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Bio-virus inhibitors with potential for retrieval of virus free plants of *Lilium* spp

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Abstract
The anti-viral property of plant extract derived from medicinal plants viz., *Asparagus*, *Centella*, *Tinospora* and *Vitex* have shown tremendous virus inhibitory potential for production of virus free plants of *Lilium* both under field conditions and *in-vitro* conditions. The extracts of these plants were prepared in different solvents using soxhlet apparatus. Serological indexing was done before applying the extract as well as after applying the extract. Only those samples were considered infected where A405nm value exceeded two times the respective healthy control. Under *in-vitro* conditions, the extract was added at two levels: one was to add extracts to standardized tissue culture medium before autoclaving other was after autoclaving. *Asparagus* extract in acetone (AA) appears to exhibit maximum inhibitory potential at 10 mg/l, when added to medium after autoclaving. However, inhibition potential was lost when added before autoclaving. *Centella* and *Tinospora* extracts in acetone (CA) and water (TW), respectively inhibited LSV significantly. Field sprays on the other hand could not eliminate virus under test. However, inhibition to certain degree was recorded.

Keywords: Medicinal plants, LSV, virus inhibition, *Lilium*

Introduction
Viral diseases of ornamental plants are numerous and of immense economic value in trade. Lily (*Lilium spp*.) is among the most important commercial flower crops of India and is highly susceptible to infection by various viruses which causes great economic losses. The Lily symptomless virus (LSV) is one of the most harmful viruses of Lily that causes severe losses in term of quality as well as quality of bulb and flower production. It may occur as single or in mixed infection along with cucumber mosaic virus (CMV) (Asjes, 2000)**.**
Virus diseases are mostly managed by controlling their vectors by using insecticides and in discriminate use of chemical insecticides, but it leads to negative impact on environment, human health and soil fertility as well as these is not economical. An alternative to this chemical pesticide is utilization of bio control agents such as botanical extracts (Waziri, 2015) [28]. The purpose of this study is to determine inhibitory effect of botanical extracts prepared in water and other solvents against LSV.

Material and Method
The research work was done in the laboratory of Department of biotechnology Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (H.P). Virus infected *Lilium* plants were selected and tagged on the bases of visual symptoms that ranged from vein clearing to chlorotic streaks, mottling and stunting. The plants were further preserved in the glass house for the research work. Identification of the virus before and after attempts of retrieving virus free plants was done through serological indexing method (DAS-ELISA) in the laboratory under controlled conditions. Scoring was done visually and in *micro titre* plate reader at 405mm wavelength. The Lilium plants found positive for LSV were maintained for further experimental purpose. After serological testing, the plants found positive for LSV were used for *in-vitro* multiplications. The bulb scales were surfaced sterilized using standard protocol and cultured on modified MS medium (Murashige and Skoog, 1962) [16].
Botanical extracts were prepared from leaves of *Asparagus adscendens* Roxb, *Centella asiatica* Linn, *Tinospora cordifolia* (Willd),*Vitex negundo* Linn by soxhlet extraction method starting from least polar to highly polar solvent i.e. acetone, methanol and finally water. The extracts so obtained were dried in oven at 25 ± 1^0C and then used for further experimentation. The infected plants maintained in the field were sprayed at various concentrations of the extracts ranging from 3 to 10mg/l. However, concentration above 5mg/l resulted in deterioration of plant health and its increased susceptibility to other pathogens, leading to plant death. Therefore, only lower concentrations i.e. 3 and 5 were tried for further experimentation.

The extracts were added to the medium at varying concentrations at two levels of media preparations i.e. before and after autoclaving the medium. The extracts were filtered through sintered glass crucible assembly under laminar airflow and then added in media after autoclaving.

Results

In this approach, plant extracts were added at two levels of media preparation to test the effect of heat on the activity of the extracts i.e. before autoclaving and after autoclaving of the medium. The extracts that resulted in death of explants are not included in table discussed here. The results and observations are as under:

Effect of spray and virus eliminations

The main OD Values of the treatments having extracts of *Asparagus* in Acetone (AA) *Asparagus* in Methanol (AM) and *Asparagus* in Water (AW) did not vary significantly amongst themselves at 3mg/l and were almost static around 0.860,0.860,0.832 and 0.860 respectively) when compared to the positive control (mean OD Value 1.458 and 1.460) after first week and second week respectively. The OD values indicated inhibition but not complete elimination of virus. The results did not vary much when spraying schedule was extended to second week in case of AW extract(mean OD value 0.822) while at higher concentration of 5 mg/l *Asparagus* acetone (AA) and *Asparagus* water(AW) extract were not much effective (mean OD 0.780 for both).

On the other hand significant reduction in virus concentration (mean OD value 0.552) was observed when plants were sprayed with *Asparagus* methanol (AM) extract at higher concentration of 5mg/l after one week. There was no change in inhibitory potential of the extract when spraying period was extended to second week (mean OD value 0.550). *Vitex* and *Tinospora* extracted in acetone gave inhibitory results at 5mg/l where the mean OD value were 0.852 and 1.087 respectively (Table 1).

Effect of *In-Vitro* addition of extracts on virus elimination

The virus infected bulb scales were maintained on MS medium supplemented with NAA 1.0 mg/l and BA 0.1 mg/l. The medium was charged with extracts of *Asparagus*, *Tinospora*, *vitex* and *Centella sp*. (Table 2).

Asparagus acetone (AA) extract was found to be prominently effective in eliminating virus at all the concentrations of 3, 5, 7 and 10mg/l when added after autoclaving. The mean OD values of tested samples were less than twice the mean OD values of healthy counterpart (0.271) indicating complete virus elimination (respective means OD value 0.289, 0.277, 0.267 and 0.264). However, extract could not retain virus inhibitory property only at 10mg/l (mean OD 0.519). In case of *Asparagus* methanol, however, when extract was added before autoclaving, the extract could retain its inhibitory property only at 10 mg/l (mean OD 0.519). In case of AM extract inhibitory response *in vitro* was observed at both the level of extract addition with mean OD values 0.731, 0.689, 0.669 and 0.665 at 3, 5, 7 and 10 mg/l respectively when added after autoclaving. There was not much difference in mean OD values when additions were made before autoclaving (mean OD value s 0.731, 0.690, 0.665 and 0.666, respectively). The AW extract at lower concentration of 3 and 5 mg/l gave OD values near to that of positive control (1.835), however, at higher concentration of 7 and 10 mg/l resulted in decreased OD value (0.898 and 0.730, respectively) indicating inhibitory response of the extract. Similar trend of change in OD values was observed when extracts were added before autoclaving. Virus inhibition was also observed with *Tinospora* extract in water (TW) at 5, 10, 15 and 20 mg/l concentration.

Discussion

Active defence mechanism against viruses exists in plants and it can be stimulated by anti-viral agents identified in few plant species. The present studies are also attempted to identify the virus inhibitory potentials of few medicinal plants. In current study, addition of plant extract to multiplication medium is considered to be one of the *in-vitro* approaches to tackle virus problem (Simpkins *et al.,* 1981) [21]. Therefore, the multiplication media was standardized.

Virus elimination via using plant extracted inhibitors:

The endogenously occurring substances of plant origin have been reported to induce systemic/localized resistance in several susceptible hosts and have been used for protecting the crops against virus infection (Verma and Barnwal, 1989) [24]. Many of these virus inhibitors are ribosome in activating proteins (RIPs) (Barbieri and Stripe, 1982) [4]. The resistance induced by such biotic moieties have been termed as acquired/induced resistance that may be localized or systemic in nature. The localized resistance is manifested only in those areas where exogenous applications of these RIP's have been made leading to prevention of virus infection (Leobenstein 1972) [13]. On the other hand endogenously occurring systemic resistance inducers do not inactivate virus *in-vitro* but activate certain host defence genes prompting the host to produce new virus inhibitory/ neutralizing agents (VIA) in susceptible hosts (Verma and Avasthi, 1980; Khan and Verma, 1990) [25, 12]. These VIA's Trans located to upper parts (not treated) of the plants and induced short and long term immunity.

Effect of virus inhibitors on plant extracts on *in vitro* cultures

There have been many attempts by scientist to find suitable virus inhibitors of plant origins (Barnwal, 1988; Gupta and Naqvi, 1991; Malhotra *et al*., 1996; Duarte *et al* 1996; Rusak *et al*., 1997) [2, 8, 14, 7, 18]. These scientists have reported anti-viral activities in plants like *Boerhaavia diffusa*, *Mirabilis jalapa*, *Clerodendrum* spp. and *Glycyrrhiza glabra*. In current study, extracts of *Asparagus adscendens* prepared in acetone gave encouraging results. The results are in line with the finding of Bhardwaj *et al*., 2000 [5], who have reported elimination of Carnation latent Carla virus and Chrysanthemum B Carla virus through extracts of *Asparagus adscendens*. However, methanol extracts had some inhibitory effect on LSV. *Ocimum* extract in acetone was observed to have failed to have any effect. *Vitex* extract in methanol was also seen to have inhibitory effect. *Tinospora* water (TW) extracts also exhibited inhibitory effect when added after autoclaving. However, the potential was reduced when extract additions were made before autoclaving.

Virus inactivation may be a consequence of virus precipitation, hydrolysis, complexing or chemical modification (Verma and Prasad, 1992) [26]. The virus inhibition may also take place due to altered metabolic environment within a cell leading to repressed viral expression or creation of secondary environment to indirectly influence virus infection and/or multiplication and/or spread in resistant tissue (Verma, 1982) [22]. In our study, the virus inactivation seems to be factor of virus host biochemical interaction explaining the differential activity a particular

extract is showing under different virus host combination. Ability of *Asparagus* extract in acetone to inhibit the virus, when added after autoclaving as well as before autoclaving indicates the possibility of active components to be heat resistant, whereas, other inhibitory extracts (TW and VM) lost their inhibitory potential when added before autoclaving indicating loss of inhibitory potential at higher temperature Since the host plant treated with plant extract is already, it is obvious that extracts which effectively inhibit or reduce viruses are systemic resistance inducers. Also the possibility of polar/non polar nature inhibitory components of the extracts can be made out from the type of solvents used in extraction procedures; however, the extensive experimentation is required to confirm the exact reaction and elimination paths to understand the intricacies of virus elimination under various virus host combination

Effect of field sprays of plant extract on virus elimination:

Extracts from number of plants have been shown to have inhibitory effects on different viruses either when sprayed or simply applied to plant surface in the field (Cheema *et al*., 1991; Barnwal and Verma, 1992; Verma and Varsha 1994; Nagaraju *et al*., 1997; Manickam and Rajappan, 1998) [6, 3, 27, 17, 15]. The inhibitory substances are present in different parts of the plant (Verma, 1986) [23] and get released during extraction. In present studies, extracts of *Asparagus* in methanol, acetone and water have been found to possess substantial amount of inhibitory substances at 3 and 5 mg/l concentration when sprayed for one week. Other extracts which were found to be inhibitory are *Vitex* and *Tinospora* in acetone. Presence of antiviral principles (AVP's) in *Vitex* was reported by Selvaraj and Narayana Samy (1991) [19]. Kannan and Doraiswamy (1993) [11] found reduction in incidence of Blackeye Cowpea Mosaic Polyvirus in cowpea by *Vitex negundo* extract. *Tinospora* sprays were found effective against PVMV in Tomato (Sircaik 2013) [20].

Table 1: Effects of field sprays on elimination/inhibition in *Lilium*

Treatment	Plant Extract	Concentrations	Mean OD Values at 405 nm	
			After 2nd week	After one Week
T1	Asparagus (Acetone)	3	0.86	0.83
		5	0.78	0.76
T2	Asparagus (Methanol)	3	0.832	0.811
		5	0.552	0.55
T3	Asparagus (Water)	3	0.86	0.822
		5	0.78	0.73
T4	Tinospora folia (Acetone)	3	1.225	1.205
		5	1.087	1.050
T5	Vitex (Acetone)	3	0.923	0.93
		5	0.852	0.831
T6	Control (+ve)	0	1.458	1.46
T7	Control (-ve)	0	0.127	0.23

Table 2: Effect of plant extract in *in vitro* Lilium multiplication medium (Before and after autoclaving)

Treatments	Plant Extract	Concentrations	Mean OD Values at 405 nm	
		R mg/l	After autoclaving	Before autoclaving
T1	Asparagus (Acetone)	3	0.289	0.725
		5	0.277	0.692
		7	0.267	0.694
		10	0.264	0.519
T2	Asparagus (Methanol)	3	0.73	0.731
		5	0.689	0.69
		7	0.669	0.665
		10	0.665	0.663
T3	Asparagus (Water)	3	1.713	1.726
		5	1.669	0.924
		7	0.898	0.732
		10	0.73	0.73
T4	Vitex (Water)	5	1.487	1.454
		10	1.454	1.439
		15	0.897	0.926
		20	0.63	0.784
T5	Tinospora (Water)	5	0.688	0.688
		10	0.730	0.824
		15	0.684	0.904
		20	0.676	0.891
T6	Centella (Acetone)	5	0.722	0.725
		10	0.726	0.724
		15	0.726	0.719
		20	0.719	0.710
T7	Centella (Methanol)	5	0.853	1.246
		10	0.860	0.854
		15	0.852	0.851
		20	0.853	0.847
T8	Control (+ve)	0	1.835	1.796
T9	Control (-ve)	0	0.271	0.268

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