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Biological and Molecular Detection of a virus infecting *Wedelia trilobata* (Linn.) Hitchc (A Medicinal Herb)

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Abstract

A Potyvirus has been isolated from *Wedelia trilobata* plants in Aligarh district of state Uttar Pradesh (India). The infected plants were showing mosaic and mottling of leaves as shown below. On host range study virus seem to infect many species of Compositae, Umblifereae, Asteraceae, Solanaceae and Euphorbiaceae. The Thermal Inactivation Point (TIP) was between 55-60°C, dilution end point was within 10^{-4} and Longevity *in Vitro* (LIV) at 20°C was within 24 hr. The virus was transmitted by *Myzus persicae (*Sulzer). Cylindrical inclusion bodies were observed in tissue of the infected plants by electron microscopy. The mean length of the flexous particle was 736.5 nm. The virus was successfully purified from infected *Wedelia trilobata* by differential centrifugation. The purified virus showed the typical UV spectrum of a nucleoprotein with maximum and minimum absorption at 260 nm and 246 nm, respectively. The A_{260} /A_{280} ratio was 1.21 ± 0.04. The symptomatic leaves were analyzed through PCR assays with specific primers.

Keywords: Morpho-biological characteristics; Potyvirus; PCR; *Wedelia trilobata*

Introduction

Wedelia trilobata is a flowering plant of sunflower family, Asteraceae. A tropical perennial medicinal herb, with deeply lobed fleshy leaves, growing up to 10 inch tall, spreading like a mat; it makes a dense cover, blossoms profusely, the flowers are orange- yellow. *Wedelia trilobata* is a medicinal plant used to treat hepatitis infections, to clear the placenta after birth and is used for menstrual pain and unspecified female complaints [1]. In a survey conducted in March-April year 2010, *Wedelia trilobata* plants were found to be heavily infected with virus disease (Figure 1A). Disease symptoms comprised of mosaic and mottling in beginning followed by distortion of leaves in later stages (Figure 1B). Attempts were made to identify the virus causing mosaic and mottling in the experimental plant. To date no reports of viral disease on *Wedelia trilobata* has been reported. This paper describes a potyvirus isolated from Wedelia in Aligarh district of Uttar Pradesh, India and reports the morpho-biological characteristics, purification, physical properties and RT-PCR based detection.

Materials and Methods

Virus source

The virus was obtained from naturally infected *Wedelia trilobata*. The virus was maintained in the seedlings of *Nicotiana tabacum* Var. (Samsun NN) grown under glass house conditions.

Host range

Wedelia trilobata leaves infected with the virus were homogenised in 0.02 M phosphate buffer, pH 7.2, and the extract was inoculated onto carborandum dusted leaves of different herbaceous plants. Sixty species belonging to 24 genera were inoculated. The presence of virus was checked with an electron microscope by the direct negative staining method.

Figure 1A: Symptoms of naturally occurring and artificially inoculated plants. Showing naturally infected plants leaf of *Wedelia trilobata*.

Figure 1B: Symptoms of naturally occurring and artificially inoculated plants. Showing healthy and infected leaf of Wedelia.

Stability in sap

Leaves of *Wedelia trilobata* inoculated with the virus were used to determine the virus properties in crude sap, Thermal Inactivation Point (TIP), Longevity *in vitro* at 20°C (LIV) and Dilution End Point (DEP) by usual techniques [2].

Aphid transmission tests

Aphid transmission tests were conducted using *Myzus persicae* Sulzer. After being starved for 1 hr, aphids were allowed to an acquisition feeding of 3-5 min on infected leaves of *Wedelia triobata*, and then allowed to feed for 24 hr on virus free seedlings of *W. trilobata*. The

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symptoms on *W. trilobata* were observed for 40 days after inoculation, after which virus transmission was checked by electron microscopy.

Purification of virus

Around one to two months after inoculation, the infected leaves were collected and were stored at -20°C until used. The leaves were processed according to the method which was for purifying procedure proposed with watermelon mosaic virus [3].

Electron microscopy

Shape and size of virus particle was studied in electron microscopy.

Leaf dip method

Method described by Brandes [4] was followed for leaf dip preparations. One drop each of 20% potassium phosphotungistic acid (PTA) and uranyl acetate was placed separately on several formvar copper grids having carbon backing. The freshly cut ends of infected leaves were dipped in the drop for 2-4 seconds. Such grids were allowed to dry for some time and there after examined under electron microscope at various magnifications.

Total RNA extraction and RT-PCR

Total RNA was extracted from the field collected samples and one of the inoculated plants that were showing symptoms of virus infection. The primers designed and used in RT-PCR which lead to the amplification of coat protein gene were as under.

First primer Dn-9502-(5' GCGGATCCTTTTTTTTTTTTTTT-3')

Cpup- (5' TGAGGATCCTGGTGYATHGARAAYGG-3')

Second primer CP F-(5'-GCTCCACATAGCTGAGAC-3')

CP R – (5'-AACGGAGCTAATCTCGAGC-3')

Reverse transcription polymerase chain reaction was performed using QIAGEN one step RT-PCR kit (promega). The reaction was set up according to manufacturers instructions. PCR amplification parameters included initial template denaturation at 94°C for 5 minutes followed by 30 cycle of PCR consisting of 1 minute of denaturation at 94°C, 1 minute for primer annealing at 53°C, 1 minute of primers extension at 72°C. Steps of denaturation and elongation were repeated and the final elongation carried out at 72°C for 10 minutes. PCR DNA marker (promega) was used to determine the size of RT-PCR amplified cDNA products [5].

Results

Host range and symptomatology

The virus had a moderate host range and infected the members of Chenopodiaceae, *Vigna sinensis*, *Vigna radiata* and *Vigna mungo* locally after 4-5 days of mechanical inoculation. It caused systemic infection in many species of Solanaceae and some species of Cucurbitaceae 10-14 days after inoculation.

The members of Chenopodiaceae, viz, *Chenopodium amaranticolor* Coste and Reyn (Figure 1C) and *C. murale* produced chlorotic local lesions. The species of Solanaceae, viz., *Datura metal* L., *D. stramonium, Lycopersicon esculentum*, *N. glutinosa, N. longiflora, N. tabaccum* Var, Samsun (Figure 1D), *N. tabaccum* Var, Samsun type Turkish and *Physalis peruviana* L. produced systemic mosaic symptoms and leaf deformation was observe in *Dahlia, Lycopersicon esculentum* and *Ricinus communis.* The host range and symptomatology are more or less similar with the ones reported by [6,7] which reported a potyvirus disease of *Cucurbita moschata*.

Properties if the virus is in plant sap

It is desirable to study biophysical properties of a virus in plant sap before an attempt is made to purify it and study its physio-chemical properties.

Parameters such as thermal inactivation point (TIP), dilution end point (DEP) and ageing *in vitro* in the identification of plants viruses. Study of these characters gives an idea about the stability, concentration and other properties of the virus in the plant sap, and provides information about the best environment in which to keep the virus and maintain its infectivity. Although these studies have restricted value, [8], still they are of utmost importance in determining the procedure for the purification and characterization of a virus.

Thermal inactivation point (TIP)

The virus in crude sap was found to be infectious after being heated for 10 min at 55°C but was found to be infective after being heated at 60°C (Table 1) Thus, the thermal inactivation point (TIP) of the virus lies between 55°C to 60°C.

Dilution end point (DEP)

The virus in crude sap was found to be infectious at a dilution of 10^{-4} but no local lesions were found at a dilution of 10^{-5} (Table 2).

Longevity *in vitro* (LIV)

Temperature had a great effect on infectivity of the virus in crude sap during storage. At room temperature 20-25°C infectivity was retained up to 2 days but was lost on the 3rd day. At 10°C the virus remained active up to 3 days and when stored at 4°C infectivity was retained up to 5 days (Table 3).

The virus was readily transmitted by *Myzus persicae* in a non-persistent manner, symptoms induced by aphid transmission were similar to those induced by mechanical transmission. Under the most favorable conditions, 100 g of tissue yielded approximately 2.90 mg of virus, as estimated spectrophotometrically, taking 2.4 as the extinction coefficient at 260 nm [9]. Flexuous rod-shaped virus particles were consistently seen in purified preparations (Figure 2 and 3). The purified

Figure 1C: Symptoms of potyvirus on some inoculated plants. *Chenopodium amaranticolor* showing chlorotic local lesions.

Figure 1D: Symptoms of potyvirus on some inoculated plants. *Nicotiana tabaccum* Var. Samsun showing severe mosaic symptoms.

virus had an ultraviolet absorption portion of nucleoprotein with a maximum at 260 nm and a minimum at 246 nm (Figure 4). Ratio of UV absorption (A_{260}/A_{280}) was 1.12 ± 0.04, indicating that 6% of nucleic acid was contained in the virus, typical of Potyvirus [10]. The modal length of the virus was 736.5 nm. Observation of the ultra-thin sections revealed the presence of typical cylindrical inclusion bodies in the leaves of artificially infected *W. trilobata* and in naturally infected *W. triobata* plants. Pin wheels, scrolls, and tube like structures were also observed (Figure 2). Potyviruses induce the formation of characteristic cylindrical inclusions in the cytoplasm of infected cells [11].

RT-PCR based detection

The electrophoresis analysis of the RT-PCR product obtained from the total RNA extraction of virus infected samples that were reverse transcripted prior to amplification with PCR using specific primers for CP designed to amplify 650 bp of the coat protein gene showed a single amplified fragment of expected size 650 bp (Figure 5).

Discussion

On the basis of symptoms produced in the host plants, the physical properties *in vitro*, the purification and absorption data, the morphology of the particles, the inclusion observed in the infected tissues, the narrow host range (one of the characteristics of potyviruses) and RT-PCR based detection, it is for the first time the virus disease has been reported in *Wedelia trilobata*. Based on preliminary investigations it was confirmed that the virus belongs to Potyvirus group of family

Temperature	Average number of lesion/ leaf *
40	32
45	19
50	11
55	06
60	00
65	00
70	00

*Average number of local lesion / plant based on three experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

Table 1: Effect of temperature on the infectivity of the virus.

Dilution	Average number of local lesion /leaf*
undiluted	40
10^{-1}	30
10^{-2}	16
10^{-3}	10
10^{-4}	04
10^{-5}	00
10^{-6}	00

*Average number of local lesions/plant bases on three experiments with 3 plant of *C. amaranticolor* having 6 leaves each.

Table 2: Effect of dilution of crud sap on the infectivity of virus.

Temperature °C	Average Number of location lesions /leaf* storage of sap in days								
	1	2	3	4	5	6	7	8	9
Room temp (20-25°C)	7	3	0	0	0	0	0	0	0
10	10	5	2	0	0	0	0	0	0
4	16	10	6	4	3	0	0	0	0

*Average numbers of local lesions/plant based on three experiments with 3 plants of *C. amaranticolor* hawing 6 leaves each.

Table 3: Longevity *in vitro* (LIV) of the virus when stored at different temperature.

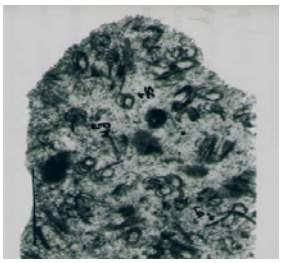

Figure 2: Showing pinwheel scrolls and tube like structures.

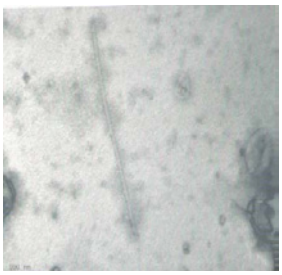

Figure 3: Flexous rod typical of potyvirus.

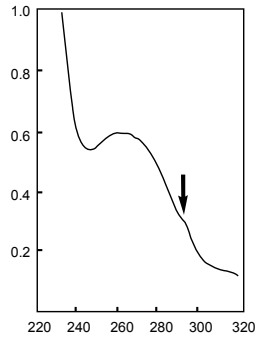

Figure 4: Absorption spectrum of purified preparation of the virus isolated from Wedelia. Arrow indicates a tryptophan shoulder at 291 nm.

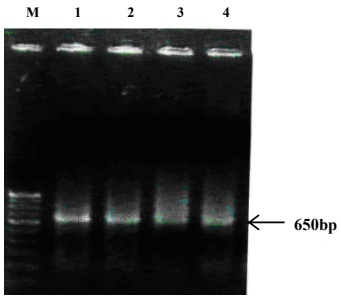

Figure 5: Gel-electrophoresis of RT-PCR amplification of a coat protein gene using specific primer pair designed to amplify 650 bp fragment in infected samples of (Lanes 2-4) with positive sample (Lane 1).

Potyviridae. The potyvirus group (named for its prototypical member, potato virus Y (PVY)) is the largest of the 34 plant virus groups and families currently recognised [12]. This group contains at least 180 definitive and possible members (30% of all known plant viruses) which cause significant losses in agricultural, pastural, horticultural and ornamental crops [12]. This is for the first time that potyvirus infecting *Wedelia trilobata* have been reported from Aligarh India.

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