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Research Article

DEVELOPING NORMAL PLANTS OF *CLERODENDRON* FROM VIRAL INFECTED STOCK THROUGH MERISTEM CULTURE

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ABSTRACT
An efficient protocol for developing normal plants of *Clerodendron inerme* (L) Gaertn from viral-infected stock (CICSV - *Clerodendrum Chlorotic spot virus* symptoms) was developed through meristem culture. About 4 mm and 6 mm long meristem from viral-infected plants of *Clerodendron* were inoculated on MS medium supplemented with BA (0.5 and 1 mg/l) and NAA (0.025 mg/l) either alone or in combinations. MS medium supplemented with BA (0.5 mg/l) and NAA (0.025 mg/l) elicited the maximum number of shoots. The regenerated shoots were rooted on half/full strength MS basal medium. The plantlets were successfully transferred to the field with 100% survival. Plantlets raised through meristem culture using explant of 4 mm size were found to be virus-free.

Key Words: *Clerodendron, Meristem Culture, Ornamental Plant, Virus-Free Plant*

INTRODUCTION
Clerodendron inerme (L) Gaertn is an important shrub belonging to the family Verbenaceae. The genus *Clerodendron* include over 450 species of tropical region. *Clerodendron inerme* is valued in landscaping as a ground cover or hedge plant. It has attractive ever-green foliage and has fragrant white flowers with delicate red protruding stamens. The plants can be regularly trimmed to a particular shape (Turner and Wasson, 1997). The plants can be multiplied by cutting and can be grown as topiary or even as a bonsai. This plant has bitter taste, so that cattle normally avoid browsing them. It is an important medicinal plant used in the treatment of skin diseases, venereal infections, elephantiasis, asthma, topical burns (Anonymous, 2001) and for rheumatism (Kirtikar and Basu, 1991). Leaves of *C. inerme* are used for treating fever, cough, skin rashes and boils in conjunction with other plant leaves (Anitha and Kannan, 2006). In Siddha medicine, it is used under the names of 'Chankan kuppi' and 'Pechagnan' (Sasikala *et al*., 1995). A glycoside ester namely verbascoside has been isolated from the roots which have analgesic and antimicrobial properties (Fauvel *et al*., 1989; Rastogi and Mehrotra, 1998).
In recent years, the *Clerodendron* plants exhibit yellow mosaic symptoms. The typical symptoms are bright yellow spots along the midrib which coalesce to give a mosaic, reduction in leaf size and stunting growth of the plant (Kothari *et al*., 2006). The leaves of infected plants have round to star-shaped (asteroid) spots or irregular yellow spots along the midrib. The spots are slightly thickened and hyaline, transmitting light when examined from the underside of the leaf. Severely infected leaves turn yellow and get abscised. The plants become disfigured and dwarfed. This disease called yellow mosaic of *Clerodendron inerme* is caused by a begomovirus CICSV (John *et al*., 2006). The virus transmitted to other plants by Insects (Kitajima, 2008). It also spread by scissors used for trimming the hedge.
Shoot meristem culture is generally used for producing virus-free material (Nehra and Kartha, 1994). When a plant is infected with virus, the titre of the virus within the plant is highly variable and most of the viruses fail to reach the meristematic tissue. Morel and Martin (1952) reported successful application of meristem culture to produce virus-free *Dahlia* from the infected stock. Meristem culture is a unique technique to eliminate various pathogens including viruses, viroides, mycoplasma, bacteria and fungi (Pierik, 1989). Benefit of using meristem culture as a means of plant regeneration is that the incipient shoot has already differentiated to establish a complete plant; only elongation and root differentiation are

required. It is important to note that such shoot meristem is devoid of vascular system and have high metabolic activity so as to be free of any virus (Bhojwani and Razdan, 1983).
The present study deals with the standardization of a protocol for establishment of virus-free plants of *Clerodendron inerme* (L) Gaertn through meristem culture.

MATERIALS AND METHODS

The 10 cm long twigs of *Clerodendron inerme* were collected from the virus-infected plants (Figure 1A) during 9-11 AM from the Botanical Garden, Gujarat University, Ahmedabad. The twigs were washed thoroughly under running tap water (l h) and treated 5 min with 0.5 % soap solution followed by repeated rinsing with distilled water. The apical shoots (5 cm) were cut into shoot-tip explants (2 cm). Explants were surface sterilized with 0.05% $HgCl_2$ (2 min) under aseptic conditions in a Laminar Airflow Hood (Lab Line, India). Finally, the explants were washed thoroughly (five times) with sterilized distilled water.
The shoot tip explants (4 and 6 mm) were inoculated on MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar-agar (SRL, India). The pH of media was adjusted to 5.8 prior to sterilization in autoclave. The cultures were incubated in a culture room with 25°C temperature and 16 hr photoperiod provided by cool white fluorescent tubes (55 µmol/m^2/sec).
The basal medium was supplemented with various combinations of BA (0.5 and 1mg/l) and NAA (0.025 mg/l) either alone or in combinations. Subcultures were made at 6 weeks interval and results were recorded. The *in vitro* generated shoots were transferred to the half or full-strength MS medium for root induction. The *in vitro* raised plantlets were washed gently to remove adhering media with 0.5 % (w/v) Bavistin solution (10 min). The treated plantlets were then planted in thermocol glasses (10X5 cm) containing a mixture of soil, sand and compost (1:1:1). The plantlets were subjected to hardening under greenhouse conditions (Jasrai *et al*., 1999).

RESULTS AND DISCUSSION

Effect of explant-size and combinations of various PGRs were validated for establishing the protocol of meristem culture to generate virus-free plants from viral-infected stock.
Meristem culture was useful for elimination of virus from a number of plant species, including *Dahlia* (Morel and Martin, 1955), Orchid (Morel, 1960), Sweet potato (Mori, 1971) and Cassava (Kartha *et al*., 1974). In present study, meristem culture was utilized for establishment of disease-free plants of *Clerodendron inerme*. Two sizes (4 and 6 mm) of meristem-tip explant (Figure 1B) were used for the development of virus-free plants. Among them, total of 10% virus free plantlets were generated from the 4 mm size meristem explants (Figure 1E). The plantlets established from 6 mm size explants showed clear diseased symptoms. The smaller explants (less than 4 mm) failed to survive during establishment on MS medium.

Table 1: Effect of shoot tip size on development of viral-free plants

Meristem size (mm)	Symptom-free plants (%)	No. of shoots	Shoot length (cm)	No. of leaves
4	10	1.23 $\pm$ 0.16	0.69 $\pm$ 0.06	4.69 $\pm$ 0.57
6	0	1.65 $\pm$ 0.14	1.75 $\pm$ 0.09	7.83 $\pm$ 0.56

In general, the larger the explants size, the better the chance for pathogen survival. Smaller meristem-tip culture with 0.5-0.7 mm and 0.2-0.4 mm was utilized to eradicate (Fig Mosaic Disease) of fig (Sahraroo *et al*., 2009). In fact, 85-93 % reduction was reported for viral symptoms of fig plant (Sahraroo *et al*., 2009). Another reports also exemplified the production of virus-free plants via meristem-tip culture of garlic (Ayabe and Sumi, 2001; Ayaso *et al*., 1981) and *Grapevine* (Salami *et al*., 2005). To overcome

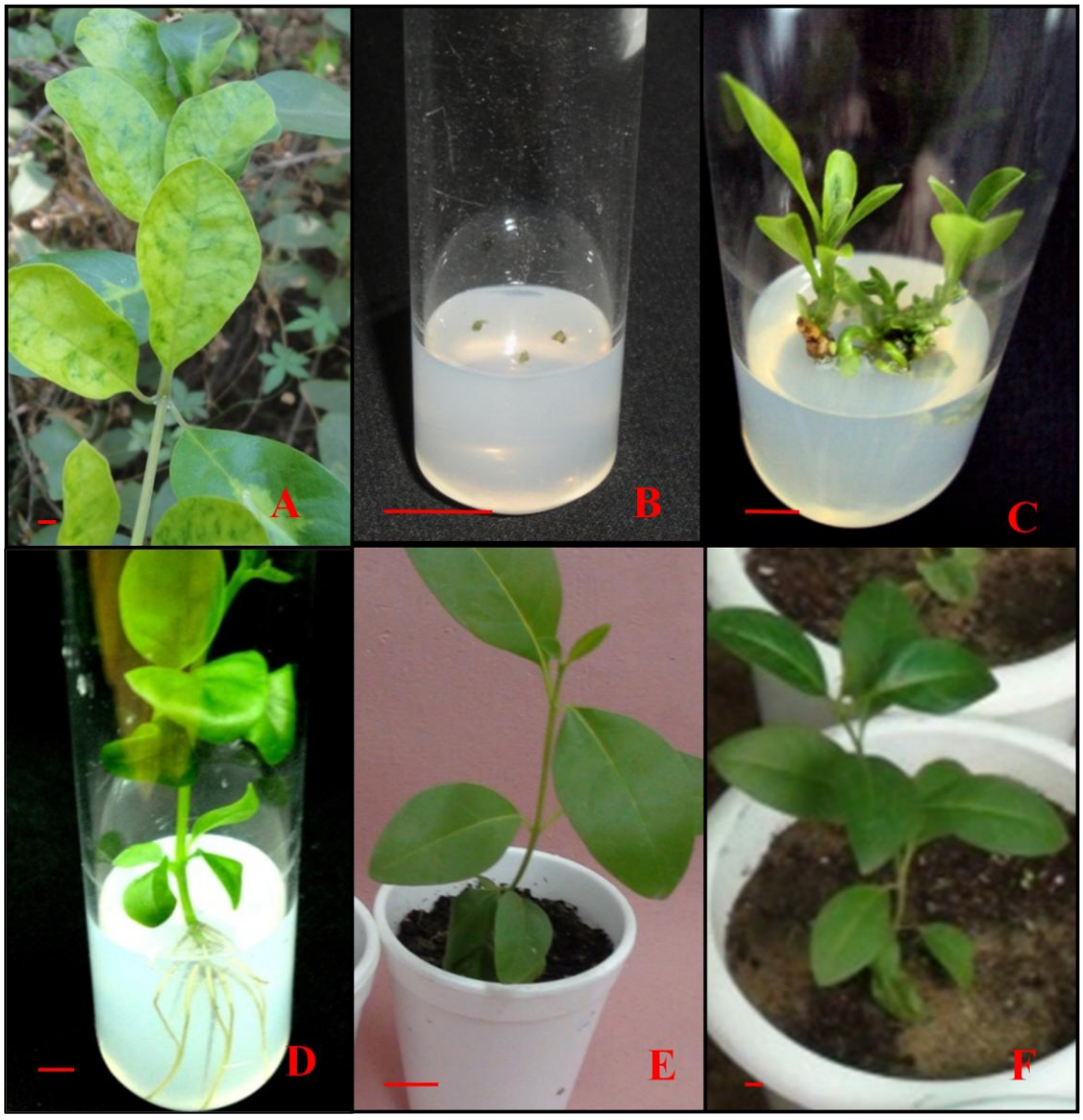

Figure 1: Micropropagation of *Clerodendrum inerme* (L) Gaertn and Developing normal plants from viral infected stock through shoot-tip cultures. (A) Field grown *Clerodendrum inerme* showing disease symptoms, (B) An initial shoot tips explants (4mm size), (C) Shoot multiplication during subculture, (D) *In vitro* generated shoots on rooting medium, (E) Symptoms free hardened plant (one week), (F) Well developed virus free plant in the plastic pot (6 weeks). Horizontal bar in each photograph is equal to 1 cm.

difficulties in removal of viral symptoms with meristem-tip culture, application of thermotherapy was demonstrated for encouraging results in propagation of Plum (Manganaris *et al*., 2003).

The size of excised shoot tip (meristem) is not only important to produce virus-free plants, but also determines the survival ability of the explants in culture (Malaurie *et al*., 1995; Manganaris *et al*., 2003; Salami *et al*., 2005; Wang *et al*., 1980). Among two different explant sizes tested (Table 1), *in vitro* shoot growth was higher with larger size (6 mm) explants than the smaller one (4 mm).

Serious problem of fungal contamination was noticed during the establishment of *Clerodendron inerme* explants on medium. Therefore, extra-care was taken to avoid contamination. First, the twigs were

washed thoroughly in running tap water followed by pre-treatment with 0.5 % soap solution (5 min). Under aseptic conditions, subtending part of twigs was removed and approximately 2 cm long explants were prepared. Further treatment with 0.05% $HgCl_2$ (2 min) was sufficient to establish explants. The explants subjected to such pre-treatment before $HgCl_2$ based surface sterilization showed excellent results. This pre-treatment helped in reducing the rate of contamination and showed maximum establishment (95%) of explants on medium (Figure 1B). *In vitro* shoot growth of *Clerodendron* was carried out with MS medium supplemented with various combinations of BA and NAA. Multiple shoot induction with maximum 3 shoots (Table 2) and shoot length of 2 cm was seen on MS medium supplemented with 0.5 mg/l BA and 0.025 mg/l NAA (Figure 1C). At higher concentration of BA, though multiple-shoot production was observed but the shoots so formed were slow in growth. Application of BA alone was not show effective for better proliferation of *Clerodendron* than combination of BA with NAA.

Table 2: Effect of growth regulators on multiple-shoot formation in nodal explants of *Clerodendron inerme*

PGRs (mg/l)		Multiple shoots	Shoot length (cm)	No. of leaves
BA	**NAA**	**Mean ±S.E.**	**Mean ±S.E.**	**Mean±S.E.**
0.5	0.0	1.5±0.43	1.2±0.23	5.7±0.43
1.0	0.0	1.25±0.22	0.9±0.05	3.5±1.09
0.5	0.025	3±0.22	2±0.05	6.25±0.43
1.0	0.025	2.3±0.4	1.5±0.08	5.3±0.46

Subcultures were carried out at intervals of every 6 weeks for 7 cycles to study shoot growth of *in vitro* nodal explants of *C. inerme*. After 6th subculture cycle there was a decline in the growth response.
Induction of roots on generated shoots was achieved with half and full strength MS basal medium (Figure 1D). After 6 weeks, *in vitro* rooted shoots were transferred for hardening in thermocol glasses (10X5 cm) containing a mixture 1:1:1 of soil, sand and compost (Figure 1E). The plantlets were irrigated with ¼ major MS medium first time and then with water (based on the soil moisture levels). The plantlets were also subjected to a fine water spray to maintain sufficient humidity. The plantlets survived successfully with 100 % survival rate. This was in line with earlier report on *C. inerme* (Kothari *et al*., 2006).

Conclusion

In present study, we report the successful elimination of *Clerodendrum Chloratic Spot Virus* (CICSV) from *Clerodendrum inerme* by meristem culture of infected plant. Only 10 % plantlets raised through meristem culture using explant of 4 mm size were found to be virus-free. Though, percentage establishment of disease free plants obtained through this technique is low, the building of virus-free plants can be archived by routine micropropagation through nodal explants.

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