Detection of viroid infecting chrysanthemum in India

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ABSTRACT: In a survey of ornamental plants conducted in fields of IARI and some private nurseries in New Delhi, a large number of chrysanthemum plants were found showing symptoms of mild chlorosis on young leaves, stunting and delayed flowering. Electron microscopic observations from infected leaves did not reveal the presence of virus particles. R-PAGE analysis of total nucleic acid extracts from symptomatic leaves from plants grown by cuttings, revealed the presence of RNA band while such a band was absent from healthy samples. The viroid nature of the RNA was confirmed by its similarity in electrophoretic mobility to potato spindle tuber viroid, resistance to high temperature and DNase but high sensitivity to RNase treatment. The nucleic acid extract from infected tomato cv. Rutgers, calendula and cineraria produced symptoms of leaf epinasty, veinal chlorosis and stunting in inoculated plants. Re-extraction of nucleic acid from inoculated tomato, cineraria and calendula leaves and its R-PAGE analysis showed the presence of RNA bands. Similar bands were also observed from nucleic acid extracts of young seedlings raised from chrysanthemum seeds collected from infected plants. The present report constitutes the first molecular evidence for natural occurrence of a viroid infection on chrysanthemum in India.

Key words: Chrysanthemum stunt viroid, Chrysanthemum chlorotic mottle viroid, detection, R-PAGE

Chrysanthemum is a highly popular cut flower of worldwide importance. It is propagated mainly by cuttings owing to which a large number of viruses and viroids have been described on this host. Most important among these are two viroid diseases chrysanthemum stunt and chrysanthemum chlorotic mottle caused by Chrysanthemum stunt viroid (CSVd) and Chrysanthemum chlorotic mottle viroid (CChMVd) respectively (Ramachandran and Mathur 1999). During 1946-47 chrysanthemum stunt disease was widespread in the United States and Canada in some greenhouses with losses estimated from 50-100% (Brierley, 1953). Infected plants showed typical symptoms of stunting, chlorosis of leaves, premature blooming and lack of root formation in cuttings. However, viroid etiology of the disease was established two decades later (Diener and Lawson, 1973). High degree of symptom variation was recorded on different host varieties and environmental conditions due to CSVd (Horst et al., 1977). CSVd has a wide host range (Lawson, 1987) while CChMVd has a narrow host range and is less contagious than other viroids. Plants infected with CChMVd show striking mottling which turns into complete chlorosis of leaves. In the later stages of the disease, plants become stunted with delay in flower development and cannot be distinguished from CSVd infection (Horst et al., 1977).

In the present studies a survey of ornamental plants was conducted in fields of IARI and private nurseries in New Delhi and it was observed that about 50% of the chrysanthemum plants were showing symptoms of mild chlorosis of young leaves, stunting of infected plants and delayed blooming (Fig.1). Cuttings from such plants were maintained in the glass house and were taken for further studies to test the presence of viroid RNA. Here we report the first evidence for the natural occurrence of a viroid infection on chrysanthemum in India.

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MATERIALS AND METHODS

Cuttings of five chrysanthemum plants, which were showing the symptoms of mild chlorosis of young leaves, stunting and late blooming, were washed in 0.5% hypochlorite solution followed by distilled water and planted on sterilised soil in the glasshouse. Three weeks later the leaves from the new sprouts were examined by electron microscope as described earlier (Gibbs et al., 1966). These observations failed to reveal the presence of virus particles. Therefore, extract from symptomatic leaves were prepared in phosphate buffer (pH 7.0) under cold conditions and rubbed inoculated on seedlings raised from healthy seeds of chrysanthemum.

Total nucleic acid (NA) was extracted from plants showing symptoms of chlorosis and stunting and analysed by electrophoresis. Young leaves (5 g) were powdered in liquid nitrogen and homogenized using TES buffer (Tris 0.1M, EDTA 0.01M, NaCl 1M, SDS 1%, DTT 0.5mM and Dieca 0.1mM) in the ratio of 1:2 (w/v). To the homogenate, chloroform:isoamyl alcohol mixture (24:1) and Tris saturated phenol were added in the ratio of 1:2 each, and left on bench at room temperature (~25°C) for 30 min. gently mixing in between. It was centrifuged at 8,500 rpm for 20 min. at 4°C in Sorvall RC 5C centrifuge. To the supernatant, half volume of ammonium acetate (7.5 M) and two volumes of ethanol were added and stored O/N at -20°C. NA was pellatized by centrifugation at 8,500 rpm for 30 min. at 4°C. The pellet was washed with 70% ethanol, vacuum dried, dissolved in 300 µl of sterilized non-ionic distilled water and subjected to re-extraction by the above procedure. The final pellet thus obtained was dissolved in 30 µl of distilled water and tested by R-PAGE (Return Polyacrylamide Gel Electrophoresis) by modifying the denaturation temperature of running buffer from 60°C as used by Schumacher et al. (1983), to 85-90°C.

Electrophoresis was carried out in a slab gel (16x14x0.15 cm) containing 7.5% acrylamide and 0.125% bis-acrylamide, 0.08% TEMED in high salt buffer and 0.07% ammonium persulphate. To 10 µl of test samples 4 µl of dye containing 0.25% each of xylene cyanol and bromophenol blue in 60% sucrose (molecular biology grade) was added and applied to the slots.

The first electrophoretic run was carried out under native conditions using high salt buffer (89 mM Tris buffer, 89 mM Boric acid, 2.5 mM EDTA, pH 8.3) at 20°C and 46 mA constant current. The run was terminated after 2.00-2.15 h when xylene cyanol migrated to near bottom of the gel. After the first electrophoresis, the buffer was exchanged for a low salt boiling buffer (1/8 dilution of high salt buffer) heated to boiling point. The gel was run at 70°C and 46 mA current with reverse polarity for 1½h. (This procedure wherein the same gel is re-run by reversing the polarity such that the samples return to the same slot positions is popularly referred to as Return-Poly Acrylamide Gel Electrophoresis -R-PAGE). The bands in the gel were resolved by silver staining (Mishra et al. 1991).

RESULTS AND DISCUSSION

Results of R-PAGE analysis showed that an RNA band in the viroid region was consistently present in NA extract from symptomatic chrysanthemum plants. No such band was detected in this region from identical preparations of healthy plants. The band was nearly similar in its electrophoretic mobility to potato spindle tuber viroid (PSTVd) (Fig. 2). The viroid nature of this band was established by its stability to high temperature treatment as well as sensitivity to RNase and insensitivity to DNase. The NA extract from seedlings tested by R-RAGE showed the presence of RNA band in the gel similar to that
from chrysanthemum plants raised from cuttings of infected plants.

The purified nucleic acid sample was found infectious on tomato cv. Rutgers, calendula and cineraria plants on sap inoculation producing symptoms of epinasty of young leaves, veinal necrosis and stunting of plants. The NA extract from these symptomatic plants showed the presence of viroid bands when tested by R-PAGE. This result indicated that the disease is not restricted to chrysanthemum.

In the present study, chrysanthemum plants showed chlorosis and yellowing of leaves when young, but as the plants grew older stunting became prominent. Such plants bloomed much later than the healthy ones (Fig.1). Sap inoculation of infectious sap from symptomatic plants produced symptoms of mild chlorosis on chrysanthemum, tomato, calendula and cineraria only. However inoculation of infectious sap from chrysanthemum plants did not produce any symptoms on two other plant species tested which are Catharanthus roseus and Chenopodium sp. Therefore, these two plant species were treated as non hosts for the causal agent under study. Cuttings raised from symptomatic plants showed a high degree of transmission of the disease. Seedlings raised from true seeds obtained from IARI, Pusa, New Delhi and some of the private nurseries in New Delhi showed chlorotic symptoms. These observations confirmed that cuttings and seeds easily transmitted the disease, a characteristic associated with viroid diseases.

According to the latest viroid classification (Flores et al., 1998), the two chrysanthemum viroids, stunt (CSVd) and chlorotic mottle (CChMVd) belong to the two different viroid families recognized so far viz. Popsiviroidae and Avsunviroidae differing in the presence and absence of the central conserved region respectively. CSVd has a wide distribution, causes pronounced stunting, and is readily transmissible vegetatively and by seeds to a number of hosts. Two sequence variants consisting of 354 (Heseloff and Symons, 1981) and 356 (Gross et al., 1982) nucleotides have been recorded. On the other hand, CChMVd appears to remain confined to its places of report, causes complete chlorosis of leaves followed by stunting in later stages and delayed flowering, with unusually long size of molecule among viroids known so far i.e. 398-399 nucleotides (Novarro and Ricardo, 1997).

In India, chrysanthemum chlorotic mottle disease was recorded as early as 1978 (Singh et al., 1978) without attributing any causal agent. Chrysanthemum plants were reported showing mottling of leaves followed by severe chlorosis and did not produce quality blooms. The disease appeared restricted to chrysanthemum and found to be graft and seed transmitted.

The findings reported here clearly demonstrate that a natural infection on chrysanthemum showing stunting and chlorosis - is caused by a viroid. Its characteristics are: prominent stunting of infected plants, high rate of vegetative, sap and seed transmission, presence of a low molecular weight (LMW) RNA band similar in electrophoretic mobility to PSTVd, its sensitivity to RNase and heat and infectivity of the purified nucleic acid to tomato, calendula and cineraria. From the present
observations it appears that the viroid recorded on chrysanthemum may be similar to *Chrysanthemum stunt viroid*. The present report constitutes the first record of detection of a viroid infection on chrysanthemum in the country. Further studies on characterization and relationship of this viroid are underway.

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