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Use of meristem tip culture to eliminate carnation latent virus from carnation plants

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A successful protocol for meristem tip culture to eliminate carnation latent virus from carnation cv. scania has been described . The virus was found to be mechanically transmissible to *Chenopodium quinoa*, *C. .amaranticolor*, *Dianthus barbatus* and *Saponaria vaccaria*. Murashige and Skoog'smedium (MS) supplemented with NAA (1.0 μM) and Kn (20.0 μM) proved best for meristem establishment and microshoots were rooted in MS medium supplemented with IBA (5.0 μM). Meristems measuring 0.1 and-0.2 mm yielded virus free plants and larger meristems were not effective.

Carnation (*Dianthus caryophyllus* L.) is an important commercial ornamental crops of the world and is popularly used as cut flowers. Returns from cut flowers mainly depend upon their quality in addition to their quantity. However, carnations are plagued by persistent infection by several viruses. Significant economic losses to carnation growers result from infection by these viruses as they are responsible for poor quality of cut flowers in terms of size of flowers, split calyces and reduced vigour in addition to lesser yield in terms of number of lateral shoots, total number of flowers and fresh weight[1-3].

Virus multiplication is so intimately associated with normal metabolic processes in plants that interfering with it selectively may not be possible as all the known virus inhibitors are also toxic to plants. Therefore, biotechnological approaches like meristem tip culture have become very effective to obtain virus free plants from infective stocks . Keeping in view the above, the present investigations have been undertaken to index the infected stocks using biological and serological (ELISA) assays and to retrieve plants free from the virus under test using meristem tip culture.

Biological indexing—Plants of carnation cv scania showing pronounced symptoms of chlorotic spotting and/or mosaic mottling due to carnation latent virus (CLV) were collected locally and used for the present experiment. Sap from young leaves of these plants extracted in phosphate buffer (0.1 *M*, *p*H 7.0), was inoculated on young leaves of various indicator plants (*Chenopodium quinoa* Willd., *C.amaranticolor* Coste & Reyn. , *Dianthus barbatus* L., *Saponaria vaccaria* L. and *Gomphrena globosa* L.) at 3-5 leaf stage and observed daily for the development of symptoms.

Serological indexing—Serological indexing using enzyme linked immunosorbent assay (ELISA) was standardized to have a quick detection of the virus before and after retrieval and also to rogue out infected plant material at early stages of growth under field conditions. Alkaline phosphatase (ALP) based direct antigen coating indirect ELISA system as described by Hobbs *et al.*[4] was used for conducting the experiments. Microtitre plates (laxbro brand) having 96 wells were used . Testing was done against Carnation latent virus using antiserum obtained from CSIR, Palampur. Microtitre plates were incubated at 37°C for a period of 3, 2 and $1^1/_2$ hr. after coating with antigen, antibody and substrate . Reaction was recorded within 30-60 min after addition of substrate using ELISA plate reader model MS5605A (ECIL Hyderabad) and absorbance was recorded at 405 nm. The results of ELISA were interpreted following Hill[5].

Meristem tip culture—The plants which showed positive symptoms for the presence of CLV were put to meristem tip culture. Meristems were excised under aseptic conditions under laminar flow cabinet and both field material and *in vitro* growing shoots were used as base material. In case of field grown material the shoots were first surface sterilized with the help of bavistin/ carbendazim (0.2% for 7 min.) and $HgCl_2$ (0.1% for 3-5 min). The tip portions of these plants were excised under stereozoom microscope and the

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meristematic region at the apex was uncovered by gradually removing of leaf primordia with eye surgery knives . The apical meristem consisting of an apical dome and one or two leaf primordia ranging from 0.1 to 1.0 mm were gently removed from the parental tissues and cultured on Murashige and Skoog's medium[6] supplemented with various combinations of two growth regulators viz. NAA (0 to 1.0 μM) and kinetin (0 to 20.0 μM) for establishment. Initial *p*H of the culture medium was adjusted to 5.8 prior to addition of 0.8 per cent (w/v) agar-agar. The meristem tips cultured in small tubes were incubated at 28°±2°C with 16 hr photoperiod provided by cool white fluorescent tubes at 1.5 kilolux light intensity.

In vitro shoot multiplication and root induction—The proliferated meristems were multiplied on MS medium fortified with best performing combination of NAA (2 μM) and kinetin (10 μM). About 2cm long shoots were transferred to MS + IBA (5μM) for root induction and plantlet formation.

Plantlet hardening, acclimatization and virus indexing—After about 4 weeks on the rooting medium the derived plantlets were transferred to soil in plastic pots (diam. 2 cm) containing a pre-autoclaved 1:1:1 mixture of soil, FYM and sand, and maintained at 28° ± 2°C, 90% RH and 16 hr photoperiod in a green house equipped with misting system. The plants that regenerated from the apical meristem were tested thrice before and once after transferring them to the field for presence of carnation latent virus using indicator plants as well as ELISA tests. The plants were initially grown in glass house and thereafter shifted to the field.

Biological indexing—As shown in Table1. Local lesions appeared on both *Chenopodium quinoa* Willd. and *C.amaranticolor* Coste & Reyn., within 5-7 days of inoculation, however, symptoms were more pronounced on *C.quinoa* Willd. These local lesions lead to veinal chlorosis in *Dianthus barbatus* L. and complete yellowing in *Saponaria vaccaria* L. Virus was, however, not found to be transmissible to *Gomphrena globosa* L.

Serological indexing—The virus was detectable at 10^{-2}, 10^{-3} and 10^{-3} dilutions of antigen, antiserum and conjugate respectively. Mean absorbance (A405 nm) value for negative control (healthy plants) was 0.277 and that of positive control (plants showing pronounced symptoms of CLV) was 0.843. Only those samples were considered infected whose A405 nm values exceeded two times the mean values of healthy control samples, whereas the samples showing mean A405 nm values in the range of 0.277 to 0.554 were considered virus free.

Meristem establishment and shoot multiplication—About 95% of the meristem tips sprouted within 8-12 days of culture in MS medium supplemented with NAA (1.0 μM) and Kn (20.0 μM), shoots differentiated in 5-6 weeks thereafter. For shoot multiplication MS+ NAA (2.0 μM) + Kn (10.0 μM) gave the best results(Fig 1A-C).

Rooting, hardening and acclimatization—*In vitro* shoots were rooted in MS medium supplemented with IBA (5.0 μM) and agar 0.6% in which 100% rooting was observed. Rooted plantlets were separated from agar medium carefully to remove traces of agar medium and hardened under insect proof glasshouse and regularly sprayed with 0.1% melathion at fortnightly interval to avoid reinfection by the vector *Myzus persicae*(Fig. 1D-E).

Effect of meristem size on its establishment and virus elimination—As shown in Fig. 2 sprouting ability of meristems increased with the increase in the size of meristems and was found to be 66.66% when the size of meristem was 0.1 mm and it reached to 100% beyond 0.7 mm of meristem tips. Plantlets as well as plant derived from meristems of size 0.1 and 0.2 mm tested negative whereas those from larger meristems still carried the virus.

Technique of meristem tip culture has been used frequently to obtain virus eradicated plants because titre of infective viruses has been found to be low or absent in meristematic regions of a large number of

Table 1—Biological indexing on various possible indicator plants

Name of Plant	Symptoms
Chenopodium quinoa Willd.	Local lesions appeared within 5-7 days of inoculation, which coalesced after 13 days and colour of leaf changed to red from green.
C. amaranticolor Coste& Reyn.	Local lesions appeared within 5-7 days of inoculations which later developed into mosaic symptoms.
Dianthus barbatus L.	Yellow local lesions and veinal chlorosis 7-15 days after inoculation.
Saponaria vaccaria L.	Chlorosis after 6 days of inoculation leading to complete yellowing of leaves within 16 days of inoculation.
Gomphrena globosa L.	No transmission

plant species may be due to a high auxin content of meristems or existence of virus inactivating system with more activity in meristematic region or due to lack of vascular differentiation in the meristem[6-7]. Present results indicate that meristem tips of carnation are highly totipotent and show establishment in all the combinations of NAA and Kn tried. However, NAA (1.0 μM) and Kn (20.0 μM) was found to be the best combination for regeneration. These results are in conformity with those of Pennazio[8]. In order to produce virus free plants through meristem tip culture, a balance has to be maintained between the size of meristem and its regeneration potential because larger meristems have the risk that they may still carry the virus while the smaller meristems show poor regeneration potential under *in vitro* conditions. Regeneration potential was found to increase with the increase in size of meristems as only 66.66% meristems could sprout when their size was 0.1 mm whereas sprouting ability reached 100 per cent beyond 0.7 mm[9-10]. However, an inverse correlation was observed between size of meristems and rate of virus elimination. Meristems of upto 0.2 mm size could produce carnation latent virus free plants larger meristems failed to do so. While working on carnation mottle virus from carnation plants, Zhola[11] could retrieve 74.3% virus

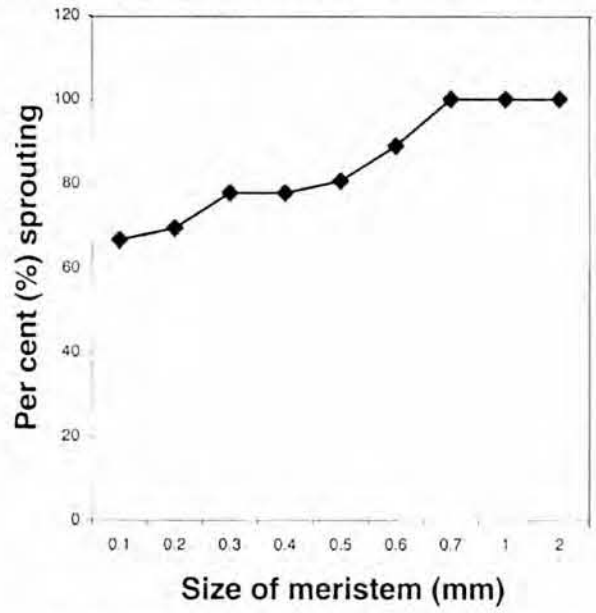

Fig. 2—Effect of size of meristem on sprouting ability

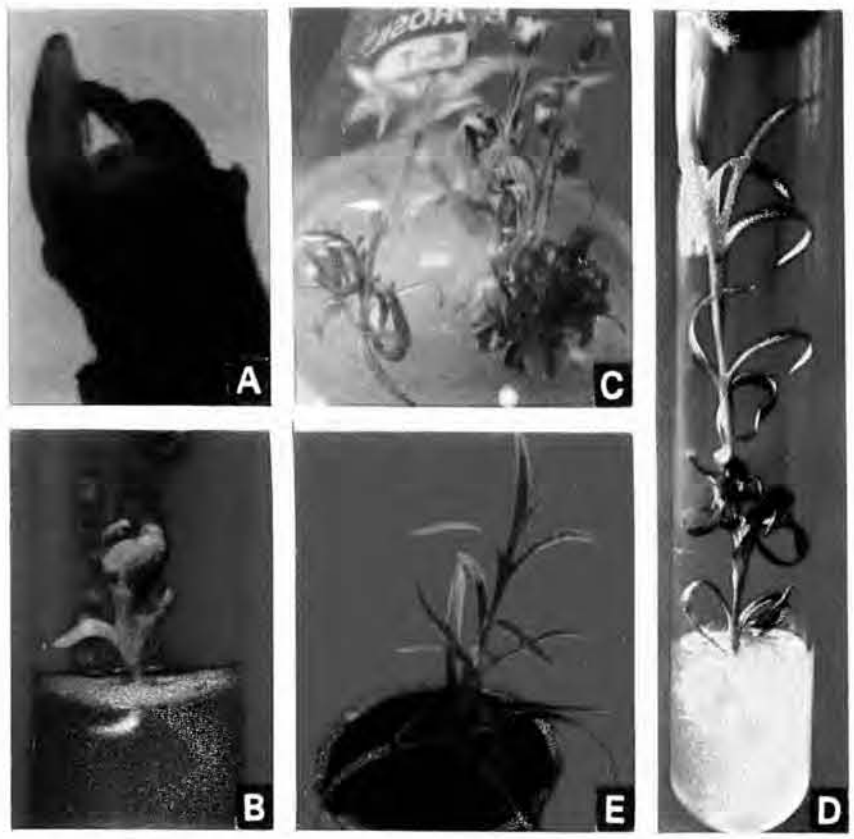

Fig. 1—(A)-Apical meristem under stereozoom microscope; (B)-Meristem after 25 days of culture; (C)-Multiplication of meristem derived shoots; (D)-Rooting of meristem derived shoot; and (E)-Meristem derived hardened plant of carnation

free plants using meristems of 0.2-0.3 mm size. However, this virus can be eliminated[12,13] by culturing meristems of much bigger size (up to 2.0 mm) but rate of virus retrieval has not been reported by these workers after culturing such large meristems. Present report is the first repot of elimination of CLV from carnations though other viruses have been eliminated from this crop by a number of workers.

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