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Micropropagation of *Russelia equisetiformis* Schlecht & Cham. through nodal segment culture

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ABSTRACT

An *in vitro* regeneration protocol of *Russelia equisetiformis* was established through nodal shoot segments cultured on Murashige and Skoog medium (MS) supplemented with different concentration and combination of cytokinins and auxins in the present study. Cent percent bud breaking response with maximum number of shoots (6.0) was observed on MS medium augmented with 1.0 mg/L 6-benzylaminopurine (BAP). Shoots multiplication further enhanced by repeated subculturing of *in vitro* shoots on MS medium with 1.0 mg/L BAP + 0.5 mg/L Kinetin (Kn) and additives. Maximum 13.2 shoots with 7.8 cm length were differentiated. Healthy shoots were rooted with high frequency (97%) on half strength MS medium containing 1.0 mg/L indole-3 butyric acid (IBA) with maximum number of roots (5.73). The *in vitro* regenerated plantlets were hardened in greenhouse and transferred to nursery with 85% survival rate.

***Keywords*:** *Russelia equisetiformis*, *in vitro*, rooting, hardening, cytokinins tested

1. INTRODUCTION

Russelia equisetiformis Schlecht & Cham. is an important medicinal plant of the family Scrophulariaceae (figwort family), belongs to the tropical America and naturalized throughout the tropical regions as ornamental garden plant due to its attractive bright red flowers (Daziel, 1948). It is commonly known as firecracker plant, coral blows or fountain plant (Watkins and

Sheehan, 1975). It is an evergreen perennial, weeping shrub with reduced scale like leaves, resembles the fern *Equisetum*, hence the species name was assigned as *equisetiformis*. This plant is reported resistant to drought and salinity. The bright red flower with tubular corolla on four angled stem with scaly bracts present attractive look to this plant year round, which pushes gardeners to cultivate it as ornamental nursery plant. It reaches up to the height of four feet and prefers sunny climate for better growth (Riaz *et al.*, 2012).

Photo 1. *Russelia equisetiformis* Schlecht & Cham.

The whole plant is used for the treatment of pain and inflammation (Olorunju *et al.*, 2012), diabetes, leukemia (Oladeinde, 2005) malaria and cancer (Kolawole and Wakeel, 2006), and promotes hair growth (Awe and Makinde, 2009). The active phytochemicals from *R. equisetiformis* are alkaloids, flavonoids, saponins, tannins, steroids and terpenoids (Lorke, 1983; Riaz *et al.*, 2012), phenylethanoid glycosides such as russectinol and russeliaoside (Awe *et al.*, 2005). About 26 phenolic compounds have been identified from this plant like,

caffeoylquinic acids, verbascoside, isoverbascoside, glycosylated flavonoids (Johnson *et al.*, 2011). In addition, this plant is reported to be rich in gallic acid (Johnson *et al.*, 2008), triterpenes, sterols (Burns *et al.*, 2001) and lupeol (Olorunju *et al.*, 2012).

This plant gains pharmaceutical important due to their broad spectrum biological activities. It is reported to possess analgesic, anti-inflammatory, antibacterial, antioxidant, antimicrobial and antinociceptive activity (Awe *et al.*, 2005; 2008; 2009; 2010), free radical scavenging activity, membrane stabilizing properties and cytotoxic activities (Riaz *et al.*, 2012). In addition, it has been reported to possess dose dependent hemolytic effect (Kolawole and Wakeel, 2006), antimalarial and prophylactic ability against *Plasmodium berghi* (Ojurongbe *et al.*, 2015), central nervous system depressant activities (Kolawole *et al.*, 2007; Kolawole and Kolawole, 2010).

Naturally this plant is propagated through root cuttings, and there is no literary reports related to *in vitro* research, tissue culture and micropopagation in this plant. This is the first report on the development of clonal propagation protocol for *R. equisetiformis* through axillary bud proliferation from nodal explants.

2. MATERIALS AND METHODS

2. 1. Plant material and surface sterilization

The fresh and young shoots were harvested from 2 year old plants of *R. equisetiformis*, maintained in the Herbal Garden of the Institute, and brought to Biotechnology laboratory. Explants were collected from January to December, 2014 to study the seasonal effect on culture induction response. Explants were prepared using sterile scissors with one or two nodes and washed with Tween-20 for 10 min by continuous agitation and washed with running tap water. The explants were treated with 0.1% Bavistin (w/v) (systemic fungicide, BASF India Ltd, India) for 6-8 min and surface sterilized with Mercuric chloride (w/v) (0.1%) for 5 min followed by repeated washings with sterile distilled water. The explants were vertically inoculated on the culture medium under aseptic conditions.

2. 2. Culture initiation and culture conditions

The explants were inoculated on nutrient medium consisted of full strength MS (Murashige and Skoog, 1967) salts with 3% (w/v) sucrose, 0.8% agar and additives (50 mg/L ascorbic acid, 25 mg/L each of adenine sulphate, L-arginin and citric acid). Different concentrations of cytokinins (BAP and Kn ranging from 0.0-2.5 mg/L) were incorporated in the culture medium to study the effect of plant growth regulators on culture induction from the nodal explants. The cultures were incubated initially at diffused light for four days and shifted to photon rich area of incubation chamber, where cultures were maintained at 25 ±2 °C temperature under 40 μmol $m^{-2}s^{-1}$ Photosynthetic Photon Flux Density (PPFD) of light for four weeks. The light source was provided by white, cool, florescent tubes.

2. 3. Multiplication of *in vitro* shoots

The *in vitro* raised shoots were continuously subcultured on fresh culture medium. Shoot multiplication frequency was assessed by various concentrations and combinations of cytokinins (BAP and Kn ranging from 0.0-3.0 mg/L) incorporated in MS medium and

additives. The cultures were maintained at 25 ±2 °C temperature and 40 µmol $m^{-2}s^{-1}$ PPFD for 12 h/d light for shoots multiplication.

2. 4. *In vitro* rooting

The multiplied healthy shoots were isolated with appropriate length (4-5 cm) and inoculated on different strength (full, half and one-fourth) of MS medium supplemented with different concentrations of IBA and Naphthalene acetic acid (NAA) ranging from 0.0-3.0 mg/L for *in vitro* root induction. The cultures were incubated under 30 µmol m^{-2} s^{-1} PPFD of diffused light for better root growth and development, and shifted to normal conditions when root induction was observed.

2. 5. Hardening and field transfer

After four weeks of incubation, the *in vitro* rooted plantlets were cautiously removed from culture vessels and washed with sterile distilled water to remove adhered medium from the roots and placed on autoclaved Soilrite® (horticultural grade perlite consists of peat moss and exfoliated vermiculite from Kelperlite, Banglore, India) contained in disposable paper cups, and moistened with one-fourth MS macro-salts between two days interval. The *in vitro* rooted plantlets were maintained for a month in the greenhouse for acclimatization of plantlets. After that the plantlets were shifted to the nursery and the survival rate was calculated.

3. RESULTS AND DISCUSSION

3. 1. Culture initiation

Since the stem of *R. equisetiformis* has small pith and angular stem, attention has been paid in surface sterilization with Mercuric chloride. The duration of sterilization has affected the percent response in culture induction. Four minutes treatment of mercuric chloride supported bud breaking response from nodal shoot segments, but the increased treatment duration had adverse effect. Maximum number of shoots (6.0) with 2.4 cm length was achieved on MS medium supplemented with 1.0 mg/L BAP and additives. Explants harvested during the months of November and December responded cent percentage in culture induction. Induction of shoots have been observed in all the concentrations of cytokinins tested with MS medium (Table 1), but there was no morphogenic response achieved on MS medium without supplementing growth regulators. Selection of suitable explants for better culture response has been extensively studied in number of plant species. Earlier reports suggest that nodal shoot segments plays significant role in culture establishments. Successful regeneration through nodal shoot segments has been reported in *Dioscorea wightii* (Mahesh *et al.*, 2010), *Rubia cordifolia* (Ghatge *et al.*, 2011) and *Passiflora foetida* (Shekhawat *et al.*, 2015b).

Comparatively less number of shoots (4.2) with 2.0 cm length was observed on 0.5 mg/L Kn. The superiority of BAP over Kn was also reported in *Caralluma edulis* (Patel *et al.*, 2014) and *Morinda coreia* (Shekhawat *et al.*, 2015a). The percentage of response in shoot initiation, number of shoots and length of shoots were increased with increasing concentration of BAP up to 1.0 mg/L, thereafter, it was gradually decreased with increased concentration of BAP.

But shoot induction was suppressed on Kn beyond 0.5 mg/L. Similar response observed in *Acalypha indica* (Saikia and Pratap, 2014) and *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012). Increased concentration of BAP and Kn had inhibitory effect in bud breaking of *R. equisetiformis*.

Table 1. Effect of MS medium with different concentrations of cytokinin on shoot induction from nodal explants of *Russelia equisetiformis*.

Conc. of BAP (mg/L)	Conc. of Kin (mg/L)	Response (%)	No. of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
0.00	0.00	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.50	-	86	4.2 ± 0.6^{e}	1.2 ± 0.4^{e}
1.00	-	100	6.0 ± 0.2^{g}	2.4 ± 0.1^{d}
1.50	-	80	5.3 ± 0.5^{f}	1.8 ± 1.0^{c}
2.00	-	77	4.1 ± 0.3^{e}	1.3 ± 0.7^{c}
2.50	-	72	2.8 ± 0.0^{c}	1.2 ± 0.5^{c}
-	0.50	85	4.2 ± 0.7^{e}	2.0 ± 0.8^{c}
-	1.00	80	3.1 ± 0.4^{d}	1.0 ± 1.2^{d}
-	1.50	75	4.0 ± 1.3^{e}	1.4 ± 0.6^{c}
-	2.00	73	3.4 ± 0.4^{d}	1.0 ± 0.2^{c}
-	2.50	65	2.0 ± 0.2^{b}	0.7 ± 0.4^{b}

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

3. 2. Multiplication of *in vitro* shoots

Shoot multiplication was achieved by repeated subculturing of *in vitro* raised shoot clumps on MS medium fortified with additives and cytokinins with four weeks of intervals. Maximum number of shoots (13.2) with 7.8 cm length was regenerated on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L Kn (Table 2). The repeated transfer of shoots to enhance multiplication frequency was also reported in *Tribulus terrestris* (Raghu *et al.*, 2010), *Mimosa pudica* (Hassan *et al.*, 2010) *Lawsonia inermis* (Ram and Shekhawat, 2011), *Ceropegia bulbosa* (Phulwaria *et al.*, 2013) and *Morinda coreia* (Shekhawat *et al.*, 2015a). The repeated subculture supports the formation of new shoots by suppressing the apical dominance, leads to the conversion of somatic cells in to meristematic cells to enhance shoot multiplication frequency (Tripathi and Kumari, 2010).
0.05%.

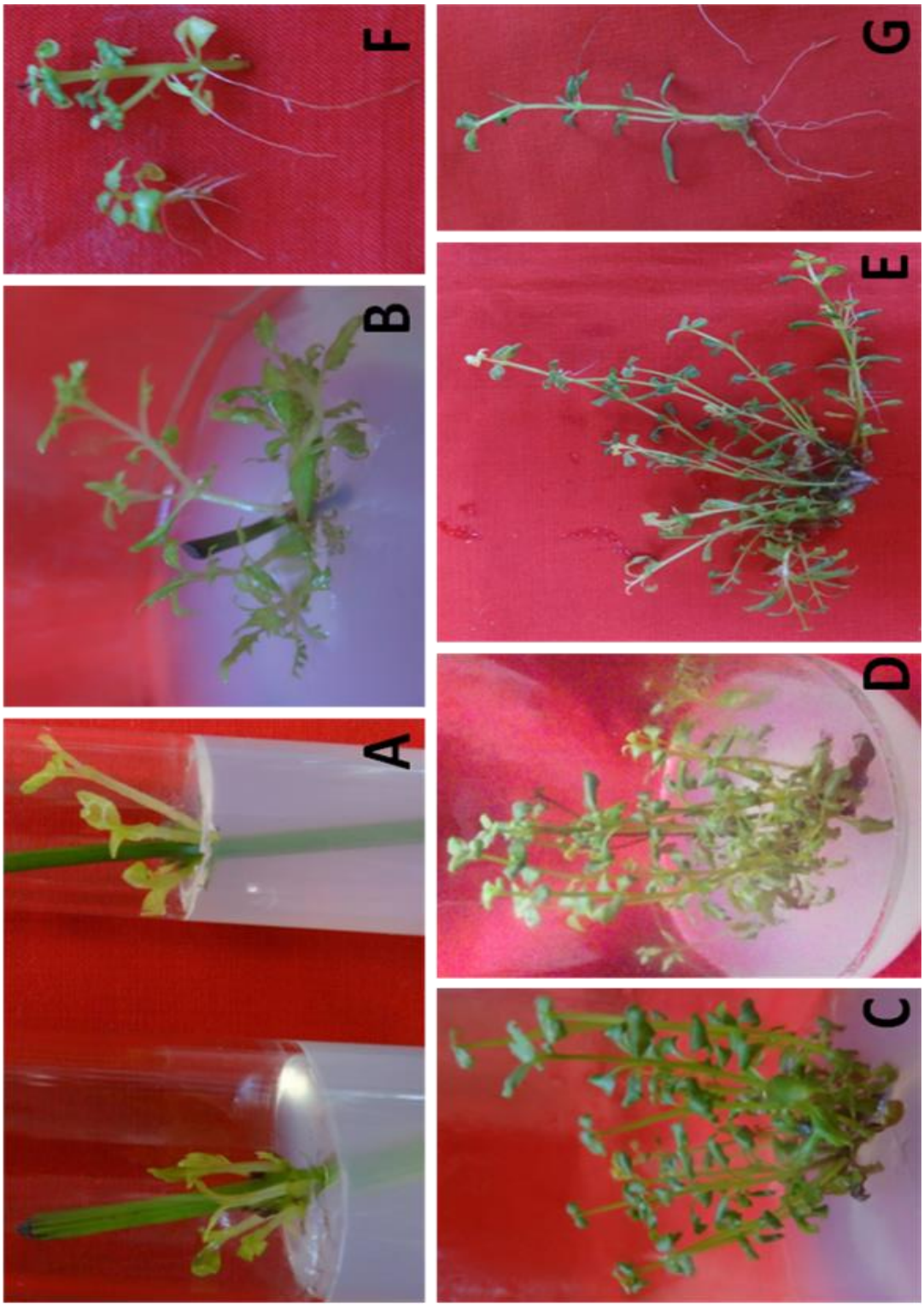

Fig. 1A and B. Induction of shoots from the nodal explants.
Fig. 1C, D and E. Multiplication of shoots on MS medium.
Fig. 1F and G. *In vitro* rooting from the cut ends of the shoots.

A significant increase in number of healthy shoots was observed when additives incorporated in the medium. Synergistic effect of BAP and Kn for shoot multiplication have been achieved in the present study. The highest frequency of shoot multiplication through combined effect of BAP and Kn have also discussed in *Acalypha indica* (Saikia and Pratap, 2014) and *Morinda coreia* (Shekhawat *et al.*, 2015a).

Table 2. Effect of cytokinins (BAP and Kn) on shoot multiplication of *R. equisetiformis*.

Conc. of BAP (mg/L)	Conc. of Kn (mg/L)	No. of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
0.00	0.00	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0.50	0.50	5.92 ± 0.53^{b}	4.43 ± 0.26^{b}
1.00	0.50	13.2 ± 0.47^{d}	7.80 ± 1.30^{c}
1.50	0.50	9.30 ± 0.39^{c}	5.01 ± 0.38^{b}
2.00	0.50	8.64 ± 0.52^{c}	4.28 ± 0.40^{b}
2.50	0.50	8.40 ± 0.36^{c}	4.15 ± 0.27^{b}
3.00	0.50	6.37 ± 0.59^{b}	4.10 ± 0.33^{b}

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

3. 3. *In vitro* root induction of micropropagated shoots and hardening of plantlets

The *in vitro* raised shootlets were transferred to half strength MS medium consisted IBA and NAA for root induction. Among the two different auxins evaluated, maximum response (97%) with highest root number (5.73) with 3.2 cm length were reported on half strength MS medium augmented with IBA 2.0 mg/L. About 82% shoots were rooted with 3.6 roots on half strength MS medium with 1.5 mg/L NAA (Fig. 1F and 1G, Table 3).

Table 3. Effect of different concentrations and combinations of auxins in root induction on half strength MS medium.

Conc. of IBA (mg/L)	Conc. of NAA (mg/L)	Response (%)	No. of roots (Mean ± SD)	Root length (cm) (Mean ± SD)
0.00	0.00	00	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0.50	-	42	3.52 ± 0.36^{e}	2.00 ± 0.61^{c}
1.00	-	54	4.06 ± 0.28^{f}	2.52 ± 0.25^{c}

1.50	-	75	5.55 ± 0.40^{h}	2.79 ± 0.38^{c}
2.00	-	97	5.73 ± 0.26^{h}	3.20 ± 0.26^{d}
2.50	-	80	5.22 ± 0.13^{g}	2.76 ± 0.44^{c}
3.00		76	4.00 ± 0.20^{f}	2.37 ± 0.20^{b}
-	0.50	29	2.84 ± 0.31^{b}	1.64 ± 0.42^{b}
-	1.00	40	3.25 ± 0.25^{d}	2.04 ± 0.36^{b}
-	1.50	73	3.67 ± 0.30^{e}	2.35 ± 0.29^{b}
-	2.00	82	3.00 ± 0.49^{c}	2.57 ± 0.40^{c}
-	2.50	67	2.91 ± 0.54^{b}	2.50 ± 0.33^{c}
-	3.00	54	2.80 ± 0.29^{b}	2.32 ± 0.22^{b}

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

Fig. 2A and B. Hardening of rooted plantlets in the greenhouse.

Similar requirement of reduced concentration of MS salts and superiority of IBA over other auxins for *in vitro* rooting were reported in *Aristolochia bracteata* (Sathish *et al.*, 2011), *Cardiospermum halicacabum* (Shekhawat *et al.,* 2012), *Heliotropium keralense* (Sebastian and Hariharan, 2013) and *Paulownia elongata* (Zayova *et al.*, 2014). The *in vitro* rooted plantlets were carefully transferred to sterile Soilrite® and moistened with one-fourth MS salts and maintained in greenhouse for four weeks (Fig. 1A and 2B).

The success of micropropagation of any plant species lies in the establishment of plantlets in soil. The plantlets with well developed shoot and root system were transferred to the herbal garden and 85% rate of survival has been recorded in present study.

4. CONCLUSION

There is no tissue culture research in *R. equisetiformis* to date. This is the first attempt in deriving stable micropropagation protocol for the horticulturally and medicinally important plant *R. equisetiformis*.

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