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Research article

AN IMPROVED PROTOCOL FOR *IN VITRO* REGENERATION OF *RUBIA CORDIFOLIA* L VIA ORGANOGENESIS

Suresh Khadke[1], Shweta Rani[2], Vithal Awad[1], Neelambika Meti[2], Elangbam Singh[2], Aniket Kuvalekar[1] and Abhay Harsulkar[1]

[1]Nutrigenomics Laboratory, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed University, Pune-Satara Road, Katraj, Pune- 411043, India.
[2]Rajiv Gandhi Institute of Information Technology and Biotechnology, Bharati Vidyapeeth Deemed University, Pune-Satara Road, Pune- 411043, India.
Correspondence address: E-mail: aharsulkar@yahoo.com

ABSTRACT: An efficient protocol for the regeneration of *Rubia cordifolia* L via indirect organogenesis was developed. The traditional harvesting procedure of plant is destructive and needs urgent conservation. Here we report, an efficient protocol for regeneration and multiplication of plantlets, through indirect organogenesis in *R. cordifolia* L. Organogenic callus and adventitious shoots were raised on filter paper bridge in MS liquid medium with supplementation of plant growth regulators. Leaf, internode and node explants were inoculated in MS medium containing 0.25 – 4.0 mg/L TDZ and 2, 4-D. Significant callus (89.3± 3.8%) and shoot induction (71.8±3.1%) was observed in MS medium with 4 mg/L TDZ. Caulogenesis was achieved on the same medium used for callus induction. An average of 24.4±2.2 shoots having 4.01±0.2 cm length were regenerated in the culture. The highest rooting response (93.7±2.0%) was observed on the medium with 1.0 mg/l IBA with a mean number of 4.9±0.7 roots per shoot with mean length of 4.7±0.2 cm. Well developed plantlets were transferred to shade net with 100% successful acclimatization. This system may prove to be beneficial for ex-situ conservation as well as for amenability of this plant for genetic manipulation. This is the first report of indirect organogenesis in *R. cordifolia* with significantly high plantlet regeneration frequency.

Key words: Conservation, Indirect organogenesis, Regeneration, *Rubia cordifolia*, TDZ

INTRODUCTION

The loss of plant genetic resource has made it necessary the development of new conservation methods. Biotechnological tools like *in vitro* organ culture, cryopreservation, and molecular markers offer ultimately conservation [1]. The biotechnological techniques used for *ex situ* conservation represent an improvement on traditional conservation methods and provide an extra means of protecting wild resources [2]. The genus *Rubia cordifolia* L., commonly known as “Indian madder”, belongs to family Rubiaceae and is represented by about 15 species in India. Some of these are Indian madder (*R. cordifolia*), Naga madder (*R. sikkimensis*) and European madder (*R. tinctorum*) [3]. *R. cordifolia* is perennial, hardy climber with long cylindrical roots covered with thin red bark [4]. It is naturally distributed in India, Japan, Indonesia, Ceylon, Malay, Peninsula, Java, and Africa in moist temperate and tropical forests up to an altitude of 3500 m [5]. The roots and stems of *R. cordifolia* are medicinally active parts of the plant and known to be rich in Purpurin and Munjistin [6]. Plant extract is cytotoxic to human larynx carcinoma, human cervical cancer and is also used in treatment against diabetes, female reproductive problems, skin disorder and urinary tract infections [7]. *R. cordifolia* exhibit number of pharmacological activities including anti-inflammatory, antistress, urinary tract disorders, antimicrobial, antifungal, analgesic, antimutagenic, hepatoprotective and blood purifier activity [8]. From ayurvedic perspective, *R. cordifolia* is an important plant species, and is regularly used in preparation of *Jwarhar mahakashya* [9].

Availability and authenticity of medicinal plant material is a major problem in Ayurved and herbal industry. Even today, only a small percentage of medicinal plants are systematically cultivated. Most of them are collected from wild sources, often in a destructive and unsustainable manner [10]. Currently, demand for *R. cordifolia* is very high as compare to the available resources. The plant is also exported to various countries adding to scarcity of genuine plant material. Over-harvesting of this plant from its natural habitat is increasing rapidly consequently threatening its existence [11, 12, 13]. For sustainable utilization and to meet the demands and its conservation, it was emphasized to resort to standardization of *in vitro* techniques for propagation and secondary metabolite production [14, 15]. In the present study, rapid and reliable protocols for multiple shoot induction and propagation of *R. cordifolia* were developed as a first step towards the *ex situ* conservation of the germplasm of this plant. Two different systems of shoot regeneration were used, caulogenesis from leaf and internode explants and development of axillary buds from nodes. The shoots obtained were efficiently rooted, and the plantlets were acclimatized with high success rate. As per our knowledge, this is the first report of indirect organogenesis of *R. cordifolia* through multiple shoot formation.

MATERIAL AND METHODS

Collection of plant material and establishment of aseptic cultures

Nursery grown plants of *Rubia cordifolia* were collected in the month of June 2010, from Jejuri, Pune, Maharashtra, India and identified with the help of standard flora of the area [16, 17] and a voucher specimen (MPCC-2303) of authentic plant species was deposited to Medicinal Plants Conservation Center (MPCC), Pune, Maharashtra, India. Collected plants were maintained in shade-net and younger leaves, node and internodes from identified plants were used as explants. Leaf, internode and nodal segment explants from *R. cordifolia* cleaned thoroughly by gently brushing under running tap water for 10 min and washing in liquid detergent (1–2 drops of Tween 10 in 1^{-1} distilled water) followed by three rinses with sterile distilled water. Surface disinfection was carried out by submerging and shaking the explants in 70% (v/v) ethanol for 30 sec and washing twice with sterile distilled water. The cleaned explants were then surface sterilized in 0.1% mercuric chloride with constant shaking inside a laminar flow cabinet for 3 min and rinsed three times with sterile distilled water. After surface sterilization, leaves, internodes and nodes were cut into small pieces (approximately 1-2 cm) and the explants were inoculated in glass culture bottles (22mm x 150mm) containing 30 ml of liquid MS (Murashige and Skoog) medium[18]. MS media was fortified with 3% sucrose, 20% Coconut water (CW) and various concentrations of different Plant growth regulators (PGR) including TDZ, KN, 2, 4-D and NAA (Table 1). The pH of the culture medium was adjusted to 5.8 ± 0.2 before autoclaving at 120ºC and at 15 lb pressure/time. Filter paper bridge was used for supporting, instead of agar. Culture bottles were maintained at 25 ± 2 ºC, 16/8 hours photo-period using cool white florescent light.

Table 1: Treatment of PGR on different explants of *R. cordifolia*

Plant growth regulators (PGRs)	Concentration (Mg/L)	Remark (Leaf, Internode & Node)
TDZ	0.25-4.0	Profuse mass of dark green Callus, adventitious shoots
KN	0.25-4.0	Profuse mass of orange Callus, axillary shoots
2,4D	0.25-4.0	Profuse mass of yellow orange Callus, adventitious and axillary shoots,
NAA	0.25-4.0	Profuse mass orange Callus, axillary shoots
Control	-	axillary shoots

Induction of shoots via organogenesis pathway

Callus induction and axillary shoot induction

Leaf, internode and node segments were excised from the shade net grown plants, surface sterilized and inoculated on MS medium fortified with different concentrations of TDZ (Table 1). MS basal medium was used as a control. Culture bottles were kept at 25 ± 2ºC, 16/8 hours photoperiod using cool white florescent light. Subculturing was done after every 3 week. Observations were recorded regularly after 1 week interval. Each experiment was replicated at least three times (n = 48).

Multiple-shoot induction and its maintenance

Callus and shoots induced in previous experiment were subcultured after 3 week on the same medium which was previously implemented for induction. Callus, along with newly induced shoots were chopped in to small pieces and subcultured on MS medium containing respective concentrations of growth regulators. The percentage of callus cultures forming shoots, number of shoots per callus and shoot length was recorded after every 3 week. Culture bottles were kept at 25 ± 2ºC, 16/8 hours photo-period using cool white florescent light. Sub-culturing was done after 3 week interval.

Root induction and acclimatization

Well developed shoots were selected and excised from callus with the help of forceps and surgical blade. Excised shoots were inoculated on MS medium supplemented with 0.5-2 mg/L of IBA, IAA, and NAA individually, on Paper bridge as well as MS medium fortified with 0.8% Agar (Himedia, Mumbai, India) and 0.1% activated charcoal. After inoculation, culture bottles were maintained at 25 ± 2 ºC, 16/8 hours photo-period using cool white florescent light. Sub-culturing was done regularly after 3 week interval to avoid browning of culture medium. Well rooted plantlets were selected, washed thoroughly in running water were transplanted in sand: compost mixture 1:2 with minimal damage to their root system and were maintained in shade net. The plantlets were covered with transparent polythene bags to prevent desiccation. After 2 week, plants were removed from sand and compost mixture and planted individually in pots. All planted pots were maintained in 80% shading for 3 week and transferred to nursery beds. Survival rate was recorded after every 2 week interval up to two months.

Histological studies

Histological studies were performed as described by Fortes and Pais (2000), one to three weeks old callus cultures of *R. codifolia* were fixed in formalin: acetic acid: 70 % (v/v) ethanol (F.A.A. 5:5:90) for 48 hours. After dehydration, transverse sections were taken stained with Aceto Orcein and observed under light microscope (Labindia, Bangalore, India). Selected stages were fixed in DPX (SRL, Mumbai, India) for further study.

Statistical analysis

The data were analyzed for analysis of variance (ANOVA) ($P>0.001$), followed by Tukey: Kramer multiple comparison test

RESULTS AND DISCUSSION

Induction of callus / shoot organogenesis

The explants swelling was observed at cut edges within first week of inoculation and green compact mass of callus was developed in third week. Leaf and internode explants showed different responses to callus induction medium dependent on the concentration of PGR in the culture media. Profuse mass of dark green callus was induced on medium containing TDZ. Callus induction in the TDZ containing media was observed to be dose dependent with maximum callus induction (89.3±3.8%) at 4 mg/L TDZ. In leaf explants, callus induction frequency was higher as compared to the internodes (Table 2, Photo Plate 1). In media supplemented with 2, 4-D, light orange pigmented callus was formed with friable mass; however, shoot induction was very low (Data not shown). Explants inoculated on MS basal medium were used as control. The role of TDZ in tissue culture is well documented. It is involved in callus formation and shoot induction at lower concentrations, while it is known to induce somatic embryogenesis at higher concentrations [19, 20]. Our results are in close agreement with the above observations. However, instead of embryogenesis at higher concentration, we observed callusing and development of multiple shoots in *R. cordifolia.* Histological studies of three week old morphogenic callus showed different stages in regeneration through indirect organogenesis (Photo Plate 3, A-F). Callus induction is considered as a first stage of morphogenic response; we observed shoot initiation within 3 week of callus induction. Initiation of organogenesis is possible at higher concentration of Adenine type of cytokinins [21].

TDZ is absorbed quickly by the explants and showed organogenesis via reduced dominance of apical meristem, resulting in formation of adventitious shoots or axillary bud response at low concentrations [22, 23, 24]. Study carried out by Gouhua [25], who reported that *Metabriggasia ovalifolia* took 5 week for callus induction in TDZ, and took efforts to optimize combinations of NAA and BA for attaining shoot proliferation. In the current study callus induction as well as shoot proliferation was obtained within 5 week. We observed that the frequency of multiple shoot initiation increases with increasing concentration of TDZ with respect to the leaf and internode explants. TDZ was observed to have dose dependent effect on the frequency of shoot induction from leaf and internode explants (Table 2). It indicates that leaf explants are better alternative as compared to node and internode segments. Our results are in agreement with Li and Filiz [26, 27] with the leaf explants performing better than node and internode explants on TDZ supplemented medium. Apart from these reports, axillary shoot induction has been reported by Radha [6]. Where 5-6 shoots/node, were observed in medium containing 1 mg/L BA and 0.02 mg/L IAA within 16-17 week. Our shoot initiation frequency was better and the time taken was significantly less, suggesting this protocol to be superior as far as clonal propagation of *R. cordifolia* is concerned.

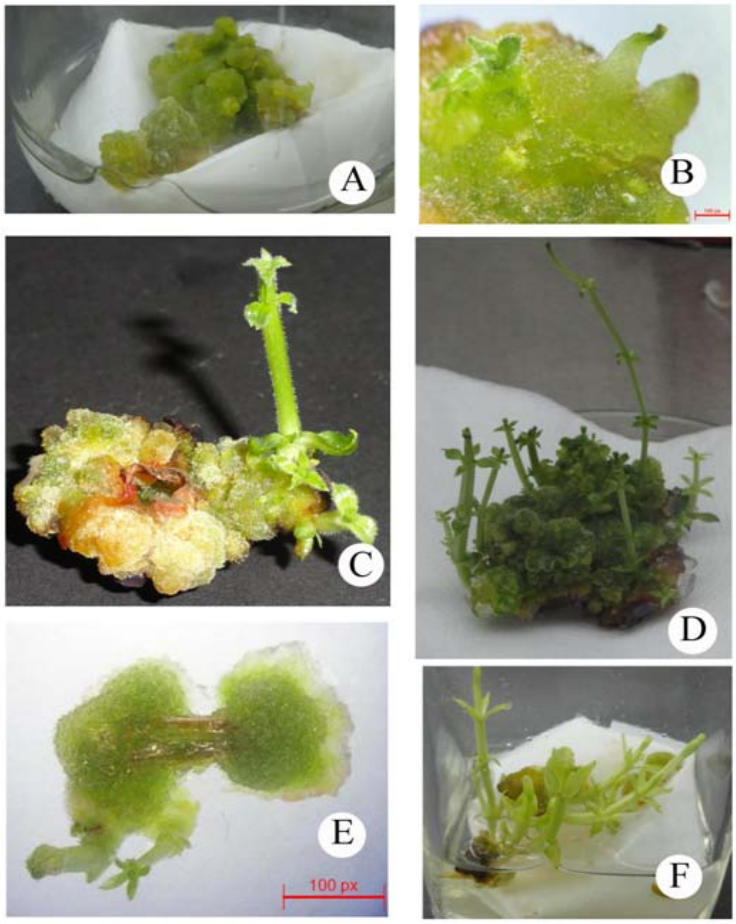

Plate 1: A.Callus formation from leaf explants on MS medium supplemented with 4.0 mg/L TDZ
B. Stereo microscopic picture of callus with micro shoots induction from leaf explants calli with prominent shoots formation
C& D callus with multiple shoot induction from leaf explants on MS medium supplemented with 4.0 mg/L TDZ
E- Stereo microscopic picture of callus with micro shoot-induction from internode explants
F- Induction of axillary shoots from nodal explants on MS medium supplemented with 4.0 mg/L TDZ

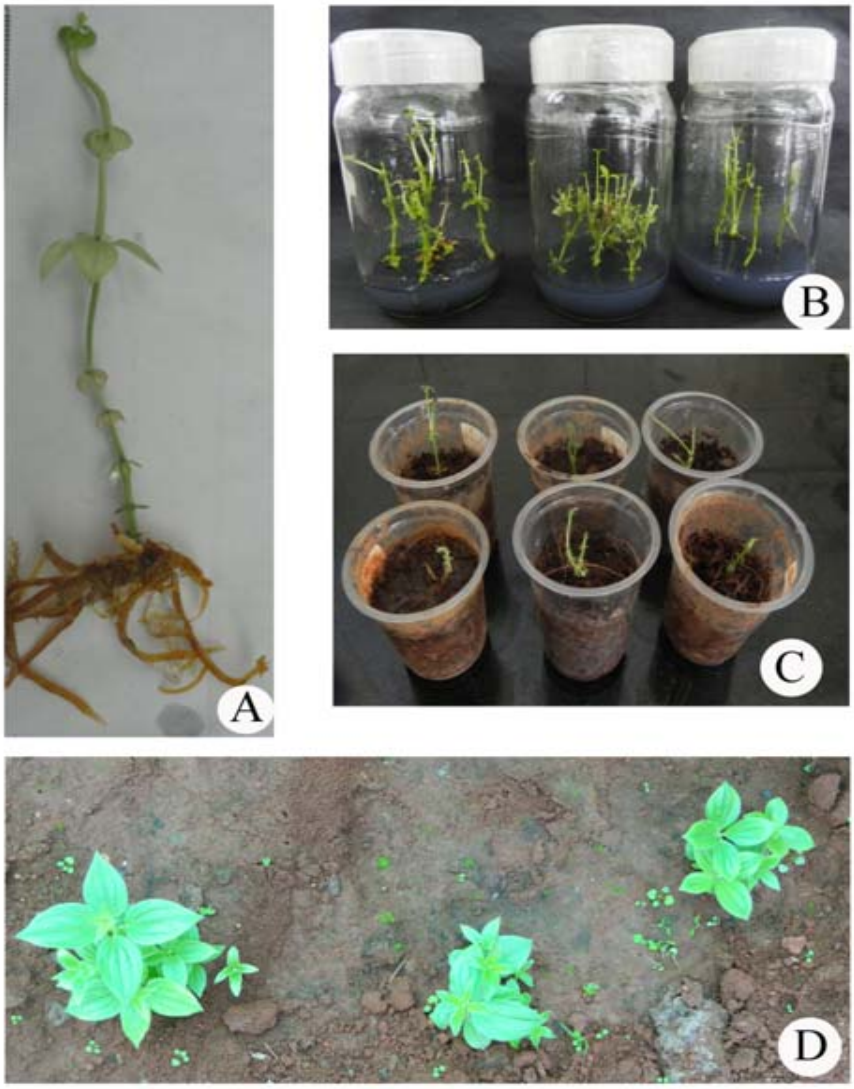

Plate 2 : A. Roots were induced on MS medium containing 1 mg/L IBA and 0.1% activated charcoal of four weeks of culture : B Well- established rooted plants 3 weeks of inoculation
C. Well- established rooted plants after 3 weeks of transfer
D. Surviving transplanted plants in a sandy bed

Shoot proliferation and its maintenance

For conventional shoot propagation, callus clump with adventitious shoots were cut into small pieces and then inoculated on same induction medium to the next 3 week for the multiplication as well as elongation (Table 2). Subcultured callus clump with shoots demonstrated differential responses according to the concentrations of TDZ in the culture media. The medium containing 4.0 mg/l TDZ was significantly different compared to other treatments, as it induced the highest percentage (71.8±3.1%) of callus cultures to form shoots, with the highest number of shoots per callus (24.4±2.2) and the highest mean length of 4.01±0.2cm (Table 2). Healthy shoots were further selected and transferred to the rooting medium. This may work an advantage to reduce time and cost in production of large-scale planting material of *R. cordifolia*.

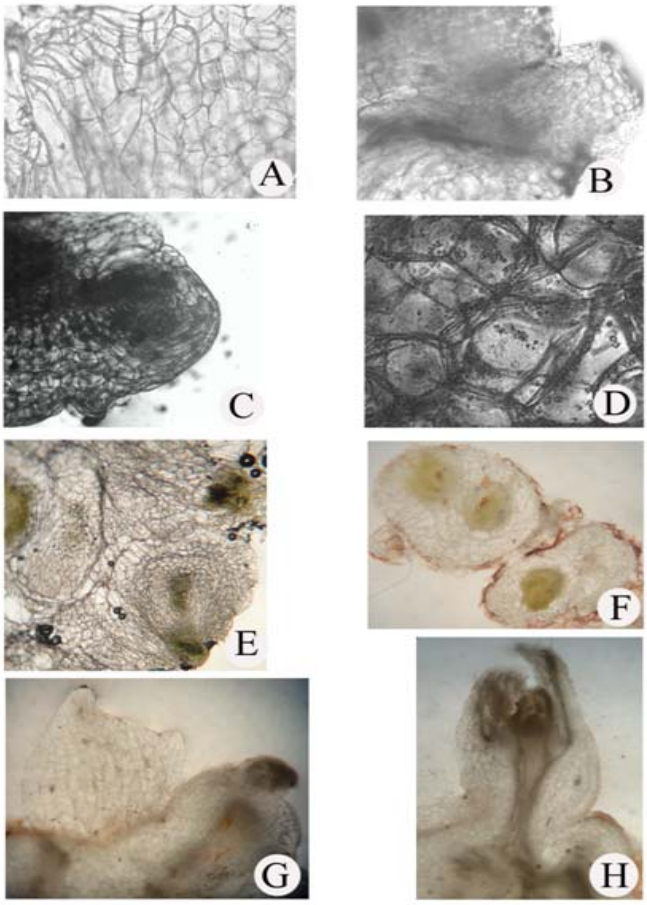

Plate 3: A -callus cells, B -Transverse sections showing initiation of prenodule,
C- Prominent nodular structures with epidermis-like tissue
D -Nodule composed of small, tightly connected cells with starch
E& F- Transverse section of nodule
G -Apical meristem
H- Apical meristem and Shoot bud regenerated from a nodule

Table 2: Effect of different concentration TDZ on regeneration of shoots from leaf and internode explants of *R. cordifolia*

Plant growth regulator PGR(mg/L)	Percentage of callus induction (%)		Percentage of callus cultures with shoots regeneration (%)		Mean number of shoots per callus		Mean shoot length (cm)	
TDZ	Leaf	Internode	Leaf	Internode	Leaf	Internode	Leaf	Internode
MS (basal)	-	-	-	-	-	-	-	-
0.25	61.6a±3.6	60.4a±2.4	41.6±3.2	45.1±3.2	2.1a±0.3	2.8a±1.2	3.3a±0.2	3.3a±0.1
0.5	68.3b±4.1	64.6b±4.7	44.3±3.2	45.8±4.2	6.3b±1.7	4.9b,c±1.2	3.5b±0.1	3.3a,b±0.1
1.0	70.7b,c,d±2.0	70.8c,d±1.8	48.6±2.0	49.9±2.0	9.2c±1.9	5.7c,d±1.3	3.5b±0.1	3.4a,b,c±0.1
2.0	72.6d±3.4	73.0d±3.3	62.4±3.5	52.7±3.1	12.7d±1.9	6.6d±1.4	3.7±b,c0.1	3.5b,d,e±0.1
3.0	78e±3.0	76.9e±2.8	68±3.2	56.2±3.6	15.3e±2.1	8.4e±1.5	3.8c±0.1	3.6e±0.1
4.0	89.3f±3.8	79.7f±3.7	71.8±3.1	61.7±3.1	24.4f±2.2	11.2f±1.6	4.01d±0.2	3.8f±0.1

Values represent the mean ± standard deviation (SD) of three replicated experiments. Within the same column, values followed by different letters are significantly different (P>0.001) according to Tukey: Kramer multiple comparison test

Induction of axillary shoots from nodal explants

Axillary shoot bud induction studies were initiated using TDZ with different concentrations ranging from 0.25-4.0 mg/L. We observed differential axillary shoot induction rate according to the concentration of PGR. However, rate of axillary shoot initiation increases with increasing concentration of TDZ (Table 3, Photo Plate 1 A). Axillary shoot buds were developed directly on nodal explants without callus formation on induction medium after two week of inoculation. Shoot bud initiation was recorded in 87.4% ±2.0 % of nodal explants and maximum (8.1±1.2) shoots per explants were observed in 4 mg/L TDZ supplemented medium. In case of 2, 4-D, KN and NAA only 3-4 shoots were observed at maximum (4 mg/L) concentration (data not shown). As low as two shoots were observed in basal MS medium, which was considered as control. In case of *Gymnema sylvestre* [28], *Holostemma annulare* [29], *Hyptis suaveolens* [30] BA was found suitable for multiple shoot induction from nodal explants but in case of *R. cordifolia* TDZ was suitable at lower (0.25 mg/L) concentration for the same in vitro response[22, 23].

Table 3: Effect of different concentrations of TDZ on auxilary shoots induction from nodal explants of *R. cordifolia*

Plant growth regulator (PGR) (mg/L) / explants (TDZ/ Node)	Percentage of nodal cultures with Shoots regeneration (%)	Mean number of shoots per node	Mean shoot length (cm)
00	93.7±2.0	2.2a± 0.4	3.2a± 0.1
0.25	87.4±2.0	3.0a,b±0.6	3.3b±0.1
0.5	86.8±3.1	4.5c±0.8	3.5c,d±0.1
1.0	80.5±3.1	5.2c,d,e,f±1.0	3.5d±0.1
2.0	81.2±2.0	5.8e,f±1.0	3.7e,f±0.1
3.0	82.6±1.2	6.0f±1.3	3.8f±0.1
4.0	87.4±2.0	8.1g±1.2	3.9g±0.1

Values represent the mean ± standard deviation (SD) of three replicated experiments. Within the same column, values followed by different letters are significantly different (P>0.001) according to Tukey: Kramer multiple comparison test

Root induction and acclimatization

Well developed shoots were derived from multiple shoot clusters and inoculated on different root induction media. Root initiation started two week after inoculation of shoots on the rooting medium. Significant root induction was recorded in 4th week of inoculation (Table 4).

Table 4 : Treatment of different auxins and activated charcoal on root formation from regenerated shoots and plantlet survival of *R. cordifolia*

Plant growth regulators (PGRs)	Concentration (Mg/L)	Percentage of root formation (%)	Mean no. of roots	Mean of root length (cm)	Percentage of Survival rate of transplanted Plants (%)
IBA	0.5	70.8±2.0	3.8a±0.9	3.1a±0.2	100
	1.0	93.7±2.0	4.9b±0.7	4.7b±0.2	100
	2.0	68.7±2.0	4.2a±0.6	3.03a±0.1	100
IAA	0.5	63.1±3.1	2.8a±0.7	2.9a±0.2	100
	1.0	70.8±2.0	4.4b±0.7	3.4b,c±0.2	100
	2.0	61.8±2.4	3.2a±0.5	3.3c±0.2	100
NAA	0.5	64.5±3.6	2.2a±0.4	2.7a±0.2	100
	1.0	65.2±2.4	3.1b±0.7	3.2b±0.1	100
	2.0	59.02±3.1	2.5a±0.6	2.8a±0.2	100
Control	MS				
	½ MS				

Values represent the mean ± standard deviation (SD) of three replicated experiments after two months of transplantation. Within the same column, values followed by different letters are significantly different (P>0.001) according to Tukey: Kramer multiple comparison test

The highest rooting response (93.7±2.0 %) was achieved on the medium supplemented with 1.0 mg/L IBA alone with a mean number of 4.9±0.7 roots per shoot with mean length of 4.7±0.2 cm (Photo Plate 2 A). Comparatively lower response was recorded in case of IAA and NAA containing medium. Giridhar [31] et al. (2001) also reported maximum rooting in IBA as compared to NAA, in case of *Vanilla planifolia*. Root initiation frequency increased at the higher (1.0 mg/L) concentration of IBA and IAA alone. Rhizogenic response in NAA was suppressed at higher concentrations. Our results regarding NAA were in agreement with the study carried out by Radha [6] et al. (2010) in *R. cordifolia*. Plantlets with well developed roots were selected for acclimatization. The complete rooted plantlets were transplanted in sand: compost mixture 1:2 with minimal damage to their root system and were maintained in shade net. Survival of 100 % of plantlet was achieved during hardening for the first 3 weeks under 80 % shading. (Table 4, Photo Plate 2). The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field [32]. The plants showed significant survival over two months and were further transferred to soil

CONCLUSION

In conclusion, an efficient protocol was developed for micropropagation of the species *R. cordifolia* by organogenesis from leaf and stem explants and induction of multiple auxilary shoots from node, completed with a high-frequency rooting protocol. These plant regeneration systems were found to be reliable protocols and may be used in future conservation programs.

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