propagation protocol is to be adopted for the conservation of germplasm. MS medium fortified with phytohormons *viz.*, BA, Kin, 2-iP, TDZ, mT, IAA, IBA and NAA singly or in combinations were used for the study. After one month of inoculation, 2.5 µM of BA and TDZ (9.37) endorsed the instigation of multiple shoots through axillary bud proliferation in a short span of time. *In vitro* produced microshoots were subjected to *ex vitro* rooting by treating 2.5 mM IBA for 5 min and planting in plastic pots containing potting mixture, to produce maximum rooting (79.17%) response. It was found to be an efficient and effective mode of rooting, since it combines both hardening and rooting. Under green house condition, all the rooted plantlets showed more than 90% survival. So direct regeneration protocol from nodal explants without the intervention of callus and thus ensure production of clonally uniform plants of *R. cordifolia*.

**Keywords:** Nodal explants, *Ex vitro* rooting, *In vitro* shoot proliferation, Benzyle adenine.

## 1. INTRODUCTION

The role of plants to uphold the harmony of nature as well as human life is legendary. Increased demand for herbal drugs, over exploitation, man-made ecological variation etc. negatively sways the distribution pattern and leads to depletion and threat of extinction of many medicinal plants. Depletion of species diversity due to overharvesting and environmental pollution affecting natural populations are strong factors that support the argument for cultivating rare and elite high-yielding medicinal plants [1]. Intensive conservation approaches like habitat protection, maintenance of seed and gene banks often become ineffective to cater to the situation. In order to produce mass number of plants within a short span of time, as well as to address the gap between supply and demand, tissue culture propagation is the most effective way.

*Rubia cordifolia* is native to Old World, found in higher elevations of tropics and subtropics; distributed in Southern Europe through African continent to Asia [2]. Paramount importance is given to its roots by pharmaceutical and textile industries, which are harvested after two years of planting at the pre-flowering stage or late fruiting stage, and the demand is increasing many fold tones/ annum every year. An intensive collection of roots from naturally growing plants leads to decreasing resource and impose a threat to the plant in the region, which can accelerate the rate of extinction. Microporpagation is one important tool for producing clones with high-yielding chemotypes especially for industrial purpose. A comprehensive literature scan on the propagation of *R. cordifolia* revealed sparse reports on *in vitro* propagation may be due to the difficulty of its aseptic culture establishment. So an effective propagation approach for the production of quality planting material and conservation through tissue culture was focused on in the communication study.

## 2. MATERIAL AND METHODS

For successful micropropagation of plants appropriate media preparation, careful selection and sterilization of explants, successful inoculation and maintenance of thorough aseptic culture conditions, subculture trials, root induction, hardening and field establishment are required.

### 2.1. Collection of material

Actively growing, disease free, young branches (15-18 cm) of *R. cordifolia,* were collected from Elappara, Idukki, Kerala (elevation 1158 m asl; Latitude 9°36', 49.89" N, Longitude 77°00', 6.59" E), and positively identified and authenticated with the help of flora. A voucher specimen (KUBH 6025) was deposited in the Herbarium, Department of Botany, University of Kerala.

### 2.2. Surface sterilization of explants

About 2.5 cm long nodal segments were excised and subjected to running water wash for 30 min and were then washed in 5% v/v polysorbitol detergent solution (Labolene, Mfg. Fischer Scientific Chemicals) for 10 min under vigorous agitation. These explants were again washed in running tap water for 10 min and then treated with 0.1% carbendazim fungicide (Bavistin, Mfg. BASF, Mumbai) for 30 min under constant agitation. After several rinsing in double distilled water, the explants were treated in 0.1% mercuric chloride (Hi Media, India) for 3 minutes and these surface sterilized explants were further repeatedly rinsed in pre-autoclaved double distilled water before inoculation.

### 2.3. Media preparation

For culture experiments, MS basal salt (Sigma-Aldrich, Germany) supplemented with 3% (w/v) sucrose and desired concentrations (µM) of various auxins *viz*., Indole Acetic Acid (IAA), Indole Butyric Acid (IBA), Naphthalene Acetic Acid (NAA) and cytokinins*viz*., Benzyl Adenine (BA), Kinetin (Kn), 6- dimethylallyl aminopurine (2-iP), Thidiazuron (TDZ) and meta Topolin (mT) singly or in combinations were used. All the phytohormones were purchased from Sigma-Aldrich, St. Louis, US. Before autoclaving the medium at 108 kPa pressure at 121°C for 15 min, pH (5.8) was adjusted and 0.7%agar (Hi Media, Mumbai) was added, melted by heating and poured in test tubes.

### 2.4. Induction of multiple shoots

Nodal segments were inoculated at different concentrations of BA (0-10 µM) and evaluated the optimum concentration of relative shoot forming capacity [3] and that concentration was taken in account for further comparative studies with other cytokinins used either singly or in combination (1-3 µM). Inoculated culture tubes were retained in a culture room at 25±2°C; 60-65% relative humidity; 16 h photoperiod at an intensity of 40 µmol $m^{-2}s^{-1}$ of PAR from cool white florescent tubes. The data were collected after one month of inoculation and subjected to subculture experiments to determine the longevity. The experiment was conducted in triplicates with 8 replicas each and the data collected were tested for statistical significance (SPSS version 20).

### 2.5. *In vitro* and *ex vitro* root induction and field transfer

For root induction, the microshoots were excised from the clumps (>2 cm) and for *in vitro* rooting, agar gelled ½ strength MS medium was used as support while for *ex vitro* rooting plastic pots (7 x 11 cm) containing sand, soil and FYM (1:1:1 ratio) was used. Auxins were used in µM (1.0, 2.5, 5.0 and 7.5) and mM (0.5, 1.0, 2.5 and 5.0) concentrations for *in vitro* and *ex vitro* rooting respectively. For *ex vitro* rooting, prior to auxin treatment (5 min), the microshoots, were dipped in 1% solution of carbendazim fungicide (Bavistin) for 10 min and directly potted in plastic pots. Pots were duly covered with polythene bags and kept in a glass house for one week, with a temperature regime of 25-34°C and after two weeks, the polyethylene bags were then removed and later one month after potting, plants were moved to nursery.

## 3. RESULTS AND DISCUSSION

Unlike other medicinal herbs, *R. cordifolia* showed a tendency to produce a single shoot per axillary bud, which creates defy in the formulation of economically viable *in vitro* multiplication protocol. BA at varying concentrations (0-10 µM) was used and it was noticed that except 5 and 10 µM in all other concentrations nodes of the nascent shoots started to produce secondary shoots within 20 day of inoculation. The concentration of BA exceeding 2.5 µM significantly ($p < 0.001$) reduced the shoot length, which may be due to the inhibition of adventitious meristem by BA at higher concentration by shifting gene activation [4]. Cultures raised in 2.5 µM BA produced 3.26 shoots per culture; shoots were grown in to 3.14 cm size accordingly with 2.51 shoot forming capacity (Table 1). The effectiveness of 2.5 µM BA was compared with other cytokinins *viz.,* Kin, 2-iP, TDZ,

meta Topolin (Table 2). The comparison of cytokinins showed superior performance of BA for *R. cordifolia*. Addition of TDZ produced 83.3 % response (Fig. 1 & 2), as reported [5], with regard to the length of shoot (2.62 cm) and the number of shoot (2.43). Previous reports [6, 7] suggest that BA alone can give a better response while in Ghatge *et al.* [5] proved BA singly neither the improved percentage of response nor the number of shoots/explant. 2-iP was found to be the least responding hormone with stunted growth and formation of compact calli. Addition of meta Topolin (mT) in the medium resulted in the development of 2.54 shoots per explants.TDZ (phenyl-N'-(1,2,3-thidiazol-5-yl) urea) is one of the several substituted urea having cytokinin-like activity, which is known to be more effective than zeatin for enhancing the growth of explants even at lower concentrations [8] but slightly inhibit root formation or may sometimes produce vitrified shoots in culture condition. In the previous works, 0.5 mg $L^{-1}$TDZ, 15.6 shoots/explant [9], 1.0 mg $L^{-1}$ 12.67 shoot/ explant [5] and at 4 mg $L^{-1}$ TDZ,8.1 shoots/ explants. In MS supplemented with TDZ (0.5 mg/L) + 0.1% PVP liquid medium about 20-25 multiple shoots were reported [10].

When the synergetic effect of auxins and BA was studied, there was no significant increase in shoot bud induction was noticed, so the combination of BA (2.5 µM) with other cytokinins (2-iP, TDZ and/or Kin) at varying concentrations (0.5- 2.5 µM) were examined.

Two way ANOVA revealed an overall highly significant ($p < 0.001$) effect of cytokinin combinations, indicates the most suitable combination of cytokinin for the efficient multiplication of *R. cordifolia* (Table 3). At BA - Kin (2.0 µM) combination, gave an inferior response featured by reduced number of shoots (1.54) shoot length (2.37 cm). BA-2-iP (1.5 µM) combination produced 2.34 shoots per culture. The combination of BA with TDZ (2.5 µM) resulted in 9.37 shoots/ explant. Microshoots obtained in the BA-TDZ combination (2.5 µM each) media subjected to continuous subculture showed a consistent number of multiple shoots where shoot number in each subculture was not varied significantly, indicating the suitability of this combination for efficient multiplication and longevity of the cultures without any deterioration cultures due to repeated subculture.

**Table 1: Effect of different concentrations of BA on shoot induction from nodal explants of *R. cordifolia* after 30 days of inoculation**

| BA Conc. (µM) | % Response | Shoot number | Shoot length (cm) | SFC |
|---|---|---|---|---|
| 0.0 | $26.85\pm5.78^{d}$ | $1.12\pm0.11^{d}$ | $0.98\pm0.14^{d}$ | $0.27\pm0.04^{e}$ |
| 0.5 | $61.58\pm5.61^{bc}$ | $1.39\pm0.21^{d}$ | $1.77\pm0.13^{c}$ | $0.86\pm0.13^{d}$ |
| 1.0 | $65.28\pm2.41^{b}$ | $1.88\pm0.13^{c}$ | $2.70\pm0.15^{b}$ | $1.22\pm0.07^{c}$ |
| 2.5 | $76.85\pm1.61^{a}$ | $3.26\pm0.28^{a}$ | $3.14\pm0.09^{a}$ | $2.51\pm0.23^{a}$ |
| 5.0 | $61.58\pm5.61^{bc}$ | $2.81\pm0.07^{b}$ | $2.76\pm0.08^{b}$ | $1.73\pm0.02^{b}$ |
| 10.0 | $54.17\pm9.11^{c}$ | $2.92\pm0.13^{b}$ | $2.63\pm0.13^{b}$ | $1.58\pm0.28^{b}$ |
| Treatment df (n-1)=5 | 27.195*** | 80.759*** | 130.15*** | 46.293*** |

*Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value*

**Table 2: Effect of different types of cytokininson shoot induction from nodal explants of *R. cordifolia* after 30 days of inoculation**

| Cytokinin type (2.5µM) | % Response (±SD) | Shoot number | Shoot length (cm) | SFC |
|---|---|---|---|---|
| BA | $76.85\pm1.61^{ab}$ | $3.30\pm0.23^{a}$ | $3.27\pm0.23^{a}$ | $2.51\pm0.26^{a}$ |
| Kin | $75.00\pm0.01^{ab}$ | $2.92\pm0.31^{ab}$ | $2.62\pm0.31^{b}$ | $2.30\pm0.14^{a}$ |
| 2-iP | $70.83\pm7.22^{b}$ | $2.04\pm0.14^{c}$ | $1.36\pm0.14^{c}$ | $1.44\pm0.14^{c}$ |
| TDZ | $83.33\pm7.22^{a}$ | $2.62\pm0.25^{b}$ | $2.43\pm0.25^{b}$ | $2.17\pm0.12^{a}$ |
| mT | $79.17\pm7.22^{ab}$ | $2.54\pm0.38^{b}$ | $2.31\pm0.38^{b}$ | $1.76\pm0.28^{b}$ |
| Treatment df (n-1)=5 | 4.051* | 8.851** | 34.008*** | 14.078*** |

*Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value, **Significant F value ($p < 0.01$), * significant at $p < 0.05$ level.*

Root initiation is a process governed by the polar auxin transport from auxin exporting tissue to the rooting zone [11]. Irrespective of auxin type and concentrations tested, initiation of adventitious root was noticed

within30 days of *in vitro* planting. It was noticed that in ½ MS medium supplemented with various concentrations of NAA, where adventitiously formed roots were thin, numerous, fibrous in nature and short. At 1 µM NAA concentration, the root formation was fast and occurred within 15 days of inoculation without any callus formation. At lower concentrations of IAA and IBA reduced numbers of roots were recorded. At 2.5 µM NAA and above unwanted basal callusing was observed at the cut ends [7, 5].

**Table 3: Combined effect of BA (2.5 µM) with other cytokinins on shoot induction in *R. cordifolia***

| Cytokinin types | | | No. of shoots | Shoot length(cm) |
|---|---|---|---|---|
| Kin (µM) | 2-iP (µM) | TDZ (µM) | | |
| 1.0 | - | - | 1.42±0.07$^{e}$ | 2.10±0.35$^{cd}$ |
| 1.5 | - | - | 1.38±0.13$^{e}$ | 2.06±0.21$^{cd}$ |
| 2.0 | - | - | 1.54±0.19$^{e}$ | 2.37±0.35$^{bc}$ |
| 2.5 | - | - | 1.46±0.19$^{e}$ | 2.27±0.31$^{bc}$ |
| 3.0 | - | - | 1.38±0.25$^{e}$ | 2.06±0.21$^{cd}$ |
| - | 1.0 | - | 1.58±0.07$^{e}$ | 1.77±0.15$^{de}$ |
| - | 1.5 | - | 2.34±0.08$^{d}$ | 2.00±.10$^{cd}$ |
| - | 2.0 | - | 1.46±0.07$^{e}$ | 2.13±0.15$^{cd}$ |
| - | 2.5 | - | 1.62±0.13$^{e}$ | 2.47±0.24$^{bc}$ |
| - | 3.0 | - | 1.42±0.07$^{e}$ | 1.47±0.06$^{e}$ |
| - | - | 1.0 | 5.42±0.14$^{b}$ | 2.70±0.10$^{b}$ |
| - | - | 1.5 | 5.45±0.18$^{b}$ | 2.63±0.15$^{b}$ |
| - | - | 2.0 | 5.04±0.19$^{c}$ | 2.40±0.44$^{bc}$ |
| - | - | 2.5 | 9.37±0.21$^{a}$ | 3.70±0.10$^{a}$ |
| - | - | 3.0 | 5.21±0.40$^{bc}$ | 2.43±032$^{bc}$ |
| Main effect F value df (n-1)= 14 | | | 547.56*** | 13.02*** |
| Cytokinin type (T) df (n-1)= 2 | | | 3181.54*** | 45.23*** |
| Cytokinin Conc. (C)df (n-1)= 4 | | | 107.42*** | 14.48*** |
| T X C df (n-1)=8 | | | 109.13*** | 4.24** |

*Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value, **Significant t value ($p < 0.01$).*

**Table 4: *In vitro* rooting of microshoots of *R. cordifolia* in ½ strength MS medium supplemented with different auxins**

| Auxin type | Conc. (µM) | % Rooting (±SD) | Root number | Root length (cm) | R/S ratio |
|---|---|---|---|---|---|
| NAA | 1.0 | 62.50±0.00$^{c}$ | 16.0±0.80$^{b}$ | 0.69±.007$^{f}$ | 0.33±.002$^{i}$ |
| | 2.5 | 62.50±0.00$^{c}$ | 18.5±0.30$^{a}$ | 0.71±.003$^{f}$ | 0.49±.016$^{h}$ |
| | 5.0 | 58.33±7.22$^{c}$ | 8.09±0.79$^{c}$ | 0.69±.005$^{f}$ | 0.25±.002$^{j}$ |
| | 7.5 | 54.17±7.22$^{c}$ | 15.9±0.40$^{b}$ | 0.83±.002$^{f}$ | 0.56±.005$^{g}$ |
| IAA | 1.0 | 66.67±7.22$^{bc}$ | 1.18±0.04$^{d}$ | 8.71±.050$^{a}$ | 3.06±.018$^{a}$ |
| | 2.5 | 62.50±12.5$^{c}$ | 1.12±0.01$^{d}$ | 5.43±.080$^{d}$ | 1.71±.024$^{f}$ |
| | 5.0 | 79.17±7.22$^{ab}$ | 1.15±.04$^{d}$ | 5.40±.070$^{d}$ | 1.68±.022$^{f}$ |
| | 7.5 | 58.33±7.22$^{c}$ | 1.03±0.06$^{d}$ | 4.96±.087$^{e}$ | 2.39±.042$^{d}$ |
| IBA | 1.0 | 66.50±7.37$^{bc}$ | 1.37±0.03$^{d}$ | 6.18±.365$^{c}$ | 1.93±.114$^{e}$ |
| | 2.5 | 79.17±7.22$^{ab}$ | 1.50±0.02$^{d}$ | 7.90±.105$^{b}$ | 2.65±.036$^{b}$ |
| | 5.0 | 83.33±7.22$^{a}$ | 1.54±0.04$^{d}$ | 7.83±.040$^{b}$ | 1.91±.010$^{e}$ |
| | 7.5 | 66.67±7.22$^{bc}$ | 1.28±0.02$^{d}$ | 6.00±.100$^{c}$ | 2.58±.043$^{c}$ |
| F value df (n-1)=11 | | 4.909*** | 1149.5*** | 1919.5*** | 1807.8*** |
| Treatment (T) df (n-1)= 2 | | 1857.96*** | 8589.0*** | 11816*** | 12.138*** |
| Concentration(C) df (n-1)= 4 | | 245.404*** | 122.99*** | 256.13*** | 3.78* |
| T x C df (n-1)= 8 | | 137.075*** | 234.86*** | 314.44*** | 3.04* |

*Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value, *Significant F value ($p < 0.05$); NS: not significant*

On prolonged maintenance (more than two months) in NAA added medium intensive callusing from the lower portion of root clusters was noticed. *In vitro* rooting response and highest number of roots were recorded in medium containing 5.0 µM IBA (Table 4). In IAA and IBA treated shoots, number of root was comparatively less, but it was robust and brown in colour. Among different hormones tried, IBA showed maximum rooting, root length, least callusing, sturdy and healthy roots. The *in vitro* produced microshoots were also subjected to *ex vitro* rooting by auxin pulse treatment (mM). The emergence of the adventitious root was noticed in *ex vitro* planted microshoots within 7 days of planting. Maximum % response was recorded at 2.5 mM IBA (79.17 %), while root number (14.43) was high in IAA (1 mM) treated microcuttings and roots were well elongated in 2.5mM IBA treatment (Table 5). Statistical analysis revealed that the F values were significant for all the parameters except root shoot ratio. When *in vitro* and *ex vitro* rooting response was compared, IBA was found to be the best hormone for root initiation at 5.0 µM IBA for *in vitro* rooting and 2.5 mM for *ex vitro* rooting. R/S ratio denotes the relation between roots and shoots length, as R/S ratio increases, plant establishment efficacy increases. Overall comparison made it clear that IBA gave good response for root induction. Earlier, 1 mg $L^{-1}$ IBA gave 98 % *in vitro* rooting within five weeks with 8.9 roots/explants and 6.4 cm length [7] was reported. MS medium with activated charcoal (0.1%) and 1.0 mg $L^{-1}$ IBA was better than IAA or NAA for root initiation [12] and 2 mg $L^{-1}$ IBA as best rooting hormone (60 %) with an average of 8.0 roots/explant and 4.6 cm length [13]. IBA (1mg $L^{-1}$) was reported to be best auxin for rooting in *R. cordifolia* [14]. Ghatge *et al.* (2011) showed that 3 mg $L^{-1}$ IBA treatment induced 100 % rooting. Earlier it was reported that the MS medium with IBA (2mg/L) developed 2-3 roots within 8-14 days [10]. In the present study 5.0 µM IBA added medium produced 83.33 % response.

**Table 5: *Ex vitro* rooting of pulse treated microshoots for 5 minutes in different types and concentrations (mM) of auxins**

| Auxin type | Conc.(mM) | % Rooting (±SD) | Root number 13.2±0.92$^{ab}$ | Root length(cm) | R/S ratio 1.12±.147$^{ab}$ |
|---|---|---|---|---|---|
| NAA | 0.5 | 50.00±12.5$^{def}$ | | 3.07±0.46$^{abc}$ | |
| | 1.0 | 66.67±7.22$^{abc}$ | 9.80±1.91$^{cd}$ | 3.10±0.50$^{abc}$ | 1.17±.196$^{ab}$ |
| | 2.5 | 75.00±0.00$^{ab}$ | 13.93±3.69$^{ab}$ | 3.37±0.95$^{ab}$ | 1.02±.225$^{b}$ |
| | 5.0 | 45.83±7.22$^{ef}$ | 7.47±0.61$^{de}$ | 2.87±0.31$^{bc}$ | 1.07±.036$^{b}$ |
| IAA | 0.5 | 37.5±0.00$^{f}$ | 6.60±0.40$^{e}$ | 3.13±0.31c$^{abc}$ | 1.31±.181$^{a}$ |
| | 1.0 | 58.33±7.22$^{cde}$ | 14.43±1.50$^{a}$ | 3.07±0.31$^{abc}$ | 1.20±.142$^{ab}$ |
| | 2.5 | 41.67±7.22$^{f}$ | 11.27±1.37$^{abc}$ | 2.83±0.35$^{bc}$ | 1.02±.116$^{b}$ |
| | 5.0 | 45.83±7.22$^{ef}$ | 6.43±0.51$^{e}$ | 2.63±0.29$^{bc}$ | 1.05±.125$^{b}$ |
| IBA | 0.5 | 66.67±7.22$^{abc}$ | 11.00±0.30$^{bc}$ | 2.50±0.27$^{c}$ | 0.72±.025$^{c}$ |
| | 1.0 | 70.83±14.4$^{abc}$ | 14.27±1.99$^{ab}$ | 2.33±0.45$^{c}$ | 0.61±.723$^{c}$ |
| | 2.5 | 79.17±7.22$^{a}$ | 11.40±1.70$^{abc}$ | 3.77±0.15$^{a}$ | 0.53±.610$^{c}$ |
| | 5.0 | 62.50±.00$^{bcd}$ | 11.36±1.40$^{abc}$ | 3.17±0.35$^{abc}$ | 0.76±.053$^{c}$ |
| Main effect F value df (n-1)=11 | | 9.506*** | 8.287*** | 2.388* | 11.315*** |
| Auxin type (T) df (n-1)= 2 | | 28.50*** | 5.540** | 0.626$^{NS}$ | 53.149*** |
| Conc. (C) df (n-1)=3 | | 9.524*** | 12.071*** | 0.948$^{NS}$ | 3.536* |
| T x C df (n-1)= 8 | | 3.167* | 7.310*** | 3.696** | 1.259$^{NS}$ |

*Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value, **Significant F value ($p < 0.01$), *Significant F value ($p < 0.05$), NS: non significant*

Cultures maintained *in vitro* relish most suitable condition with minimal stress facilitating maximum multiplication and growth. Thus *in vitro* grown plantlets characterized by leaves with reduced cuticle and abnormal stomata, poor root system [15], when transferred directly to fields, became susceptible to transplantation shock. Hardening and acclimatization were essential steps for *in vitro* grown plants because the ideal conditions provided in the culture makes them hard to acclimatize the rigor of natural condition. For

this purpose, the potted plantlets were initially maintained in a glass house (27±1°C; 65% RH), nurtured with ¼ MS macro and micro nutrient solution at an interval of 7 days. After 2 weeks, the polythene bags were removed and replanted to large earthen pots containing potting mixture sand: soil: FYM (1:1:1) and watered regularly. These plantlets were then shifted to an agro net shade house for 2-3 weeks and finally transferred to field. Acclimatized plants were found to have vigorous growth and no abnormalities were noticed at any stage of acclimatization process. Auxin pulse treated microshoots for *ex vitro* rooting, were planted in sand, soil and FYM (1:1:1 ratio) mixture prepared in plastic pots and covered with polyethylene bags and maintained under glass house conditions produced profound rooting within two weeks of planting (Fig 3 & 4). These plantlets were irrigated with ¼ MS salt solution every week. When establishment of *in vitro* and *ex vitro* rooted plants were compared, e*x vitro* rooted plants were established within 6 weeks where as *in vitro* rooted plants took more than 12 weeks to complete the weaning process. So the *ex vitro* rooting and hardening was considered to be comparatively cheap and less time consuming thus recommended for the *R. cordifolia*. The plantlets with ample number of leaves were survived (90%) and were planted in the field.

## 4. CONCLUSION

The destruction of habitats, fragile ecosystems, restricted distribution, lower fruit set coupled with poor seed germination, illegal trade by over exploitation etc. are posing serious threat to *R. cordifolia*. At this juncture, the *in vitro* propagation protocol developed in the present study offers an opportunity for the rapid and effective multiplication of *R. cordifolia* as a pharmaceutical crop.

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***Conflicts of interest***

There is no conflict of interest about the publication on the manuscript

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