Journal of Advances in Science and Technology

Vol. 11, Issue No. 22, May-2016, ISSN 2230-9659

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AN INTERNATIONALLY INDEXED PEER REVIEWED & REFEREED JOURNAL

www.ignited.in

*Journal of Advances in Science and Technology*
*Vol. 11, Issue No. 22, May-2016, ISSN 2230-9659*

# Identification and Characterization the Virus Isolate Prevalent In Pea Growing Localities by DAC ELISA Technique

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***Abstract – The present investigations were undertaken to identify virus responsible for causing mosaic disease in pea (Pisum satium L.) under natural condition in Shahjahanpur. An attempt was also made to find out the resistant sources through field screening of available germplasm. The virus isolate was transmissible through sap, seed and aphid vectors Acyrthosiphon pisum and Myzus persicae. Seed transmission of virus isolate checked by grow out test and DAC ELISA technique revealed that virus is seed transmissible with a transmission rate of 20 percent. It was detected in embryo not in seed coat of seeds harvested from infected plants (var. PB-89). Thus, the virus under report is transmissible through sap, seed and aphid vectors.***

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## INTRODUCTION

Like other vegetable crops, this crop is also known to be a victim of many plant pathogens like fungi, bacteria and viruses. Routine surveys conducted for observing the natural occurrence of various diseases in different pea growing areas of Shahjahanpur revealed the presence of mosaic symptoms on plants, typical of viral etiology. Mosaic disease in pea is known to be associated with a number of viruses belonging to different genera such as Potyvirus Enamovirus, Alfamovirus, Comovirus, Potexvirus, Luteovirus, Carlavirus, Nanavirus, Tobravirus, Cytorhabdovirus, Cucumovirus and Tospovirus. Mosaic disease was present variably in different pea growing localities of state. Thus, it became imperative to carry out investigations on characterization and identification of virus isolate(s) associated with mosaic disease besides finding out resistant sources against the prevalent isolate in available germplasm.

## REVIEW OF LITERATURE

Pea (Pisum sativum L.) is an important vegetable crop and it is reported to be infected by a large number of viruses both under natural and experimental conditions. A critical screening of the available literature on the occurrence of various plant viruses infecting pea under natural conditions in different parts of the world revealed that this crop is infected by about 22 viruses and their strains. Major viruses along with their genera/families are listed below:

1. Alfalfa mosaic virus (AMV) Family: Bromoviridae, Genus: Alfamovirus
2. Bean leaf roll virus Family: Luteoviridae, Genus: Luteovirus
3. Bean yellow mosaic virus(BYMV) Family: Potyviridae, Genus: Potyvirus
4. Beet western yellow virus (BWYV) Family: Luteoviridae, Genus: Luteovirus
5. Clover yellow mosaic virus Family: Alphaflexiviridae, Genus: Potexvirus
6. Clover yellow vein virus (CYVV) Family:Potyviridae, Genus: Potyvirus
7. Lettuce mosaic virus (LMV) Family: Potyviridae, Genus: Potyvirus
8. Milk vetch dwarf virus (MVDV) Genus: Nanavirus
9. Muskmelon vein necrosis virus (MuVNV) Family: Betaflexiviridae, Genus: Carlavirus.
10. Pea early browning virus (PEBV) Family: Virgaviridae, Genus: Tobravirus.

11. Pea enation mosaic virus (PEMV) Family: Luteoviridae, Genus: Enamovirus

12. Pea mild mosaic virus (PMiMV) Family: Comoviridae, Genus: Comovirus

13. Pea mosaic virus (PMV) Family: Potyviridae, Genus: Potyvirus.

14. Pea seed borne mosaic virus (PSbMV) Family: Potyviridae, Genus: Potyvirus

15. Pea stem necrosis virus

16. Pea streak virus (PeSV) Family: Betaflexiviridae, Genus: Carlavirus

17. Peanut stunt virus (PSV) Family: Bromoviridae, Genus: Cucumovirus

18. Pisum virus (PisV) Family: Rhabdoviridae, Genus: Cytorhabdovirus

19. Soybean dwarf virus (SDV-D) Family: Luteoviridae, Genus: Luteovirus

20. Subterranean clover red leaf virus Family: Luteoviridae, Genus: Luteovirus

21. Subterranean clover stunt virus (SCSV) Genus: Nanavirus

22. Tomato spotted wilt virus (TSWV) Family: Bunyaviridae, Genus: Tospovirus

## PEA SEED BORNE MOSAIC VIRUS (PSbMV)

Pea seed borne mosaic virus (PSbMV) was first reported in Pisium sativum L. by Musil in 1966 from Europe. Virions are filamentous, not enveloped and usually flexuous with clear modal length of 770 nm and 12 nm wide. Virus is serologically related to bean yellow mosaic virus. The biophysical properties include thermal inactivation point (TIP) of 55 0C, longevity in vitro (LIV) of 1 day (in leaves), or 4 day (in roots) and dilution end point (DEP) of 10-3 to 10-4 . Pea seed borne mosaic virus (PSbMV) is reported to be transmitted by more than 20 aphid species in non-persistent manner but most efficient aphid vector are green peach aphid (Myzus persicae) and pea aphid (Acyrthosiphon pisum). It naturally infects pea however, plants from more than 9 families are susceptible to PSbMV under experimental conditions (Brunt et al., 1997).

Virions are filamentous, not enveloped, usually flexuous, with a clear modal length of 770 nm and 12 nm wide. The characteristic symptoms of pea seed borne mosaic virus produced in P. sativum soon after infection are transitory vein clearing followed by mosaic, rosetting of stem and branches, dark green leaves, adaxially folding of leaflets, flower malformation, often sterile and small pods. The predominant seed symptoms including necrotic rings, line markings on the seed coat, malformation, reduced size and splitting (Esfandiari et al., 2005; Coutts et al., 2008).

## PEA MOSAIC VIRUS

Pea mosaic virus was first reported in Pisum sativum L. by Doolittle and Jones (1925) from Wisconsin, U.S.A. It is also known as common pea mosaic virus. Virions are filamentous, flexuous and non-enveloped with a clear modal length of 785-800 nm and 13 nm width. Biophysical properties of particles include TIP of 55-60°C, LIV of 1-2 days and DEP of 10-3 to 10-4. Virus is serologically closely related to bean yellow mosaic virus, bean common mosaic and clover yellow vein viruses. Distinct mottling of foliage is most conspicuous symptom along with yellowing of tissue between veins, intermingled yellow and green patches and stunted plant. Pods may be fewer and smaller than normal under natural field conditions. Virus is transmitted in a non-persistent manner by nine species of aphids namely, Myzus persicae, Aphis laburni, A. citricidis, Pentatrichopus tetrarhodus, Canariella aegopodii, Macrosiphum gei, M. rosae, A. gossypii, and Rhopalosiphum pseudobmssicae. Virus is also transmitted by mechanical inoculation (Norris, 1943; Gofflot et al., 1991; Brunt et al., 1996).

## BEAN YELLOW MOSAIC VIRUS

Bean yellow mosaic virus (BYMV) was first reported to infect Phaseolus vulgaris L. by Doolittle and Jones (1925) from U.S.A and Netherlands under natural condition and there are reports of natural infection of pea from worldwide. Virions are filamentous, not enveloped, usually flexuous, with a clear modal length of 750 nm and 12-15 nm wide. The virus is serologically related to clover yellow vein and bean common mosaic viruses.

## CLOVER YELLOW VEIN MOSAIC VIRUS

Clover yellow vein virus (CIYVV) was first reported in Trifolium repens by Holling and Nariani in 1965 from U. K ( Brunt et al., 1997). It was also reported to infect pea (Pisum sativum L.). The virions are filamentous, not enveloped and usually flexuous with a clear modal length of 760 nm and 1 2 - 1 5 nm wide. The virus is serologically related to bean yellow mosaic, pea mosaic virus, buckeye cowpea mosaic and soybean mosaic viruses. The biophysical properties of virus include a TIP of 65°C, LIV of 2-8 days and DEP o f 10-3 to 10-5. Host range of CYVV under natural field conditions is quite vast including, Coriandrum sativum, Daucus carota, Glycine max, Limonium sinuatum, Lupinus luteus, Lupinus spp., Pisum sativum and Trifolium hybridum, T. incarnatum, T. pratense, T. repens and T. subterraneum, Symptoms developed on infected pea plant include

mosaics, mottles, streaks, vein yellowing or netting in field condition. The virus is transmitted efficiently by aphids including Acyrihosiphon solani, Macrosiphum euphorhiae and Myzus persicae in non-persistent manner. (Plant viruses online. 2010). Clover yellow vein virus is also known as dendrobium mosaic virus (Pratt, 1961), pea mottle virus, pea necrosis virus (Bos et al., 1977).

## LETTUCE MOSAIC VIRUS

Lettuce mosaic virus (LMV) was first reported to infect Lactuca sativa by Jagger in 1921 from Florida, U.S.A. (Description of Plant Virus. 2003). This virus is distributed worldwide. Virions are filamentous, not enveloped, usually flexuous with a clear modal length of 750 nm and 1 5 nm wide. Biophysical properties of virus include a TIP of 55-60 °C, LIV of 1 - 2 days and DEP 10-2 to 10-3. Virus is serologically related to bean yellow mosaic virus, clover yellow vein, potato Y and tobacco etch virus (Brandes and Bercks, 1965; Dinant and Lot, 1992). This virus has been found to infect Lactuca spp., Senecio vulgaris, Sonchus spp, Cicer arietinum and Pisum sativum under field conditions. It produces chlorotic mottling on infected pea plant. Transmission of virus through aphids includes Aphis gossypii, Macrosiphum euphorbiae, M. gei and Myzus persicae in a non-persistent manner. Virus is also transmitted by mechanical inoculation and infected seeds (Brunt et al., 1996).

## MATERIAL AND METHODS

COLLECTION AND MAINTENANCE OF VIRUS ISOLATES

The cultures of mosaic isolates were collected from three different pea growing localities of Shahjahanpur. The isolates collected on the basis of symptoms and localities were maintained on healthy seedlings of pea variety “PB-89” by mechanical sap inoculation under insect proof glasshouse conditions.

RAISING AND MAINTENANCE OF TEST PLANTS

Healthy seedlings of pea and other test plant species were raised in earthen pots filled with sterilized soil and FYM mixture (3:1 ratio v/v). Generally fifteen to thirty days old seedlings of the test plants at 2-3 leaf stage were used for inoculation. The pots containing test plants were maintained under insect proof glasshouse where the temperature varied between 25-35oC during experimentation.

PREPARATION OF INOCULUM

The inoculum of each isolate was prepared from the young leaves of infected plants showing pronounced symptoms. Leaves were harvested and washed thoroughly first with tap water and then washed with distilled water to remove the extraneous material. Moisture of leaves was removed by keeping them between the folds of blotting papers. The dried leaves were triturated in phosphate buffer (0.1 M pH 7.2) in a sterilized pestle and mortar. The slurry thus obtained was strained through two layers of muslin cloth in sterilized Petri plate. The standard extract thus prepared was used for mechanical inoculations.

## TRANSMISSION

### Mechanical transmission

Healthy test plants of the same age and uniform size were raised by sowing seeds for mechanical transmission studies. Test plants of pea were inoculated at 2-3 true leaf stage by usual leaf rub method. The standard extract was applied by rubbing the sap with fore finger or by cotton swab method. Inoculated leaves were washed thoroughly with distilled water immediately after inoculation to eliminate the excess of inoculum and abrasive from leaf surface. During the mechanical transmission test, every possible care was taken to avoid lethal injury to leaves by abrasive or through hand pressure.

### Transmission by aphid

For the aphid transmission tests, virus free colonies of aphid species viz., Myzus persicae Sulz. and Acyrthosiphon pisum most commonly encountered in and around pea fields were examined for their possibility to act as vectors. Few adults of these species were collected from their healthy host plants and maintained on pea var. PB-89 in isolation chambers of 3’x3’x3’m size covered with nylon net of 80 mesh.

Apterous form of each aphid species were removed from their colonies with gentle tapping and by moist camel hair brush in separate Petri dishes. These were then given one hour pre acquisition access. Sections of leaf tissue infested with 6-10 aphids were placed on the leaf of the test plants (pea). Ten plants were inoculated and kept in separate insect proof cage. After 24 hours of inoculation access, the plants were sprayed with 0.1 per cent metasystox to kill the aphids. These plants were observed for 2-3 weeks for symptoms development under glasshouse conditions.

### Seed transmission

To check the possibility of seed borne nature of isolate two different methods were used. In first method twenty seeds of variety PB-89 from pods of infected plants were collected during 2016. These seeds were sown during the growing season of February 2016 in pots having sterilized potting mixture. The plants thus germinated were allowed to grow under insect proof conditions and were

observed for symptom expression up to 40 days. Plants raised from seeds collected from healthy plants were kept as control.

Serological detection of virus isolate in seed through ELISA as described by Masmoudi et al.(1994) with little modification, if any. Ten mature seeds (Avg. weight of one seed 260 mg) were collected from mechanically inoculated plants. Seed coat and embryo of virus infected seeds were manually separated with help of sterilized forceps and washed 2-3 times with distilled water. The seed coat and embryo homogenized in 5 ml extraction buffer (PTA buffer). Further virus presence in seed coat and embryo was detected by DAC-ELISA using potyvirus specific immunoglobulin following manufacture protocol and O.D. value noted down.

**Soil transmission**

To study the soil transmission four types of soils were used which included (a) fresh soil from the rhizosphere of healthy pea plants (b) fresh soil from the rhizosphere of diseased pea plants infected with respective isolates (c) autoclaved soil mixed with fresh virus infected leaves of respective isolate and (d) only autoclaved soil. Seeds of pea were sown in earthen pots containing above mentioned different soils under glasshouse conditions. Observations on the development of symptoms on the plants grown in these pots were recorded till the plants attained a flowering stage.

## SEROLOGY

Serological detection and identification of virus(es) under present investigation was carried out by following enzyme linked immunosorbent assay (ELISA) technique with the use of commercially available immunoreagents (BIOREBA-AG Switzerland and SEDIAG SAS- France) and by following protocols of suppliers of ELISA Kits with little modification, if any. Detail of the procedures of DAC and DAS forms of ELISA used are given below:

**Potygroup specific DAC- ELISA**

Potyvirus group specific ELISA Kit, purchased from M/s BIOREBA AG, Switzerland was used to detect the presence of potyvirus infection in isolates under investigation. Direct DAC-ELISA as per the protocol supplied by the manufacturer of kit was used in the detection. Test sample (leaves) of approximately 0.5 g were homogenized in 5 ml extraction buffer (PTA buffer). Extract was then distributed in portion of 200 µl into the wells of a microtiter plate (NUNC maxisorp certified microplates). The plate was then covered with parafilm and incubated overnight at 4-6oC. The plate was washed with PBST for 2-3 times. The appropriate portion of the IgG was diluted (1:1000) in conjugate buffer (e.g. for one plate 20 µl in 20 ml conjugate buffer) and dispersed @ 200 µl per well. Plate was again covered as described above and incubated for 2 hours at 37oC, followed by washing of the plate with PBST for 2-3 times. Goat antimouse IgG conjugate was diluted in conjugate buffer (i.e. 1: 1000) and dispersed @ 200 µl per well. Cover the plate as described above and incubated for 2 hours at 370C, followed by washing of the plate with PBST for 2-3 times. Substrate buffer along with the paranitrophenyl phosphate (pNPP) tablet @ 5 mg pNPP tablet in 5 ml of 1X substrate buffer was added to the washed microtiter plate @ 200 µl per well and the plate was again covered with parafilm and was incubated for 30 to 60 minutes at room temperature in dark. The absorbance was then recorded in ELISA plate Reader for interpretation of the results at 405 nm. The results of ELISA for the detection were interpreted as per Lemmettry, 1988 and Dijkstra and Jager, 1998. Test samples were considered infected if their absorbance values (A405 nm) exceeded two times the mean values of the respective healthy control samples.

## DAS-ELISA

Serological detection of viruses through DAS-ELISA was carried out by following the protocol of manufacturers of ELISA reagents (SEDIAG SASFrance). Wells of the microtitre plate (NUNC maxisorp certified microplates) except those of the top and bottom rows and rows on the extreme left and right, were filled with 200 µl aliquots of coating antibodies diluted in 1X coating buffer (1:1000 ratio v/v). The plate was incubated in humid box for 4 hours at 37oC. The coating of antibodies suspension was removed shaking out the plate over the washbasin. The wells were filled with 1X PBS-Tween (washing buffer) and plate was emptied and filled again with PBS-Tween. The washing was repeated three times. The test samples were ground in 1X general extraction buffer (1:10 v/v). All coated wells were filled with 200 µl aliquots of test sample (each sample duplicate) besides positive and negative control wells. The plates were incubated at 4±1oC overnight (at least 12 hrs.). The washing steps were repeated as mentioned above. The alkaline phosphate (ALP) conjugated antibodies were filled in each well with 200 µl aliquots after diluting it in 1X ECI (enzyme conjugated immunoglobulin) buffer at ratio of 1:1000 v/v. The plate was incubated in humid box for 2 hours at 37oC. The washing was done three times as mentioned above. The paranitrophenyl phosphate (pNPP) substrate was dissolved in 1X substrate buffer by dissolving 5 mg pNPP tablet in 5 ml of 1X substrate buffer. Each well was filled with 200 µl aliquots of substrate. The plates were kept in humid box in the dark condition at room temperature until a yellow colour was clearly visible in the positive control (usually between 30 to 60 minutes). If desired the reaction was stopped by adding 50 µl of NaOH to each well. The results were assessed in the same manner as for DAC- ELISA.

## RESULTS

Three isolates were collected from different fields of pea from different locations depending upon the presence of the virus like symptoms on the infected plants observed during the surveys and were designated as I1, I2 and I3, respectively. These isolates were maintained on healthy pea (var. PB-89) plants under insect proof glasshouse conditions. The reaction of three virus isolates (I1, I2 and I3) was observed on certain indicator plants . All the isolates were found to infect the test indicator plant species viz. Chenopodium album L., C. amaranticolor Coste and Reyn, C. quinoa Wild, C. murale L., Vicia faba L. but not Nicotiana glutinosa L., N. tabacum var. White Burley, N. debneyii L., N. occidentalis "37B", Datura metel L. and D. stramonium L. Hence, these isolates were assumed to be similar. A representative isolate I1, "pea virus isolate" of Nauni was selected for further detailed investigations on symptomatology, transmission, host range, biophysical properties, electron microscopy, serology .

## SYMPTOMATOLOGY

The pea (var. PB-89) plants were inoculated mechanically at first true leaf stage by usual leaf rub method using the infectious sap extracted from a diseased pea plant. Visible symptoms on the leaves of inoculated pea plants were noticed in the form of vein clearing and mild mosaic within 15-20 days of inoculation under glasshouse conditions. Later mosaic, typical transient clearing and swelling of vein symptoms were developed on the leaves which turned into severe mosaic with the advancement of infection. As the infection progressed, stunting of plant with shortening and downward rolling of the leaflets were observed and growth of leaf lamina was also impaired. The infected plants also exhibited curling of tendrils along with rosetting of apical portion of plant. Severely infected plants either fail to bear pods or give rise to small distorted pods as compared to healthy plants. Different symptoms observed on the inoculated plants of pea are shown in Plate-3A&3B.

## TRANSMISSION

### Mechanical sap transmission

Healthy test plants of pea were sap inoculated with the standard extract of the virus prepared from infected leaves of pea var. PB-89 having prominent symptoms. The sap inoculated plants were kept under observations for 4 weeks for the development of symptoms. The results of the mechanical sap inoculation experiment revealed that the virus isolate was easily sap transmissible. Symptoms exhibited by the test plants were almost similar to those observed under field conditions.

### Transmission through insect vectors

Two aphid species viz. Acyrthosiphon pisum Harris and Myzus persicae Sulz, mostly encountered in and around pea crop under field conditions were tested for transmission of the virus isolate under studies.

Thus, A. pisum was found to be comparatively more efficient in transmission of the virus isolate than M. persicae.

### Seed transmission

Fifty seeds collected from the pods harvested from infected plants (PB- 89) were sown in pots containing sterilized soil mixtures in the insect proof glasshouse. Observations were recorded on germination and for symptoms development on germinated plants upto 40 days after germination. Only 30 out of 50 seeds germinated and developed into plants. Out of these 30 plants 6 showing stunting and mosaic symptoms after 15-20 days after germination. Samples drawn from symptomatic plants gave positive reaction in ELISA test with the antibodies. Thus, the results medicated the seed borne nature of the virus 12 percent.

### Soil transmission

Healthy seeds of pea were sown in the autoclaved soil mixed with fresh virus infected leaves as well as in fresh soil from the rhizosphere of diseased and healthy plants. Seedlings raised in autoclaved soil served as control. Observations on the plants upto 40 days after germination of seeds could not reveal the virus symptoms in any of the plants grown in soil mixtures mentioned above. Thus, the virus under study was not found transmissible through soil.

### Serological detection of virus in the inoculated plants through DAS ELISA

Different species of plants which were tested for the host range studies by mechanical sap transmission were also tested to confirm the association of PSbMV. Sap inoculated plants of eleven plant species tested under host range were subjected to DAS-ELISA.

The data indicate that the plants of different species includes: Chenopodium album, C. amaranticolor, C. quinoa, C. murale, Pisum sativum, Vicia faba exhibiting virus symptoms after inoculation had PSbMV association as the plants reacted positively with PSbMV antisera by giving desired optical density values. However, Brassica campestris spp. rapa, Datura metel, Datura metel L. var. festuosa, Nicotiana glutinosa, Nicotiana tabacum L. var. "White Burley", failed to react with PSbMV antisera as evident from the respective O.D. values which are

less than twice the value of negative and healthy control.

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