Micropropagation of *Clerodendrum aculeatum* through adventitious shoot induction and production of consistent amount of virus resistance inducing protein

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Rapid micropropagation through adventitious shoot induction from *in vitro* raised leaf explants of *Clerodendrum aculeatum* (Verbenaceae), was successfully achieved for the first time. Basal portion of the leaves showed highest regeneration potential when grown on MS medium supplemented with BA (5.0 mg/l) and NAA and IBA (0.5 mg/l of each). Shoots after elongation in growth regulator-free medium, were rooted in MS medium containing 0.5 mg/l of NAA and IBA. Aqueous leaf extract of *in vitro* raised plants, induced high degree of resistance against viruses in susceptible healthy hosts when applied prior to virus inoculation. Upon purification from leaves of cultured plants, the resistance inducing protein, showed molecular mass of 34 kDa. Amount of resistance inducing protein obtained from leaves of cultured plants, was consistent throughout the year, as compared to the protein isolated from leaves of field grown plants, which showed marked seasonal fluctuation. The purified 34 kDa protein from *in vitro* raised plants, was serologically related to field grown plants and possessed similar characteristics. The micropropagated plants were successfully established in earthen pots under greenhouse conditions.

**Keywords:** Adventitious shoot, *Clerodendrum aculeatum*, Micropropagation, Resistance inducing protein, Virus resistance

Many angiospermic plants have ability to resist attack of virus infection. The immunity against viruses is usually associated with two types of proteins present in the healthy plants. One category of the proteins influence virus infection by inhibiting eukaryotic protein synthesis. The second category of proteins prevent virus infection by invoking systemic resistance in susceptible healthy plants. The leaf extract of *Clerodendrum aculeatum* (CA) when applied on the leaves of susceptible hosts, prior to virus challenge, induced strong systemic resistance at the site of application (local induced resistance) as well as away from the site of application (systemic induced resistance). Purification and characterization of *C. aculeatum* systemic resistance inducing protein (CA-SRIP) yielded a protein of molecular weight 34 kDa. The induction of resistance was a host mediated phenomenon. Application of CA-SRIP resulted in formation of a new virus inhibitory agent (VIA—a protein) in treated plants which actually checked the virus infection/replication in susceptible hosts producing any phytotoxic effect in susceptible plants. Cloning and characterization of gene encoding the systemic resistance inducing protein from *C. aculeatum* has been done. Antiviral protein was isolated from callus culture of *Boerhaavia diffusa* and plantlets established under *in vitro* conditions.

*In vitro* propagation of the genus *C. aculeatum* (containing systemic resistance inducing protein) has been done for the first time, in view of the antipathogenic importance of the leaf extract of this plant. *C. aculeatum* plant is mainly grown for hedge purpose and can be easily macropropagated through cuttings. Since there is great fluctuation in the amount of CA-SRIP obtained from the leaves of this plant in different seasons, there is an urgent need to micropropagate this plant and to see whether micropropagated plants also contain this SRIP, if so, in what quantity and whether the amount of protein is consistent in micropropagated plants.

The present work describes a regeneration system for the plant from *in vitro* raised leaf explants, to

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**Abbreviations**

BA—Benzyol adenine; 2,4-D—2,4-Dichloro phenoxy acetic acid; IAA—Indole acetic acid, IBA—Indole butyric acid; IPA, Indole propionic acid; GA—Gibberellic acid; NAA—Naphthaline acetic acid
demonstrate that micropropagated plants in modified MS medium, contained higher amount of the resistance inducing protein (CA-SRIP) as compared to field grown plants, and also the micropropagation protocol of *C. aculeatum* could be exploited for its mass propagation in short period of time as an alternative approach for consistent and uniform production of high amount of virus resistance inducing protein throughout the year.

**Materials and Methods**

**Maintenance of cultures**—Shoot culture of *Clerodendrum aculeatum* was established as axenic micropropagated culture from nodal segments. Single shoot cultures were maintained by subculturing nodal segments and shoot tips every four week interval on Murashige & Skog (MS) medium. The pH of the medium was adjusted to 5.8 with 0.1 M NaOH before autoclaving. The cultures were kept at 27±2°C under white fluorescent light for 16/8 hr photoperiods per day.

**Regeneration medium**—Newly formed leaves were excised from vigorously growing micropropagated shoots from 4 week old cultures. Leaves were cut into 4-5 mm² segments and placed on the medium under aseptic conditions. Regeneration experiments were conducted in test tubes or in flasks using 25 or 50 ml MS medium. The medium was supplemented with different concentrations of BA or kinetin in combination with NAA and IBA. Sucrose (3%) was used as the carbon source. Effect of different concentrations of GA (2.5, 5.0 and 10 mg/l) was tested on the elongation and further growth of shoots. The pH of the medium was adjusted to 5.8 prior to sterilization by autoclaving. The cultures were maintained at 27±2°C and 16 hr light/ 8 hr dark photoperiod. Explants (20) per treatment were taken and each experiment was replicated 5 times.

**Rooting and hardening**—Individual shoots of 3-4 cm length were transferred to the root induction medium (modified from original MS medium (mg/l) - NH₄NO₃=500; KNO₃=1250; CaCl₂.2H₂O=220; KH₂PO₄ = 340). The rest of the ingredients were same as in the original MS medium.

Modified MS medium was supplemented with a range of combinations of each NAA and IBA from 0.25 to 1.0 mg/l. The plantlets after 30 days of rooting were removed from the culture flasks and washed under running tap water to remove the adhering agar. Washed plantlets were transplanted in potting mixture of sand and soil (1:2) in 10 cm nursery pots after acclimatization for 2 weeks in culture room at 27±2°C. Fully acclimatized *in vitro* raised plants of *C. aculeatum* after six weeks were transferred to big earthen pots.

**Comparison of CA-SRIP from leaves of micropropagated and field grown plants**

A comparative study of proteins isolated from the leaves of field grown and micropropagated plants was done, using column chromatography, spectrophotometry, SDS-PAGE and Western blot techniques following the protocol of Verma et al.

**Purification of Clerodendrum aculeatum**—Details of techniques for protein purification using column chromatography were followed from Verma et al. For purification, leaves (25 g) were taken. Basic protein sample purified from SP-Sepharose fast flow cation exchange column was used for comparative studies.

**Quantitative analysis**—Quantitative comparison of basic proteins from both field and culture samples was done by comparing protein bands on SDS-PAGE using markers obtained from Pharmacia (α-lactalbumin, Mr= 1404 kDa; soybean trypsin inhibitor, Mr = 20 kDa; carbonic anhydrase, Mr = 30 kDa; ovalbumin, Mr = 43 kDa; BSA, Mr = 67 kDa; and phosphorylase b, Mr = 94 kDa) and estimating the protein concentration by adopting Lowry’s procedure taking bovine serum albumin (BSA) as standard on Bausch and Lomb Spectronics 2000 spectrophotometer.

**Qualitative analysis**—Qualitative comparison was done by testing the resistance inducing activity using *Nicotiana tabacum* cv. Samsun ‘NN’ host against Tobacco mosaic virus following methodology used by Verma et al. (1996). Serological testing was done by using Agar gel diffusion and Immunoblotting.

**Results and Discussion**

First experiment was directed to maintain single shoot cultures of *C. aculeatum* from nodal segments on MS medium. Leaves for adventitious shooting for *in vitro* propagation were taken from these *in vitro* established shoots. Development of first adventitious shoot on leaf explants from vigorously growing four week old culture took place without any intermediate callus phase. Differentiation was independent of factor that which side
of leaf was in contact with the medium. Formation of adventitious shoots occurred from either side\(^{15}\). Basal and middle portions of leaves were more suitable for regeneration. The present investigations have thus demonstrated that MS medium was suitable for shoot differentiation in leaf explants of \textit{C. aculeatum} (Table 1, Fig. 1).

Explants on growth regulator-free medium did not produce any new shoots (Table 1). Leaf explants were grown on shoot regeneration medium containing BA and NAA+IBA. Within 3-4 weeks, adventitious shoot formation was observed at the mid-vein of the basal portion of the leaf (Fig. 1a). This indicated that meristematic activity might be located in the transition zone area between leaf and stem tissue or specific cells at the transition zone might be responsible for initial adventitious shoots\(^{15}\).

BA was the most effective cytokinin for shoot bud differentiation, whereas kinetin was ineffective for differentiation. Superior effect of BA over kinetin was noticed by Siril and Dhar\(^{17}\). BA treatment resulted in high number of multiple shoot formation. Shoot regeneration from leaf explants increased with increasing concentration of BA. Of the three concentrations of BA (1.0, 2.5 and 5.0 mg/l), maximum number of shoots (10±1) were recorded from leaf explants on medium supplemented with 5.0 mg/l of BA. On further increasing the concentration of BA, the shoot production and number of explants forming shoots was reduced. Negative effect of higher concentration

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>Frequency of explants response (%)</th>
<th>No. of shoots per explants ±SEM</th>
<th>Growth of shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA+IBA</td>
<td>BA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0+0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>10±1.0</td>
<td>1±0.10</td>
<td>++</td>
</tr>
<tr>
<td>2.5</td>
<td>50±5.0</td>
<td>6±0.35</td>
<td>+++</td>
</tr>
<tr>
<td>5.0</td>
<td>80±9.0</td>
<td>10±1.0</td>
<td>+++</td>
</tr>
<tr>
<td>0.5+0.5</td>
<td>1.0</td>
<td>15±2.5</td>
<td>++</td>
</tr>
<tr>
<td>2.5</td>
<td>58±6.3</td>
<td>9±0.5</td>
<td>+++</td>
</tr>
<tr>
<td>5.0</td>
<td>83±8.8</td>
<td>15±1.9</td>
<td>++++</td>
</tr>
</tbody>
</table>

(-) No response; (+) growth response; Medium MS

Table 1—Effect of different growth regulators on shoot initiation from leaf explants of \textit{in vitro} raised \textit{C. aculeatum} after four weeks
[Values are mean ± SD of 5 replications]
of BA on shoot production was also reported by Vardja and Vardja\textsuperscript{18}. Incorporation of auxins along with BA helped not only in early shoot bud differentiation but also improved the number of shoots per explant. Combination of auxins and cytokinin was also very effective for the growth of regenerated shoots and for proper shape of leaves in differentiated shoots. Positive effect of cytokinins and auxins for shoot differentiation has been observed by several workers in other plant species\textsuperscript{19}. Maximum number of shoots were obtained in medium containing 5.0 mg/l of BA along with 0.5 mg/l of each NAA and IBA after four weeks (Fig. 1a; Table 1).

Multiple shoots produced on BA supplemented medium required subculturing after every 3-4 weeks otherwise shoots became necrotic. Therefore, cultures were maintained as multiple shoots in subsequent passages when the multiple shoots were transferred to MS medium devoid of growth regulator specially BA (Fig.1b). Shoot deterioration in presence of higher concentration of BA was noticed by many workers in other plants. According to Yi \textit{et al.}\textsuperscript{20} inhibitory effect of BA might be due to extensive endogenous accumulation of cytokinin. Negative effect of BA on elongation of axillary buds indicated that high cytokinin was only needed for shoot initiation and varying ratio of auxins and cytokinins may be required to enhance axillary shoot proliferation as was also reported by Blakesley and Constantine\textsuperscript{21}. This indicated that one of the possible role of auxin in shoot multiplication stage may be to nullify the suppressive effect of high cytokinin concentration on shoot elongation and restore normal shoot growth as stated by Lundergan and Janick\textsuperscript{22}.

GA was not necessary for shoot induction, but 2.5 mg/l of GA was needed for shoot elongation (Fig. 1c). Further, higher concentration of GA influenced negatively the growth of the developed shoots suggesting that it was not necessary for shoot proliferation as observed by Anchora \textit{et al.}\textsuperscript{23} for shoot elongation and hastening the growth of already formed organs. In \textit{vitro} developed shoots were better rooted on modified MS medium containing combination of auxins (Table 2). Rooting behaviour of subcultured shoots was affected by medium composition, auxin type and concentration used. Among different auxins used (NAA, IAA, IBA, 2,4-D and IPA), the retrieved shoots rooted best in medium containing IBA followed by NAA and other auxins. Superior effect of IBA has also been suggested by several earlier workers\textsuperscript{17,24}. Optimum rooting was obtained in medium

<table>
<thead>
<tr>
<th>Auxins</th>
<th>Per cent rooting ±SEM</th>
<th>Root initiation Days ±SEM</th>
<th>No. of roots/shoot ±SEM</th>
<th>Length of roots (cm) ±SEM</th>
<th>Growth of shoots</th>
<th>Callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 (mg/l)</td>
<td></td>
<td></td>
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<tr>
<td>NAA</td>
<td>80±10.0</td>
<td>15±1.5</td>
<td>20±2.5</td>
<td>3.0±0.2</td>
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<tr>
<td>IAA</td>
<td>60±9.5</td>
<td>17±1.0</td>
<td>12±1.2</td>
<td>2.0±0.1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
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<td>12±1.0</td>
<td>20±2.5</td>
<td>3.0±0.5</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>2,4-D</td>
<td>10±0.5</td>
<td>20±2.5</td>
<td>4.0±0.5</td>
<td>0.5±0.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IPA</td>
<td>50±3.5</td>
<td>17±1.2</td>
<td>8.0±1.0</td>
<td>1.0±0.5</td>
<td>+</td>
<td>+</td>
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<tr>
<td>0.5±0.5 (mg/l)</td>
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<td></td>
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<tr>
<td>NAA+IBA</td>
<td>100±8.0</td>
<td>15±1.5</td>
<td>35±2.5</td>
<td>5.0±0.1</td>
<td>+++++++</td>
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<tr>
<td>IAA+IBA</td>
<td>80±5.0</td>
<td>15±1.0</td>
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<td>3.0±0.5</td>
<td>+++</td>
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</tr>
<tr>
<td>IPA+IBA</td>
<td>60±5.0</td>
<td>17±2.0</td>
<td>15±1.5</td>
<td>2.0±0.2</td>
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<tr>
<td>NAA+IBA (mg/l)</td>
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<tr>
<td>0.25</td>
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<td>20±1.0</td>
<td>4.0±0.5</td>
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<td>0.5</td>
<td>100±9.0</td>
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<td>35±3.0</td>
<td>5.0±0.5</td>
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<td>10±1.0</td>
<td>2.0±0.1</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

(-) No response; (+) Growth response
containing low levels of salts. High salt concentration, regardless of type of growth regulator present, was ineffective in root initiation as also experienced by Polistty et al.\textsuperscript{26}. Combination of IBA and NAA significantly improved the rooting response. Hundred percent rooting was obtained in modified MS medium containing 0.5+0.5 mg/l NAA+ IBA (Table 2, Fig. 1d). Audus\textsuperscript{28} and Weaver\textsuperscript{27} have reported that combination of auxins is more effective in induction of rooting than either of them used individually. It is necessary to avoid treatments of auxins which causes callusing of shoots to be rooted. This agrees with the assumption that low level of auxins has synergistic effect on shoot growth, while higher concentration was not beneficial because of callusing\textsuperscript{28}. Excessive callusing at the basal end of the shoots resulted in low survival rate of hardened shoots. This could be due to poor vascular connection of shoots with roots which resulted in improper nutrient supply. In vitro raised plants after proper washing in tap water were transferred into \textit{1/4} strength of liquid modified medium. Plants were covered with polythene bags perforated with minute holes to maintain humidity and kept in culture room for 12 days and then transferred to pots in glass house conditions. Hardening of potted plants was essential for better survival and growth of culture raised plants. Survival percentage of potted plants was about 70%. Six month old plants propagated through tissue culture did not show any morphological abnormality when compared with naturally field grown plants (Fig. 1e).

Tests for systemic resistance inducing virus inhibitory activity in micropropagated plants was done in total protein fraction obtained after precipitation in ammonium sulphate (60%) followed by dialysis in 0.02 M of sodium acetate buffer. On SDS-PAGE, the sample showed the presence of 34 kDa protein band as reported by Verma\textit{et al.}\textsuperscript{4} in field grown plants (Fig. 2). Amount of protein (mg/ml) and the resistance inducing activity in the protein fraction obtained from leaves was very high. Protein fractions from stem and roots showed lesser activity as compared to leaf samples. Protein (20 µg/ml) from leaf was required for complete inhibition of virus while 120 and 200 µg/ml of proteins was needed from stem and root respectively for hundred per cent inhibition of virus. Amount of protein in leaves (25 g) was 2.0 mg/ml, while in stem and root was 0.5 and 0.5 mg/ml respectively. Presence of high amount of 34 kDa protein on SDS-PAGE in leaf sample than stem and root sample could be responsible for its high virus inhibitory activity.

On highly sensitive western blot test, all stem, leaf and root samples reacted positively against 34 kDa CA-SRIP antiserum. However, in less sensitive agar gel diffusion test, positive reaction was seen only with
sample from leaf, which could be because of presence of high amount of CA-SRIP in the leaf samples as compared to stem and root samples. Results showed same qualitative behaviour of resistance inducing protein isolated from cultured leaves, as obtained from field grown plants. However, in micropropagated plants the resistance inducing protein was also present in the stem and roots.

Proteinaceous antiviral substances have also been reported from tissue cultured plants like Phytolacca americana\textsuperscript{29}, Agrostema githago\textsuperscript{30}, Mirabilis jalapa\textsuperscript{31}. These proteins have not been shown to induce resistance against viruses, but inhibit the virus replication by inactivating the host ribosomes, thereby blocking the protein synthesis. Because of their non-systemic protective effect, they did not find wider use in virus disease control. Mechanism of protection through CA-SRIP is a host mediated response, it acts as signal molecule to produce defensive proteins known as virus inhibitory agent (VIA)\textsuperscript{9}.

For comparative analysis, the total basic protein fractions eluted from SP-Sepharose cation exchange column were pooled. Elution profile of field grown sample showed low absorbance (70%), while that of micropropagated sample showed high absorbance (100%) during isolation of basic fractions indicating presence of high amount of protein in micropropagated sample. Basic protein isolated from field grown and culture sample showed very high systemic virus inhibitory activity on non-treated remote site leaves of Nicotiana tabacum cv. Samsun ‘NN’ against TMV. The same amount of basic protein from field grown plants decreased lesion number by 70%, whereas the protein from micropropagated plants decreased lesion production by 98%. The amount of protein was three times higher in cultured samples as compared to the field samples.

Protein samples from micropropagated and field grown plants when loaded on SDS-PAGE, a high intensity and broader band was observed in cultured plants than field grown plants (Fig. 3) which indicated higher amount of resistance inducing protein in cultured plants. The purified protein sample from field and micropropagated plants gave positive reaction on immunoblot (western blotting) against 34kDa CA-SRIP antiserum (Fig. 4). Thus, serologically also the CA-SRIP isolated from leaves of in vitro raised plants and field grown plants were similar.

Thus, the amount of CA-SRIP from leaves of in vitro raised plants was consistent throughout the year (1.0 mg/ml) whereas as reported by Verma et al.\textsuperscript{9}, there was marked seasonal variation in the amount of protein observed in field grown plants. Results thus, have shown that CA-SRIP obtained from in vitro raised plantlets was qualitatively same as in field grown plants but quantitatively its amount in micropropagated plants was higher.

The higher amount of protein in micropropagated plants may be because of changed nitrogen status (inorganic nutrients) of the medium. In Scots pine callus when KNO\textsubscript{3} was the exclusive inorganic nitrogen nutrient, protein synthesis was more abundant\textsuperscript{32}.
Requirement of high potassium as well as ammonium nitrate was reported for diosgenin\textsuperscript{33} and berberine\textsuperscript{34} production. Pissara \textit{et al.}\textsuperscript{35} reported role of nitrogen in reduction or diminishing of protein content whereas Erikson and Blobel\textsuperscript{36} reported the role of high potassium in improvement of protein synthesis. According to them high potassium was required for translation.

Protocol for micropropagation and purification of CA-SRIP from culture raised plants described in this communication are highly reproducible and efficient. This protocol could be used for high CA-SRIP production, conservation of germplasm, propagation of high frequency generating multiple shoots in leaf segments and production of consistently high amount of antiviral resistance inducing protein throughout the year.

References

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