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In vitro Multiple shoot induction directly from Different Explants of *Gardenia resinifera* Roth

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The study has provided a protocol for rapid *in vitro* propagation by multiple shoot induction of *Gardenia sinifera*, an important medicinal plant belonging to Rubiaceae family. Multiple shoots were induced *in vitro* from the nodal segments and immature fruit segments on Murashige and Skoog (MS) medium containing 6-benzylaminopurine (BAP) alone or in combination with naphthalene acetic acid (NAA), IAA, IBA and 2, 4–D (2, 4-dichlorophenoxy acetic acid). Internodal segments in combination with BAP (2mg/l) + NAA (0.25 mg/l) observed two shoots and in immature fruit segments maximum number of shoots (4 shoots per explant) was observed on the medium containing BAP (2 mg/l) in combination with NAA (0.5mg/l). Regenerated shoots were rooted on 1/2 MS supplemented with IAA (0.5) proved best rooting from nodal segments. The rooted *in vitro* raised plantlets were acclimatized in growth chamber and successfully transferred to greenhouse with 80% survival rate. The regeneration protocol developed in this study provides a basis for germplasm conservation and for further investigation of medicinally active constituents of the elite medicinal plant.

Key words: Acclimatization, Germplasm conservation, *in vitro* propagation, Nodal segments, Immature fruit segment, *Gardenia resinifera*

Plants valued with medicine are of great interest to the researchers in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds (Chand *et al*., 1997). Herbal medicines are the precursors of many common drugs prescribed in clinical practice in many countries today. Furthermore, herbs and herbal products are still an important part of the primary health care systems in many parts of the world (Jawahar *et al*., 2008). *In vitro* culture techniques offer a viable system for true-to-type rapid mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora & Bhojwani, 1989; Sharma *et al*., 1991; Sudha & Seeni, 1994; Sahoo & Chand, 1998; Karuppusamy & Pullaiah, 2007; Mallon *et al*., 2010).

In vitro micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations (Nehra and Kartha, 1994).Outstanding findings in plant cell and tissue culture - an important discipline of biotechnology have generated great optimism both in developed and developing countries (Mehra, 1993; Popelka *et al*., 2004).

Gardenia resinifera Roth. is related to Rubiaceae family, which parades a long list of plants of medicinal importance. It is shrub or small tree and bears small leaves and secretes gummy matrix or sap at the detached end of ear and stem. This substance known as Dikamali or cumbi-gum. Cumbi gum is antispasmodic, expectorant, diaphoretic, carminative, antihelmintic, relieves

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constipation, pain treat worms. Gum is antimicrobial, anthelmintic; used in skin diseases. According to Ayurveda it increases appetite, astringent to bowels, relieves pain of bronchitis, vomiting and constipation.

The aim of the present study was to establish an effective protocol for multiple shoot induction from *G.resinifera.*

MATERIALS AND METHODS

The explants material (Shoot tip, node internode, leaf and immature fruit segments) were collected from Kakatiya arboretum, Warangal district, A.P. Fruits were procured from Tirumala foot hills, Thirupathi, Chittor district, A.P. The healthy seeds were separated and stored for the experimental work. Presoaking of explants in anti oxidant solution (100mg/l PVP + 100-500mg/l Ascorbic acid + 50-100mg/l Citric acid) for 30 minutes for removing gum substances and phenolic compounds.

The explants were taken in a 500ml clean sterile Erlenmeyer flask and washed with 1% (v/v) tween-20 solution for 20 minutes and then washed with tap water thoroughly. Further operations were carried out in the laminar flow chamber. Seeds were subjected to 70% alcohol for 30 seconds and washed with sterile distilled water. Then the explants were disinfected with 0.1% (w/v) mercuric chloride ($HgCl_2$) for different time intervals (2 or 3 min) followed by thorough washing with sterile distilled water.

The surface sterilized explants were then aseptically inoculated on sterile MS medium having a wide range and combinations of plant growth regulators (2, 4-D, IAA, IBA, NAA, BAP, KN and TDZ) and pH of the medium was adjusted to 5.8 before autoclaving at 121^0C for 15 minutes. 0.5-3% of Activated Charcoal added to the culture medium to remove Phenolic compounds from the explants produced during the culture period.

All the cultures were initially kept in dark for 2 days to avoid the problem of releasing phenolic compounds and gum resins from explants inoculated. These were shifted to a growth chamber maintained at 25 ± 2^0C 16h photo period provided by cool white florescent tubes (Philips, India) (Ca.2000-2500 lux) for further observations.

Frequent observations were done and contaminated tubes were removed carefully. For callus studies, observations were recorded after 30 days of incubation for each experiment minimum of 50 tubes were maintained and all experiments were repeated thrice. The average data for all the above experiments were tabulated and was analyzed statistically by Analysis of variance (ANOVA) was applied for signifying the results.

For root induction, *in vitro* developed shoots were excised and cultured onto MS medium supplemented with different concentrations (mg/l) of IAA, IBA and NAA. The rooted plantlets were taken out from the culture tubes and washed to remove adhered agar and traces of medium. Plantlets were then transferred to plastic pot containing vermiculite and sand (3:1). These plants were maintained inside the growth chamber or 15 days and irrigation was gently perforated once per day. Afterwards, the plants were grown under greenhouse conditions.

RESULTS AND DISCUSSION

In order to establish an efficient *in vitro* micropropagation for *G. resinifera* from explants (Shoot tip, node and immature fruit segment) the experiments were conducted. Explants were incubated on MS medium supplemented with varying levels of BAP, KN (0.1 to 10mg/l) and TDZ (0.05, 0.5 and 1.0 mg/l).

Shoot tips were cultured on MS medium supplemented with BAP (2 mg/l) given one shoot with average shoot length 4.58 cm (Fig 1) and percentage of response was 70. In case of nodal segments, fortified with BAP (2mg/l) produced single shoot with average shoot length 5cm (Fig 2) and percentage of response was 85. In immature fruit segment, BAP (2 mg/l) resulted one shoot with average shoot length 1cm and percentage of response was 35.

The results showed that in internodal segments in combination with BAP (2mg/l) + NAA (0.25 mg/l) observed two shoots with average shoot length 3.75cm (Fig 3). In BAP (2mg/l) + IAA (0.1 mg/l) also given two shoots with average shoot length 3.53cm (Table1).

When immature fruit segments cultured on MS medium in the presence of BAP (2mg/l) + NAA (0.5mg/l) showed four shoots with average shoot length 1.79cm (Fig 4). BAP (2mg/l) + IAA

Table 1. Effect of BAP (2mg/l) with the combination of various auxin concentrations on multiple shoot formation from nodal segments cultured on MS medium in *Gardenia resinifera*

BAP	NAA	IAA	IBA	2,4-D	% of Response	No.of Shoots *Mean ± S.D	Shoot length *Mean ± S.D
2	0.1				70	3.22 ± 0.14	4.10 ± 0.13
2	0.25				60	2.95 ± 0.20	3.75 ± 0.43
2	0.5				65	2.55 ± 0.38	3.65 ± 0.13
2	1.0				60	2.53 ± 0.29	3.62 ± 0.17
2		0.1			50	2.50 ± 0.27	3.53 ± 0.08
2		0.25			55	2.45 ± 0.19	3.51 ± 0.13
2		0.5			50	2.40 ± 0.17	3.50 ± 0.22
2		1.0			60	2.33 ± 0.12	3.45 ± 0.19
2			0.1		45	2.23 ± 0.10	3.40 ± 0.13
2			0.25		50	2.20 ± 0.27	3.25 ± 0.15
2			0.5		45	2.16 ± 0.40	3.16 ± 0.16
2			1.0		50	2.15 ± 0.07	3.12 ± 0.06
2				0.1	35	2.15 ± 0.07	3.10 ± 0.07
2				0.25	-	-	-
2				0.5	30	2.14 ±0.07	3.05 ± 0.03
2				1.0	-	-	-

*While looking at the results of ANOVA, it can be said that the F-value is found to statistically significant at the 1% level. Therefore it can be concluded that the null hypothesis is rejected and the alternative hypothesis of significant response is accepted.
- No response

Table 2. Effect of BAP (2mg/l) with the combination of various auxin concentrations on multiple shoot formation from immature fruit segment cultured on MS medium *Gardenia resinifera*

BAP	NAA	IAA	IBA	2,4-D	% of Response	No.of Shoots *Mean ± S.D	Shoot length *Mean ± S.D
2	0.1				60	2.00 ± 0.21	1.56 ± 0.17
2	0.25				65	3.00 ± 0.53	1.72 ± 0.15
2	0.5				60	4.23 ± 0.38	1.79 ± 0.13
2	1.0				70	5.00 ± 0.49	1.85 ± 0.08
2		0.1			55	2.00 ± 0.21	1.55 ± 0.16
2		0.25			-	-	-
2		0.5			50	1.00 ± 0.00	1.52 ± 0.16
2		1.0			45	1.00 ± 0.00	1.10 ± 0.10
2			0.1		-	-	-
2			0.25		-	-	-
2			0.5		40	1.00 ±0.00	1.30 ± 0.16
2			1.0		-	-	-
2				0.1	-	-	-
2				0.25	-	-	-
2				0.5	-	-	-
2				1.0	-	-	-

*While looking at the results of ANOVA, it can be said that the F-value is found to statistically significant at the 1% level. Therefore it can be concluded that the null hypothesis is rejected and the alternative hypothesis of significant response is accepted.
- No response

Fig. 1. BAP (2mg/l) on shoot regeneration from shoot tip explant cultured on MS medium

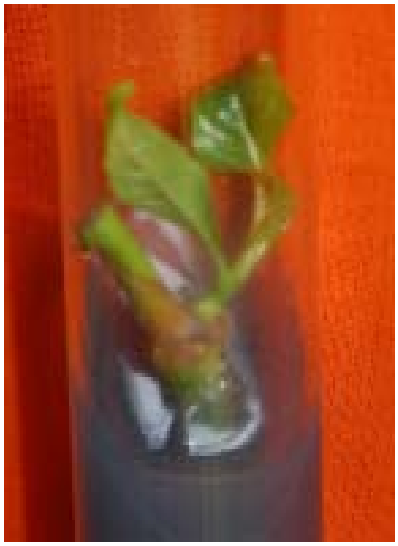

Fig. 2. BAP (2mg/l) on shoot regeneration from nodal explant on MS medium

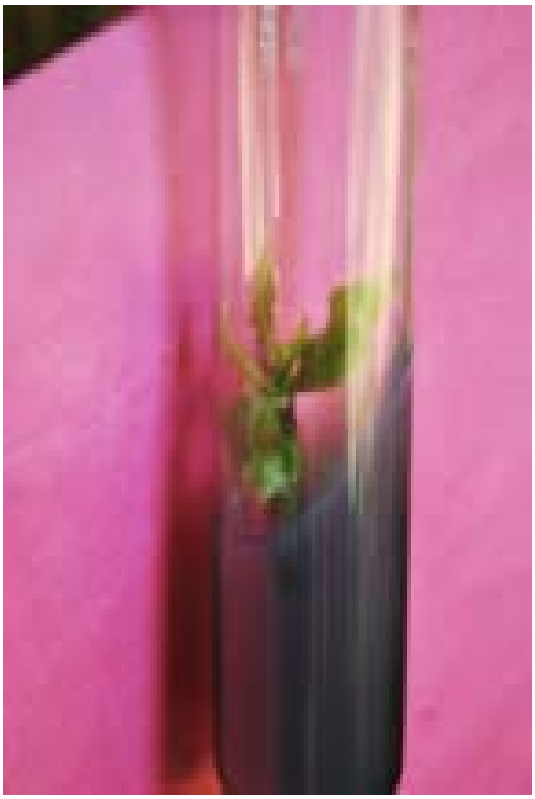

Fig. 3. BAP (2 mg/l) in combination with NAA (0.1 mg/l) on shoot regeneration from nodal segment

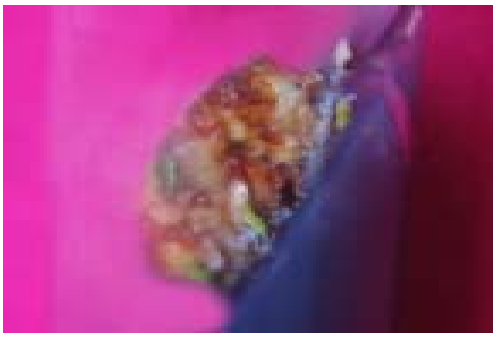

Fig. 4. BAP (2 mg/l) in combination with NAA (0.5 mg/l) on shoot regeneration from immature fruit part without pericarp

Fig. 5. Rooting from i*n vitro* regenerated shoot from nodal explants on IAA (0.5mg/l)

Fig. 6-7: *In vitro* raised plants transferred to plastic containers filled with vermiculite

(0.1 mg/l) responded two shoots with average shoot length 1.55cm. Among all auxins NAA showed more response followed by IAA, IBA and 2, 4–D (Table 2).

Multiple shoots were reported in several plants such as *Veronia amygdalina* (Mohmed *et al*., 2007), *Solanum nigrum* (Sridhar and Naidu, 2011), *Ocimum basilicum* (Daniel *et al*., 2010), *Bacopa monnieri* (Sudir Sharma *et al*., 2010), *Plumbago zylanica* (Chinnamadasamy *et al*., 2010), *Magnolia obovata* (Nakamura *et al*., 1995), *Aegle marmelos* (Das *et al*., 2008), *Vitex negundo* (Mohmmad *et al*., 2008).

Promotion of shoot bud regeneration by BAP either alone or in combination with IAA, has been reported in several other species such as *Spilanthus acmella* (Anjali *et al*., 1997), *Withania somnifera* (Saritha, 2004). The effect of BAP and NAA on direct shoot regeneration was also reported in *Bacopa monnieri* (Tiwari *et al*., 1998) and *Clerodendrum inerme* (Baburaj *et al*., 2000). Among the auxins tested in combination with BAP and IAA proved to be better for providing number of shoots were reported in *Cajanus Cajan* (Misra, 2002), *Plumbago* species (Das and Rout, 2002), *Lilium* (Bacchetta *et al*., 2003) and *Spilanthus acmella* (Saritha *et al*., 2003).

The regenerated shoots were excised and placed on the 1/2 MS medium supplemented with different concentrations of auxins IAA, IBA and NAA. The small outgrowths were observed after one week and they gradually grew into roots. Nodal segments when fortified with 1/2 MS + IAA (0.5) proved best rooting with average shoot length 3.84cm (Fig 5) followed by IBA and NAA.

The hardening process of *G.resinifera* was carried out by transferring rooted plantlets shifted to plastic containers containing vermiculite and sand (3:1) (Fig 6 & 7) and covered with big polythene bag and kept for 15 days. Small holes were made on the polythene bag to reduce the relative humidity. Slowly the width of the holes was increased until the relative humidity inside the polythene bag and out-side the chamber comes to equal. Finally polythene bags were removed and the pots were kept in diffuse light for 15 days and finally plants were shifted to pots for acclimatization and they were exposed to the sunlight. The regenerated plants did not show any noticeable morphological variations when compared with mother plant. Frequent observations were made and they were growing very healthy than *in vivo* raised plants. The comparative physio-morphological studies are to be carried out in vivo plants with the plants raised by *in vitro*.

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