SCIFED Publishers

Awasthi LP, SF J Immunol, 2017, 1:1

SciFed Journal of Immunology

Research Article Open Access

Studies on the Production of Virus Inhibitory Agent (VIA) in Different Hosts Treated with Partially Purified Phytoprotein Isolated from Leaves of Clerodendrum Aculeatum

*[1]Awasthi LP, [2]Ahmad Najam, [3]Verma HN

*[1]*Amity Center for Research and Innovation (Amity University Uttar Pradesh), Varanasi- 221007, India*

[2]*Mumtaj P.G. College, Lucknow University, Lucknow-226007, India*

[3]*Jaipur National University,Jaipur-302017, India*

Abstract

The synthesis of virus inhibitory agent (VIA), could be induced, in treated as well as nonrated leaves of hypersensitive and systemic host plants, by the application of partially purified phytoprotein isolated from the leaves of *Clerodendrum aculeatum* plants (CAP). Exvivo inactivation of virus using LS-VIA (*Lagenaria siceraria* -Virus Inhibitory Agent), CS-VIA (*Cucumis sativus* -Virus Inhibitory Agent) and CJ-VIA (*Crotolaria juncea*-Virus Inhibitory Agent) revealed that each of the VIA sample isolated either from of LS- VIA, CS-VIA or CJVIA posses strong ability of **ex vivo** inactivation of viruses.

It was revealed that the effectiveness of VIAs isolated from different sources was host dependent. VIA isolated from *Lagenaria siceraria* plants, pre-treated with CAP (LS-VIA) gave best response when assayed after incubation with SHRV. The VIA at a concentration of 1:10 was found to be optimum which could not enhance its effect on the ex vivo inactivation of Virus.

Keywords

Phytoprotein; Virus Inhibitory Agent; Hypersensitive; Systemic; Inactivation

Introduction

Viral diseases are of immense importance considering the extensive damage and severe losses they cause to crops. Because of their peculiar nature and characteristic association with hosts and vectors, no therapeutic method to completely control them has been found successful [1]. The worldwide losses caused by viral diseases are estimated at about US $220 billions per year [2]. However, certain preventive measures can be of great help in avoiding virus diseases [3, 4].

Many higher plants have the ability to resist pathogen attack including virus infection [5-8]. Some of the plants are known to contain endogenous proteins that act as antiviral agents [9-11]. There is no indication, however, that all the plants contain same type of inhibitor or that the antiviral mechanism is the same in all cases. Although, attempts have been made to explain the mechanism of antiviral action of plant products [12] no mechanism could

***Corresponding author:** Awasthi LP, Amity Center for Research and Innovation, Varanasi- 221007, India. E-mail: lpawasthi14@gmail.com; Tel: +91-9415718904

Received June 18, 2017; **Accepted** November 20, 2017; **Published** December 06, 2017

Citation: Awasthi LP (2017) Studies on the Production of Virus Inhibitory Agent (VIA) in Different Hosts Treated with Partially Purified Phytoprotein Isolated from Leaves of Clerodendrum Aculeatum. SF J Immunol 1:1.

fully and satisfactorily explain the phenomenon of virus inaction. In most of the cases, the proposed mechanism for the antiviral action of the widely occurring proteinacious inhibitors seems to be the result of the inhibitor ribosome-specific-N glycosides activity in-vitro [13, 14].

Endogenously occurring substances, in a few higher exotic plants have been reported to induce systemic resistance in susceptible hosts against virus infections [15-17]. Such plant extracts have been used for protecting economically important crops against virus infections [3, 11, 18, 19]. The leaves of *C. aculeatum* and roots of *B. diffusa* have been shown to contain potent endogenous virus inhibitory phytoproteins called as CA-SRIP and BD-SRIP, respectively [17, 20]. These phytoproteins ,referred as systemic resistance inducing protein (SRIP), could induce the production of potent virus inhibitory agents (VIAs), when applied on to the leaves of different susceptible healthy host, and confer strong systemic resistance in such plants against a number of plant viruses [7 , 14, 20-28].

The present investigations were carried out to study the production of Virus Inhibitory Agent (VIA), in different hosts, treated with partially purified phytoprotein, isolated from the leaves of *Clerodendrum aculeatum* plants.

Materials and Methods

Procedure for raising of test plants, maintenance of virus culture, preparation of virus inoculum and the induction of systemic resistance were the same as described earlier [29, 30].

Preparation of Virus Inhibitor and the Isolation and Partial Purification of Phytoprotein

The leaves of *C. aculeatum* were harvested and ground in freshly prepared 0.2 M phosphate buffer (PB) of pH 6.6 containing 0.1 % β mercaptoethanol in the ratio of 1:2. It was then squeezed through double-layered muslin cloth. The extracted sap was centrifuged at 8,000 g for 10 minutes to remove the cell debris. A saturated solution of ammonium sulphate was added to the supernatant with continuous stirring and then left overnight at 4OC. The mixture was centrifuged at 8,000 g for 15 minutes and the precipitate in the form of thick pellets was collected. It was then suspended in a small amount of buffer [20g fresh weight/ml of 0.2M PB (pH 6.6)] and then dialyzed, in a dialysis bag, against running water for overnight, to obtain total phytoprotein fraction. The dialyzed phytoprotein fraction was either diluted as per requirement or was ˚concentrated through freeze-drying. Lyophilized phytoprotein sample, stored at –20 C ˚ was dissolved in sodium acetate buffer and .further purified by elution through a Sephadex G- 25 (Pharmacia Fine chemicals, Uppsala, Sweden) column, following the procedure as described Earlier [31]. The eluted fractions, showing antiviral activity, referred as "Partially purified phytoprotein" were used for VIA induction in susceptible host plants, viz. *Lagenaria siceraria, Cucumis sativus, Crotolaria juncea and Cyamopsis tetragonoloba* plants.

Induction of Systemic Resistance and its Purification

The systemic resistance inducing partially purified *C. aculeatum phytoprotein* (CAP), was applied on *Cyamopsis tetragonoloba* plants. The leaves from *Cyamopsis tetragonoloba* plants, sprayed 24 hours earlier with CAP, and also the leaves from untreated control plants(treated with distilled water)were harvested separately, weighed, washed well with distilled water and frozen immediately. After 24 hours frozen leaves were homogenized in 0.2 M sodium acetate buffer, pH 5.2 containing 0.1% β mercaptoethanol. The homogenates thus obtained were squeezed through two fold of muslin cloth and the solutions in each case were centrifuged at 10,000g for 15 minutes. The pellets were discarded and supernatant was precipitated with a saturated solution (60 % w/v) of ammonium sulphate. Following overnight of precipitation, the sample was centrifuged at 5,000g for 15 minutes and the pellet obtained was dissolved in minimum volume of 0.02M sodium acetate buffer, pH 5.2 containing 0.01% β mercaptoethanol. The dissolved pellet was cleared by centrifugation at 8,000g for 15 minutes and clear supernatant was collected. The supernatant thus obtained was assayed for VIA activity in the samples against SHRV. The samples were incubated with SHRV inoculum, in equal ratio for 4 hours and then assayed for antiviral efficacy on the leaves of hypersensitive host.

Production of Virus Inhibitory Agent (VIA)

Partially purified phytoprotein isolated from the leaves of *C. aculeatum* was sprayed with the help of a small atomizer on vigorously growing 4-6 young and healthy seedlings of *Lagenaria siceraria, Cucumis sativus, and Crotolaria juncea*. The leaves of each plant treated 24 hours earlier with phytoprotein from *C.aculeatum* were harvested separately,weighed, washed and homogenized

using standard protein purification protocol, and centrifuged at 10,000g for 15 minutes. Supernatants, in each case, collected separately were screened for their antiviral state and designated as LS-VIA (*Lagendaria siceraria* -Virus Inhibitory Agent), CS-VIA (*Cucumis sativus -Virus Inhibitory* Agent) and CJ-VIA (*Crotalaria juncea-Virus Inhibitory Agent*).

Each of the VIA sample isolated either from of LS- VIA, CS-VIA or CJ-VIA posses an ability of ex vivo inactivation of viruses. The samples of LS- VIA, CS-VIA and CJ-VIA were incubated, with an equal amount of virus inoculum (V/V), for 4 hours at room temperature, then assayed on to the leaves of *Cyamopsis tetagaonoloba* plants. An equal number of identical plants of same age group, height and vigor, treated with distilled water, served as control. Leaves of all the plants (treated and control), treated 24 hours earlier, either with VIA or distilled water alone were challenge inoculated with Sunnhemp rosette virus. Local lesions appeared, on the leaves of treated and control plants, in the form of necrotic spots, four to six days following virus inoculation were counted separately. Biological activity, in terms of percent reduction in local lesions production, by Sun hemp rosette virus (SHRV) challenge inoculation, on the leaves of *Cyamopsis tetragonoloba* plants was calculated for the degree of the induction of systemic resistance. The percent reduction in local lesion production / percent decrease in virus infectivity was calculated, separately for treated site and remote site (non-treated site) in comparison to control/ untreated plants, by the following formula.

$$= \frac{C - T}{C} \times 100$$

Where: C=Average number of local lesions produced on control plants and

T = Average number of local lesions produced on treated plants

The percent reduction corresponds to antiviral activity; hence it is supposed to be due to systemic antiviral resistance induction and concomitant VIA induction as well. Data obtained were analyzed statistically for the significance of results [32].

Results

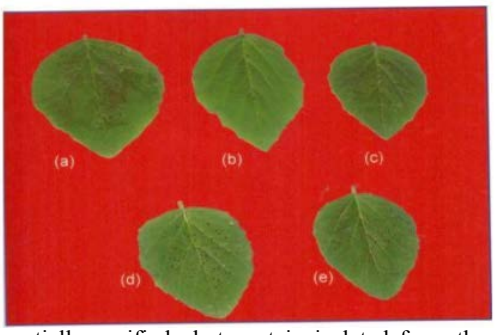
Resistance Induction by Partially Purified CA Protein

The partially purified CA phytoprotein, sprayed on to the leaves of *Cyanosis tetagaonoloba* plants, revealed a tremendous reduction in the number of local lesions produced by SHRV on the leaves of *Cyanosis tetagaonoloba* plants treated earlier with VIA . The percent reduction in lesion number was drastically reduced by about 92%. The reduction of virus infection to this extent was most probably due to the induction of virus inhibitory agent (VIA) in the test host plants (Plate -1).

Ex-vivo Inactivation of Virus using VIA

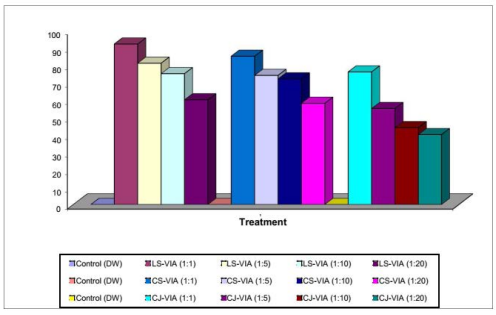
Results presented on the ex-vivo inactivation of virus using VIA from different source hosts and the screening of antiviral efficacy of LS-VIA (*Lagenaria siceraria -Virus Inhibitory Agent*), CS-VIA (*Cucumis sativus -Virus Inhibitory Agent*) and CJ-VIA (*Crotolaria juncea-Virus Inhibitory Agent*) have clearly indicated that each of the VIA sample isolated either from of LSVIA, CS-VIA or CJ-VIA posses an ability of ex- vivo inactivation of viruses. Data, on comparison with DW-SHRV control plants, clearly indicated significant reduction of about 92%, 85% and 76% .

Plate -1



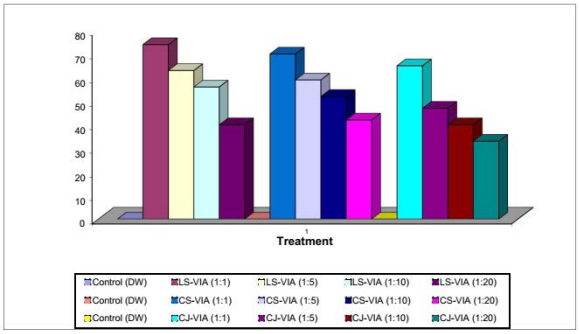
Effect of partially purified phytoprotein isolated from the leaves of Clerodendrum aculeatum (CA) on the production of Lagenaria siceraria virus inhibitory agent (LS-VIA) in Cyamopsis tetragonoloba
(a) Control distilled water (DW)
(b) LS -VIA (1:1)
(c) LS – VIA (1:5)
(d) LS – VIA (1:10)
(e) LS – VIA (1:20)

Graph 1: Ex-vivo inactivation of SHRV using LS-VIA, CS-VIA and CJ-VIA on *Cyamopsis tetragonoloba*



*Challenge inoculation was made using SHRV 1:50 (V/V) dilution

Graph 2: Ex-vivo inactivation of SHRV using LS-VIA, CS-VIA and CJ-VIA on *Cyamopsis tetragonoloba*



*Challenge inoculation was made using SHRV 1:100 (V/V) dilution (LS-VIA = *Lagenaria siceraria* -Virus Inhibitory Agent, CS-VIA = *Cucumis sativus* -Virus Inhibitory Agent, CJ-VIA = *Crotolaria juncea* –Virus Inhibitory Agent)

Table 1: Ex-vivo inactivation of SHRV using LS-VIA on *Cyamopsis tetragonoloba*

Treatment	Local Lesion at the Site of Application				Average Number of Local Lesions ±SEM	Percent Reduction in Local Lesion Number
	L1±SEM	L2±SEM	L3±SEM	L4±SEM		
Control (DW)	305 ± 3.60	316 ± 2.16	297 ± 3.68	294 ± 2.16	303 ± 3.68	0 ± 0
LS-VIA (1:1)	21 ± 1.06	26 ± 2.06	29 ± 1.38	24 ± 0.68	25 ± 0.88	92 ± 0.92
LS-VIA (1:5)	53 ± 1.06	50 ± 2.16	61 ± 2.22	67 ± 1.67	58 ± 3.60	81 ± 2.98
LS-VIA (1:10)	71 ± 1.60	74 ± 1.80	81 ± 1.96	76 ± 1.42	76 ± 1.36	75 ± 0.98
LS-VIA (1:20)	108 ± 0.26	115 ± 0.76	132 ± 0.78	125 ± 0.78	120 ± 0.76	60 ± 0.60

+ SEM = Standard Error of Mean
LS – VIA = *Lagenaria siceraria* -Virus Inhibitory Agent
L1, L2, L3 and L4 represent the number of leaves treated
1.0g of SHRV infected leaf tissue in 50ml distilled water [virus inoculum (1:50)]

Table 2: Ex-vivo inactivation of SHRV using LS-VIA on *Cyamopsis tetragonoloba*

Treatment	Local Lesion at the Site of Application				Average Number of Local Lesions ± SEM	Percent Reduction in Local Lesion Number
	L1±SEM	L2±SEM	L3±SEM	L4±SEM		
Control (DW)	305 ± 3.16	316 ± 3.22	297 ± 1.98	294 ± 2.21	303 ± 1.16	0 ± 0
LS-VIA (1:1)	72 ± 2.20	78 ± 2.38	81 ± 1.16	85 ± 2.28	79 ± 1.76	74 ± 2.26
LS-VIA (1:5)	95 ± 1.78	109 ± 1.68	117 ± 1.72	121 ± 1.68	111 ± 1.18	63 ± 2.16
LS-VIA (1:10)	128 ± 1.68	132 ± 1.21	130 ± 1.38	137 ± 2.16	132 ± 2.36	56 ± 2.02
LS-VIA (1:20)	182 ± 1.72	191 ± 1.76	172 ± 1.80	184 ± 1.82	182 ± 1.84	40 ± 1.04

+ SEM = Standard Error of Mean
LS – VIA = *Lagenaria siceraria* -Virus Inhibitory Agent
L1, L2, L3 and L4 represent the number of leaves treated
1.0g of SHRV infected leaf tissue in 100ml distilled water [virus inoculum (1:100)]

Table 3: Ex-vivo inactivation of SHRV using CS-VIA on *Cyamopsis tetragonoloba*

Treatment	Local Lesion at the Site of Application				Average Number of Local Lesions ±SEM	Percent Reduction in Local Lesion Number
	L1±SEM	L2±SEM	L3±SEM	L4±SEM		
Control (DW)	308 ± 2.68	303 ± 3.16	311 ± 3.16	314 ± 3.14	309 ± 3.22	0 ± 0
CS-VIA (1:1)	41 ± 1.66	45 ± 1.23	54 ± 1.54	44 ± 1.44	46 ± 1.23	85 ± 1.60
CS-VIA (1:5)	68 ± 1.44	78 ± 1.38	83 ± 1.26	86 ± 1.26	79 ± 1.78	74 ± 2.68
CS-VIA (1:10)	81 ± 1.68	86 ± 1.60	89 ± 2.00	91 ± 1.76	88 ± 1.72	72 ± 1.78
CS-VIA (1:20)	111 ± 1.76	119 ± 1.76	136 ± 1.24	148 ± 1.68	129 ± 1.23	58 ± 2.18

+ SEM = Standard Error of Mean
CS-VIA = *Cucumis sativus* -Virus Inhibitory Agent
L1, L2, L3 and L4 represent the number of leaves treated
1.0g of SHRV infected leaf tissue in 50 ml distilled water [virus inoculum (1:50)]

Table 4: Ex-vivo inactivation of SHRV on using CS-VIA on *Cyamopsis tetragonoloba*

Treatment	Local Lesion at the Site of Application				Average Number of Local Lesions ±SEM	Percent Reduction in Local Lesion Number
	L1±SEM	L2±SEM	L3±SEM	L4±SEM		
Control (DW)	308 ± 2.16	303 ± 3.16	311 ± 2.28	314 ± 2.14	309 ± 3.16	0 ± 0
CS-VIA (1:1)	86 ± 2.16	91 ± 3.90	95 ± 2.16	101 ± 2.14	93 ± 2.90	70 ± 3.06
CS-VIA (1:5)	118 ± 1.18	126 ± 1.44	129 ± 1.68	133 ± 2.20	127 ± 1.68	59 ± 1.72
CS-VIA (1:10)	136 ± 1.66	155 ± 1.66	142 ± 2.16	161 ± 1.68	149 ± 1.56	52 ± 1.68
CS-VIA (1:20)	176 ± 1.68	179 ± 2.72	181 ± 2.72	184 ± 1.72	180 ± 1.68	42 ± 1.32

+ SEM = Standard Error of Mean
CS-VIA = *Cucumis sativus* -Virus Inhibitory Agent
L1, L2, L3 and L4 represent the number of leaves treated
1.0g of SHRV infected leaf tissue in 100 ml distilled water [virus inoculum (1:100)]

Table 5: Ex-vivo inactivation of SHRV using CJ-VIA on *Cyamopsis tetragonoloba*

Treatment	Local Lesion at the Site of Application				Average Number of Local Lesions ±SEM	Percent Reduction in Local Lesion Number
	L1±SEM	L2±SEM	L3±SEM	L4±SEM		
Control (DW)	299 ± 2.86	314 ± 3.66	321 ± 3.16	309 ± 2.19	311 ± 2.68	0 ± 0
CJ-VIA (1:1)	68 ± 1.88	74 ± 2.16	79 ± 2.76	80 ± 1.08	75 ± 2.38	76 ± 1.76
CJ-VIA (1:5)	123 ± 2.60	136 ± 3.60	147 ± 1.72	159 ± 1.68	141 ± 1.72	55 ± 1.00
CJ-VIA (1:10)	167 ± 1.62	171 ± 1.92	176 ± 1.82	179 ± 1.68	173 ± 0.98	44 ± 1.12
CJ-VIA (1:20)	185 ± 1.72	182 ± 1.82	190 ± 1.60	193 ± 1.78	188 ± 1.98	40 ± 1.76

+ SEM = Standard Error of Mean
CJ-VIA = *Crotolaria juncea* -Virus Inhibitory Agent L1,
L2, L3 and L4 represent the number of leaves treated
1.0g of SHRV infected leaf tissue in 50 ml distilled water [virus inoculum (1:50)]

Table 6: Ex-vivo inactivation of SHRV using CJ-VIA on *Cyamopsis tetragonoloba*

Treatment	Local Lesion at the Site of Application				Average Number of Local Lesions ±SEM	Percent Reduction in Local Lesion Number
	L1±SEM	L2±SEM	L3±SEM	L4±SEM		
Control (DW)	299 ± 2.16	314 ± 2.32	321 ± 1.88	309 ± 2.98	311 ± 1.78	0 ± 0
CJ-VIA (1:1)	101 ± 1.78	105 ± 2.16	111 ± 2.16	113 ± 1.16	108 ± 2.76	65 ± 1.16
CJ-VIA (1:5)	143 ± 2.68	166 ± 1.68	179 ± 1.78	171 ± 1.72	165 ± 1.65	47 ± 1.32
CJ-VIA (1:10)	181 ± 1.16	187 ± 2.18	184 ± 2.22	190 ± 1.98	186 ± 1.86	40 ± 1.06
CJ-VIA (1:20)	199 ± 1.16	208 ± 2.60	211 ± 2.54	218 ± 2.68	209 ± 2.18	33 ± 1.04

+ SEM = Standard Error of Mean
CJ-VIA = *Crotolaria juncea* -Virus Inhibitory Agent L1,
L2, L3 and L4 represent the number of leaves treated
1.0g of SHRV infected leaf tissue in 50 ml distilled water [virus inoculum (1:100)]

Discussion

Virus Inhibitory agent (VIA) was induced in host plants, following the treatment with phytoprotein, isolated from extract of a non-host plant, endogenously produced in them [33, 34]. Such phytoproteins mediated induced systemic resistance showed partial resemblance with SAR. VIA, which is an inducible gene product likewise AVF, IVR and PR-proteins [35-37]. VIA induction in host plants following application of endogenously produced plant protein extracts, are capable of exvivo virus inactivation [38-40]. Efforts were made to screen the production of VIA in different hosts [41, 42]. The sources used for VIA production were bottle gourd (*Lagenaria siceraria*), cucumber (*Cucumis sativus*) and sun hemp (*Crotolaria juncea*) plants [29]. These antiviral proteins may induce the host for the production of virus inhibitory agents. Probably some protein (VIA) diffuses to surrounding tissues and other plant parts [17]. The VIA have been isolated from leaves of plants treated with antiviral agents and they have been shown conclusively to inactivate the viruses in vitro [43-47]. The release of resistance seems to be an activation of a pre-existing system and hence is easily stimulated. It is probably able to move from one leaf to another through the vascular system of the plant [48-51].

Verma and Awasthi [43] reported that the synthesis of VIA is inhibited, if Actinomycin D was applied soon after phytoprotein treatment. The VIA synthesized was neither virus specific nor host specific [15, 16, 52]. Extracts containing VIA when incubated with the viruses reduced their infectivity [53]. VIAs from a few hosts have been characterized [20]. The VIA synthesized in the leaves of *N.glutinosa* treated with *B.diffusa* root extract reduced infectivity of TMV on *N.glutinosa*, *Datura stramonium* and *D. metel* [38]. It was, however, less effective in inhibiting TRSV and GMV on *C. amaranticolor*. The VIA production was maximum after 24 hours of treatment with the extract. The VIA, synthesized in *C. tetragonoloba* plants ,following treatment with *B. spectabilis* leaf extract , was proteinacious in nature and could prevent the infection of tobamoviruses in seven hypersensitive hosts [44].

These experiments resulted in the conclusion that VIA produced in different host plants bottle gourd (*Lagenaria siceraria*), cucumber (*Cucumis sativus*), sunnhemp (*Crotolaria juncea*) and (Guar) *Cyamopsis tetragonoloba*, can serve as an effective control measures for the management treatment of viral diseases of economically important plants.

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Citation: Awasthi LP (2017) Studies on the Production of Virus Inhibitory Agent (VIA) in Different Hosts Treated with Partially Purified Phytoprotein Isolated from Leaves of Clerodendrum Aculeatum. SF J Immunol 1:1.