ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Partial Characterization and Serological Relationship of a Tospo Virus Infecting Sunflower (*Helianthus annuss*. L) In South India.

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ABSTRACT

A tospo virus infecting sunflower in India was characterized by host range studies, the virus was purified and polyclonal anti serum was produced. The virus infecting sunflower reacted with homologous antiserum, and PBNV antiserum is DAC-ELISA, and failed to react with TSWV, INSV, IYSV and PSNV. The purified virus resolved as four polypepticles species of 331, 58, 52, 31 kD in SDS PAGE. In electro blot immuno assay, all four polypeptides reacted with homologous antiserum, and only nucleocapsid protein of 31kD reacted with recombinant nucleocapsid antibodies raised against PBNV and none of the polypeptides reacted with TSWV, INSV and IYSV. The RNA from purified virus resolved into three bands of L RNA of 9 Kb, M RNA of 5 Kb and S RNA of 3.5 Kb in agarose gel electrophoresis.

Keywords: Host range, Purification, Polyclonal antibodies, DAC ELISA, SDS PAGE and Immunoblot assay

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ISSN: 0975-8585

INTRODUCTION

Oilseeds are very important of tropical agriculture. America and Europe occupies first two places in oil production respectively [1]. India occupies third place to produce oil seeds in the world. Sunflower was originated in South-West USA and Mexico area. Later the sunflower crop was introduced from Europe to North America in the nineteenth century as cultivated plant species [2]. Sunflower as oilseed crop was introduced in India during 1969. Sunflower is known to be infected by fungal diseases and phytoplasma like organism. In addition to fungal, bacterial and phytoplasma diseases, viral diseases are also very serious problem in limiting the production of sunflower. The common viruses infecting sunflower were reviewed by Brunt *et al.,*[3] and the and recently reported viruses are Sunflower necrosis virus [4]; Sunflower necrosis Tospo virus [5]; Sunflower Tospo virus [6]; Sunflower Poty Virus [7] and Sunflower Ilar virus [8]. Sunflower showed symptom of virus disease characterised by severe mosaic, necrosis of leaves, necrosis along the stems and necrotic rings on floral parts malformation of young leaves, markedly reduced leaves and stunting of plants. The virus isolate have not been properly characterized and typed and no sensitive techniques for virus detection in the crops have been developed. This diseases incidence is very high in all sunflower growing states of India; no sensitive virus detection techniques in sunflower have been developed. The development of practical, sensitive, rapid techniques for detecting and identifying plant viruses have been useful in controlling the disease Many important agricultural, horticultural and floricultural crops are now routinely assayed by ELISA for a range of virus disease [9]. Therefore the present work was initiated to known the cause of the disease.

MATERIALS AND METHODS

Collection, Establishment and Maintenance of Virus Culture

Systemically infected sunflower (Helianthus annus L.) leaves showing characteristic mosaic with light green areas and necrosis in leaves, stem and floral heads were collected from commercial fields in and around Tirupati region of Andhra Pradesh. The samples were tested by DAC-ELISA (Enzyme Linked Immunosorbant Assay) with CMV, PBNV, PSNV (Ilar virus), TSWV-T, INSV and IYSV antisera and positive samples were mechanically inoculated on healthy sunflower seedlings and maintained in the wire mesh (insect proof) house by periodical mechanical inoculation. The virus was maintained by periodical sap inoculation of the 8 to 13 days old healthy sunflower plants at primary leave stage. The virus culture on sunflower was maintained and propagated on sunflower (Cargil, MHSF-8) and *Gompherena globosa* for further studies.

Host Range Studies

A host range study was undertaken to find suitable experimental, differential and propagating hosts. The following plants were grown in earthen pots kept inside the wiremesh house and sap inoculated - *Arachis hypogaea, Cajanas cajan, Chenopodium amaranticolor, C. quinoa, Citrullus lanatus, Commelina , Cymopsis tetragonaloba, Cucumis melo, Cucumis pepo, Cucumis sativum, Cucumis mosechata, Dolichos lablab, Datura metal, Glycene max, Gomphrena globosa, Helianthus annus, Hibiscus esculantus, Luffa acutangula,*

Luffa cylindrica, Lycopersicon esculantum, Mimordica chrantia, Nicotiana glutinosa, N. tobacum Var. Harrison special, Phaseolus auerus, P. mungo, P. vulgaris Petunia hybrida, Solanam melangena, Tridax , Tagetus , Vigna uniguculata and Catheranthus rosea.

Purification of Virus

The virus was purified by using Potassium Phosphate buffer 0.1 M pH 7.6. The virus from infected sunflower leaves was purified according to the procedure of Satyanarayana *et al*., [10] with slight modifications. 100 g of virus infected leaf material was macerated in 3 volumes of (w/v) 0.1 M potassium phosphate buffer pH 7.6 containing 0.2% 2-mercaptoethanal, 225 mg of DIECA and 1% of Triton X –100. The homogenate was filtered through, four fold muslin cloth and volume of the filtrate was measured. To the filtrate 1% Non Diet P-40 was added and stirred for 10 min at 4^0C. The clarified extract was centrifuged at 8000 rpm for 20 minutes at 4^0C in RC5C (Sorvall) high speed refrigerated centrifuge using fixed angle rotor and the supernatant was collected. The supernatant was centrifuged at 35,000 rpm for 1 1/2 hr at 4^0C in Combiplus (Sorvall) ultra centrifuge by using fixed angle rotor. The pellet was collected and dissolved in minimal volume of 0.01 M Potassium phosphate buffer pH 7.0 and left overnight at 4^0C. The sample was centrifuged at 6000 rpm for 5min. The supernatant was over layered on 10% to 40% preformed linear sucrose gradient column and centrifuged at 25,000 rpm for 2 hrs at 4^0C. The gradient tubes were observed under light in dark room and light scattering zone was collected carefully and diluted twice with 0.01M Potassium phosphate buffer pH 7.0. The virus was pelleted at 35,000 rpm for 1 1/2 hrs at 35000 rpm at 4^0C. The pellet was suspended in minimal volume of 0.01 M Potassium phosphate buffer pH 7.0 and stored at –80^{o}C in deep freezer for further use. The purified virus samples were scanned in U.V visible (Hitachi2000) recording spectro photometer from 220 to 300 nm. The A_{max}/A_{min}, A_{260}/A_{280} ratios were determined assuring a specific extinction coefficient of 5 cm^{-1} mg^{-1}(uncorrected for light scattering zone)[3] Purified virus samples were quantified spectrophotometrically by measuring the absorbance at 260 and 280 nm concentration of purified virus was determined by taking 5 OD=1 mg of virus at 260nm.

Analysis of Nucleo capsid protein

Virus Nucleo capsid protein was analysed by SDS-PAGE as described by Laemmli [11]. Purified virus suspended in 0.01 M potassium phosphate buffer, pH 7.0, (equivalent to 10-12 μg) was diluted with sample buffer (1:5 v/v). The viral polypeptides were disrupted by heating the samples on water bath at 95 ^{0}C for 3 min. Then the samples were immediately transferred to cold water. The purified virus and marker protein samples were loaded into different gel slots. The distance migrated by virus proteins and marker proteins was recorded. The molecular weight of virus nucleo capsid proteins was calculated based on a calibration curve drawn for marker proteins on a semi log graph paper.

Isolation of RNA from Purified Virus

The viral nucleic acid was isolated from purified virus preparations by following the method of Sambrook *et al*., [12]. The purified virus was suspended in 0.01 M phosphate buffer pH, 7.0, to this 10%SDS and 200 mM EDTA were added to 1% and 20 mM,

respectively. The contents of the tube were mixed and incubated at 65 ^{0}C for 10 min. The tubes were cooled to room temperature and proteinase K was added to 1 mg/ml and incubated at 37 ^{0}C for 10 minutes. Afterwards, the sample was extracted with an equal volume of Tris-saturated phenol. Aqueous phase was collected by centrifugation at 10,000 rpm for 10 min at 4^{o}C and reextracted with equal volume of phenol: chloroform-isoamyl alochol (24:1v/v). Again the upper aqueous phase was collected and an equal volume of chloroform:isoamyl alcohol only was added and extracted. The nucleic acid in the final aqueous phase was precipitated by adding 2.5 M sodium acetate to 250 mM and ice-cold absolute ethanol to 2.5 volumes and kept at –20^{0}C for overnight. The precipitated nucleic acid was pelleted by centrifugation at 10,000 rpm for 15 min at 4^{o}C. Pellet was washed twice with 70% ethanol. The final pellet was dried and dissolved in minimal volume of sterilized TE buffer.

Agarose gel Electrophoresis of Viral Nucleic acid

Agarose gel electrophoresis was performed to resolve viral nucleic acid as described by Sambrook *et al*., [12] using TBE buffer system. Purified virus sample was mixed with dissociation buffer (1:1, v/v) and kept in water bath at 65 ^{0}C for 10 min. immediately the sample was transferred onto ice- bath. In another eppendorf tube the isolated viral nucleic acid sample was mixed with sample buffer (1:1, v/v). The isolated viral nucleic acid and marker samples were loaded into different gel slots. The distance migrated by nucleic acid and marker was recorded.

Production of Polyclonal Antiserum

Healthy New Zealand white rabbit was taken for Immunization. 1 mg of the purified virus was emulsified with an equal volume of incomplete Freund's adjuvant and injected into the thigh muscle of the rabbit. Five injections were given at one week interval. Seven days after the last injection, the rabbit was test bleed by making a cut on the marginal ear vein with a sharp sterile blade. Blood was allowed to clot for 3 hours at 4^{0}C. The tubes were centrifuged at 6,000 rpm for 15 minutes and antiserum was collected into 1ml aliquots and was stored in deep freezer. The rabbit was further bleed at weekly intervals.

Enzyme Linked Immunosorbent Assay (ELISA)

The direct antigen coating (DAC) form of indirect ELISA described by Hobbs *et al*.,[13] was adopted to determine antiserum titer and serological relationships of the virus. The samples were prepared in carbonate buffer and 0.2ml was added to each well of the plate (Tarson, India) and incubated far 2 hours at 37^{0}C. The plate was washed 3 times with PBS-T (by keeping 3 minutes interval between each wash). Antiserum dilutions were prepared with PBS-TO (1: 500, 1:1000, 1:2000, 1:5000, 1:10000) and added to the wells (0.2ml/well). The plate was incubated at 37^{0}C for 2 hours. After incubation the plate was washed 3 times with PBS-T and goat antirabbit antibodies labelled with ALP (Sigma) was diluted (1:1000) with PBS-TO and added to the plate. The plate was incubate at 37^{0}C for 2 hours and washed with PBS-T for 3 times. The substrate P-nitrophenyl phosphate(Sigma) was added to the wells and incubated at room temperature for 30 min. The reaction was terminated by

adding 50µl of 3 M NaOH solution to each well. The reactions were noted according to yellow colour intensity.

Electro Blot Immuno Assay: (EBIA)

Immuno electroblot detection of viral proteins was described by Koenig and Burgermeister [14]. Approximately 40 µg of virus coat protein was boiled with sample buffer (1:1 v/v) for 3 minutes in boiling water and electrophorosised by 12% SDS-PAGE as described earlier. Electro blotting was carried out using semi dry blot apparatus (Millipore). The unstained gel was soaked in cathode buffer and shaken for 20 minutes at room temperature. Seven sheets of whatman No.3 filter papers were cut in the size of gel and soaked 2 sheets in anode buffer I, 2 sheets in anode buffer II and 3 sheets in cathode buffer. The PVDF membrane (Millipore) was cut in the gel size and soaked in 100% methanol for 15 seconds and then soaked in deionized water for 15 seconds. Membrane was equilibrated in anode buffer II for 5 minutes and sheets were arranged in semi dry blot unit (Millipore). The unit was connected to power pack and current was adjusted to 54 mA per 1 hour. After the electrophoretic transfer of proteins to the membrane and it was incubated in a blocking solution for 2 hours at room temperature. The membrane was incubated by shaking on a rocker Later the membrane was cut into two equal halves and one half was soaked in 1:1000 dilution of homologous antiserum and another half into solution of Peanut bud necrosis virus antiserum in antibody buffer separately. After 3 washings of each 5 minutes in TBT-T, the membrane was incubated for 1 hour in 1:5000 dilution of horse radish peroxidase labelled goat antirabbit antibodies in antibody buffer. The membrane was washed thrice by 5 minutes interval each in TBS-T and transferred to a substrate solution. Colour development was recorded visually and the reaction was stopped by washing the membrane in distilled water. The membrane was dried and photograph was taken and kept under dark condition.

RESULTS

The sunflower infected leaf samples were collected from commercial fields in and around Tirupati of Rayalaseema region in Andhra Pradesh. The sunflower plants showing chlorotic, mosaic, and severe necrosis on leaves followed by necrosis along the leaf lamina, petiole, stem and the necrotic and chlorotic concentric rings on floral heads of the infected plants. These samples were checked for virus detection by DAC-ELISA using different antisera available in our lab, such as different isolates of CMV, TSWV-T, PBNV, PSNV (ICRISAT, Hyderabad), INSV (IIHR, Bangalore) and IYSV (Netherlands). In DAC-ELISA, the field samples reacted positively with PBNV antiserum and negative reaction with the remaining antisera. This isolate has been taken for a further study of the virus which is causing severe yield loses in sunflower crop.

Symptoms on Sunflower

The virus was maintained by inoculating the same virus sample on to sunflower seedlings of the same variety (MSHF-17) for the maintaince of the virus culture. The virus has been identified and characterized as follows. Initially chlorotic lesions were appeared on inoculated primary leaves of sunflower after 7-8 days of inoculation. Later small chlorotic

spots developed on young emerging leaves and gradually these chlorotic spots developed in size and to form large chlorotic areas there by giving mosaic appearance, (Morden variety MHSF-8, MHSF-17 and Cargil variety). After ten days necrotic symptoms were observed on the leaves as necrotic rings, necrosis spread along the veins and leaf lamina (Fig.1). Systemic necrosis was observed along the stem, (Fig.2) petioles of the leaves and the pedicle of the floral head. Concentric necrotic rings were observed on the calyx (Fig.3) and spots on the petals of the ray florets in the infected plants (MHSF-8 and Cargil variety). Finally necrosis (death) of the plant occurs. Early infected plants show severe necrotic symptoms and chlorosis of the plant. Later the plants were stunted and die due to necrosis.

Fig.1 Fig:2 Fig.3

Fig.1 Infected sunflower leaf showing chlorotic spots, rings and necrosis
Fig. 2 (a) Healthy stem (b) Infected stem of sunflower showing necrosis
Fig.3 Infected sunflower floral head showing necrosis and necrotic rings

Host Range

In the host range studies plant species belongs to families Chenopodiaceae, Solanacae, Amaranthacea, Cucurbitaceae, Asteraceae, Leguminaceae was tested. Six plants of each species were used for inoculation. Reaction of various host plants to the virus was observed. The results showed that the virus has a narrow host range producing chlorotic and necrotic local lession on member of family chenopodiaeae i.e, *Chenopodium amaranticolor* (Fig.4), local chlorotic lession on *Datura metal* (Fig.5), on *Gompherena globosa*, local chlorotic, spots were observed intially later turn to chlorotic to necrotic ring spots on inoculated leaves and turns systematic mosaic on developing leaves (Fig.6a,6b), tar like symptoms produced on the leaves of *Catheranthus rosea* (Fig.7), chlorotic spots on *Cowpea* CV 152 were observed later turned to chlorotic ring spots and finally necrotic ring spots were observed (Fig.8). Chlorotic symptoms produced on inoculated leaves of *Petunia hybrida*. Brown spots produced on the inoculated leaves of *Cassia tora,* Cucumber long plants produced systematic symptoms like bronzing of leaves and stunting of plant and the tip of plant die back. *Watermelon* produce systemic symptoms like mosaic, necrosis along the stem and leaves and finally the terminal bud die back due to necrosis [3,15].

ISSN: 0975-8585

Fig 4

Fig 5

Fig 6a

Fig.4. Infected *Chenopodium amaranticolor* leaf showing chlorotic and necrotic spots
Fig.5. Infected *Datura metal* leaf showing Chlorotic spots
Fig.6a. Infected *Gomphrena globosa* leaves showing chlorotic spots, mosaic symptoms

Fig 6b

Fig 7

Fig 8

Fig.6b and chorotic rings
Fig.7. Infected *Catheranthus rosea* leaf showing tar like symptoms
Fig.8. Infected *Cowpea cv.152* leaf showing chlorotic and necrotic ring spot symptoms

Purification of Virus

The virus was successfully purified from infected sunflower leaves by extracting in 0.1M of Sodium Phosphate buffer pH .7.0 containing 0.2% of 2-mercaptoethanol, 0.02% of Sodium Sulphate and 225 mg of DIECA was added to extraction buffer before extraction of virus. Clarification of sap was done with 1% nonidet P-40, concentration of virus was done by high speed pelleting and sucrose gradients centrifugation of concentrated virus, single light scattering zone was observed in the density gradient tube at the height of 4 to 4.3 cm from the bottom of the tube (Fig.9). Purified virus was proved to be infectious on sunflower, *Gomphrena globosa* and *C. amaranticolor* when inoculated on healthy plant. The virus culture was maintained and propagated on sunflower and *Gomphrena globosa*. The purified

virus obtained form sunflower leaves was scanned in UV Vis spectrophotometer from 220nm to 300 nm (Fig.10). The virus has maximum absorption at 258.2nm and minimum absorption at 240 nm respectively. A_{260}/A_{280} and A_{max}/A_{min} ratios were 1.547 and 1.419 respectively. The virus yield was range from 37 mg / 100g freshly infected sunflower leaves.

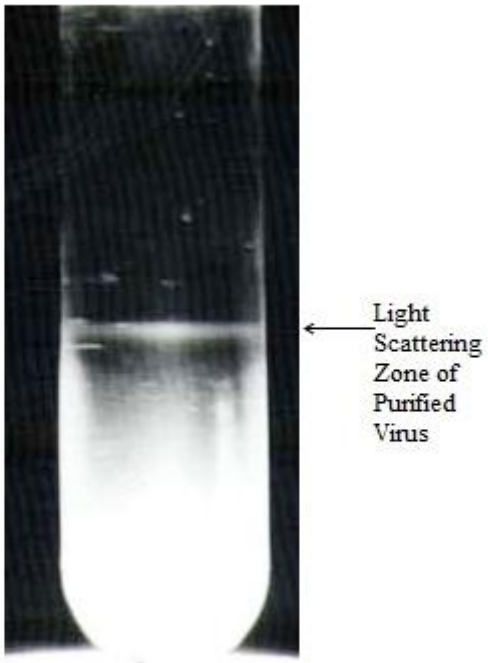

Fig.9
Light scattering zone after density gradient centrifugation.

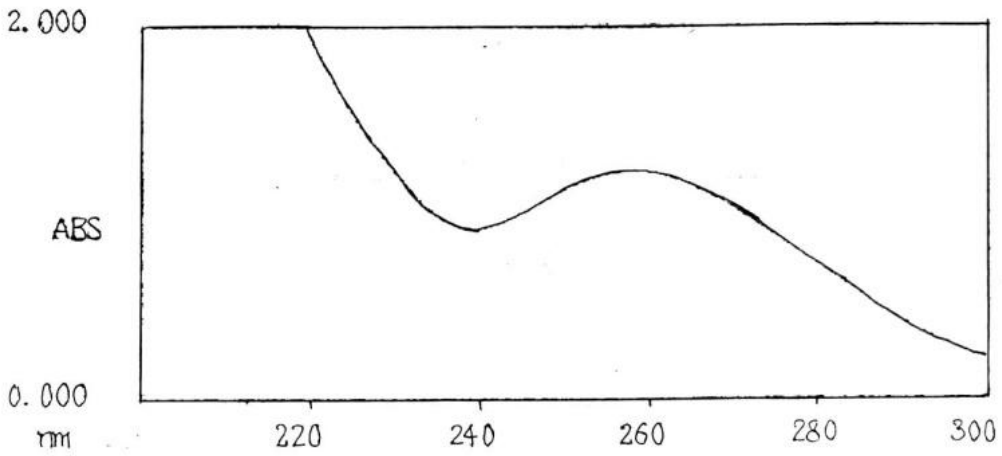

Fig.10. U.V. absorption spectrum of purified virus

After four successive injections of the purified virus, the test bleed was done by cutting the vein on ear of the rabbit and the serum was collected. Antiserum was collected week after week and the titre was detected by performing DAC-ELISA. The positive reaction was upto 1/5000 titre of antisera of IVth bleed was observed, but in case of 1st bleed reaction was observed upto 1/2000 dilution. In DAC-ELISA almost all the samples from sunflower, *C.amaranticolour, Cowpea, Gomphrena globosa, Catheranthus rosea, Datura metal, Petunia hybrida* are indexed with different antiserum of CMV isolates (Department of Virology), PBNV, PSNV (ICRISAT) TSWV-T, (Georgia), INSV (IIHR, Bangalore) IYSV [Netherlands] and also

ISSN: 0975-8585

with homologous antiserum. In ELISA all the samples reacted positively with sunflower mosaic homologous antiserum and also with PBNV antiserum and negative reaction with INSV, TSWV-T, IYSV and CMV antisera. There was slight healthy back ground reaction with homologous antisera.

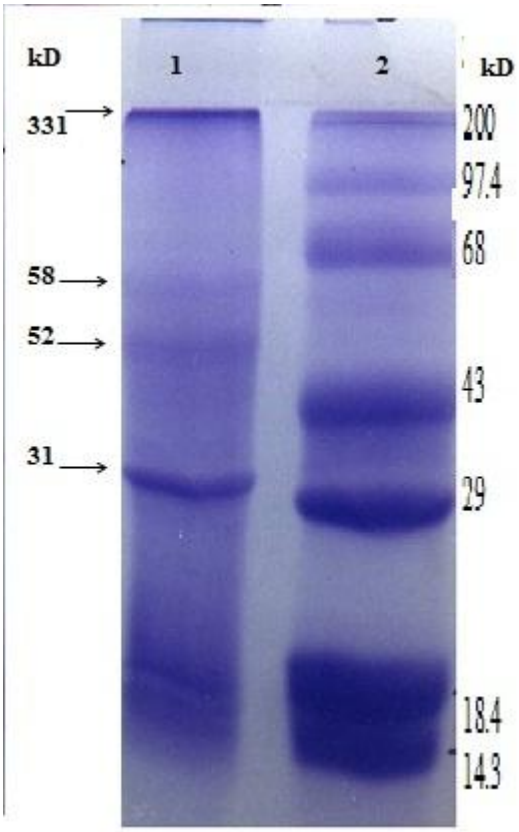

Fig.11. SDS-PAGE analysis of coat protein
Lane.1: purified virus
Lane.2: Protein marker(GIBCO-BRL)

Molecular weight of virus coat protein was determined by SDS-PAGE. The virus when analysed soon after purification resolved as 4 major polypeptide of Mw. 31Kd, 52 kd, 58 kd, 331 kd (Fig.11).

The RNA isolated from purified virus was run in 1% agarose gel electrophoresis, the RNA resolved as three bands which as the molecular weight same as that of reported tospoviruses. The three bands as the following molecular weight L-RNA-9.0Kb, M-RNA-5.0Kb and S-RNA-3.5Kb (Fig.12).

In Electro blot Immuno assay all the bands transferred from gel to PVDF membrane and reacted strongly with homologous antiserum and with PBNV antiserum. The band coressponding to the coat protein was reacted strongly with PBNV antisera produced against recombinant N capsid protein of virus (ICRISAT) (Fig.13).

ISSN: 0975-8585

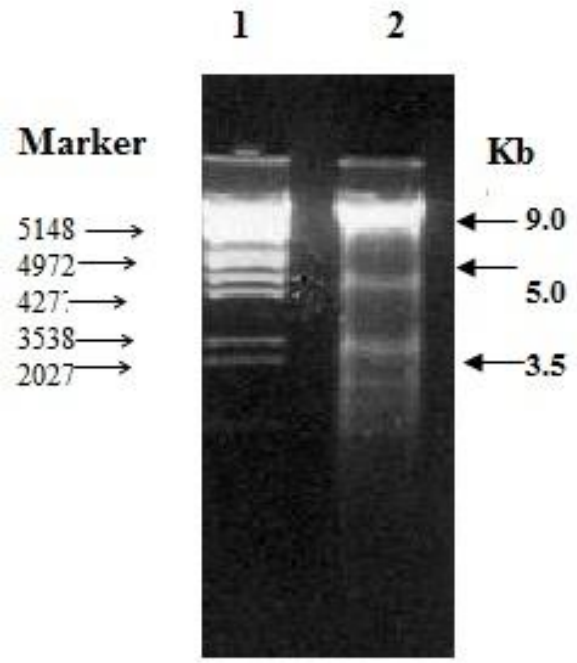

Fig.12. Agarose gel electrophoresis of Nucleic acid
Lane.1.RNA marker(GIBCO-BRL)
Lane.2.Isolated RNA from purified virus

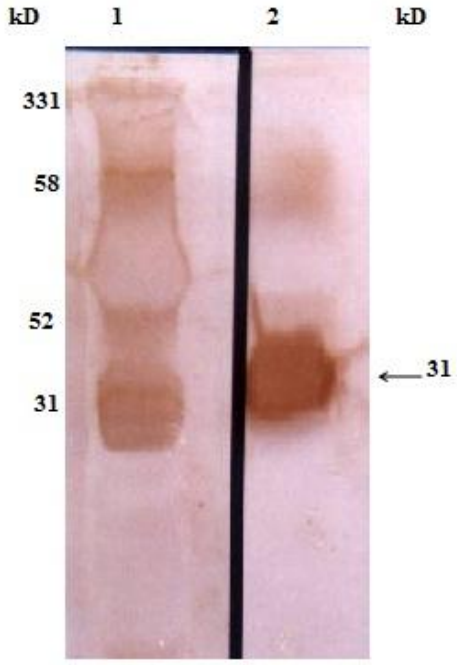

Fig. 13

Fig.13 Immuno electroblot analysis of virus coat protein
Lane1. Homologous antiserum
Lane.2. PBNV antiserum

ISSN: 0975-8585

DISCUSSION

Sunflower (*Helianthus annuus*. Linn) is one of the most important edible oilseed crops in the world. It is not a season bound crop and can be cultivated at any time of the year. Due to continuous cropping and wide adoptability the sunflower crop suffers with severe fungal, bacterial and viral diseases and these diseases cause immense yield losses. Viral disease occupies an important position as they caused severe yield losses from time to time [3, 16]. Tospo viruses are the new emerging plant viruses and belong to family Buniyaviridae. Currently more than 800 different plant species within 82 families of monocots as well as dicots are known to be susceptible to these viruses. Tospo virus named after the type species tomato spotted wilt virus (TSWV).

Tospo virus infecting sunflower has been reported from India [5,6]. In the present study the systemically infected sunflower leaves showing characteristic mosaic followed by necrosis was observed in commercial fields in and around Tirupati region of Andhra Pradesh. The virus infected field samples were collected and inoculated on the sunflower plants and maintained in the insect proof mesh house.

In the present study on a virus infecting sunflower, produced symptoms of chlorotic, severe mosaic, necrosis, necrotic ring spots on leaves followed by necrotic steaks on stem, petioles of the leaves and concentric necrotic rings were observed on the floral heads of the infected plants [5,16,17]. Both the natural symptoms and artificial induced symptoms in the glass house were more or less identical. All the symptoms were similar to the earlier reported tospo viruses with the present virus infecting sunflower [5,18]. The present virus differs with respect to symptoms like, mosaic, leaf distortion, leaf curl and yellowing which symptoms caused by cucumber mosaic virus (CMV); Poty virus [7], Tabocco streak virus and sunflower Ilar virus [8]. Whereas the sunflower tospo virus does not produced the above symptoms.

The occurrence of tospo virus infection on sunflower has been reported by several workers [5,18] and identified as a Tospo virus based on transmission, host range, physical properties, serological relationships, nucleic acid, protein and blotting analysis. Earlier TSWV considered as the single virus present in the tospo virus group. Presently there are increased in many viruses reported in tospo virus group and studies on vector transmission, serology and N protein relationship has indicated the presence of several tospo viruses. For sap transmission studies, 0.1M Potassium phosphate buffer pH 7.0 containing 0.2% 2-mercaptoethanol and 0.2% sodium sulphite was found better for mechanical inoculation. Prasad Rao *et al.,* [19] was also used 0.5M potassium phosphate buffer containing 0.02% 2-mercaptoethanol for successful transmission.

Sunflower tospo virus has caused chlorotic, necrotic local lesions on *C.amaranticolor* and chlorotic spots on cotyledens of cucumis sativas [20], local cholorotic lesions on *Datura metal* , local chlorotic and necrotic ring spots on inoculated leaves of *Gomphrena globosa*, tar like symptoms on *Catheranthus rosae* [15], Chlorotic and necrotic ring spots on Cowpea CV-152 [21], cholorotic spots on inoculated leaves of Petunia hybrida, brown spots on Cassia tora [3], Watermelon produced systemic symptoms like mosaic, necrosis along the stem, petioles of leaves and finally terminal bud with die back [17].

These host range study were similar to the earlier reports [6, 15, 17, 19, 20, 22]. The virus produced both local and systemic symptoms on *Gomphrena globosa*. The present virus differs with peanut bud necrosis. Sunflower tospo virus do not infect Cowpea CV-152 systemically, where as PBNV infects [22] . Sunflower tospo virus also does not infect peanut, therefore it is different tospo virus from PBNV. Sunflower tospovirus was easily detected by inoculating to indicator plant such as Cowpea CV-152 and Immunological techniques such as Indirect DAC-ELISA and DIBA.

The immuno assays are very sensitive as the virus could be detected even in 1:5000 dilution of infected sap. The results of the virus detection were similar to the earlier findings of tospo viruses detection [23,24]. Despite of variable symptoms expression in the field tospoviruses have certain unique biological properties that are useful for diagnosis. The mechanically sap transmissible as found in the present investigation to the indicator plants such as *Chenopodium amaranticolor*, *Petunia hybrida, Datura metel*, *Vigna unguiculata, Cassia tora, Gomphrena globossa* and *Catheranthus rosea* [25-30].

Several scientists have tried the purification of tospo viruses [10,15,21, 31-34]. The isolation of tospo virus was difficult [31] and the first purified the tomato spotted wilt virus, was TSWV-Tx isolate infecting peanuts in Texas [15] . Reddy *et al.* [21] purified PBNV from groundnut which was later modified by Satyanaryana *et al.,* [10] for the purification of Nucleo capsid protein of tospo virus. Yeh *et al.,* [34] purified Nucleo capsid protein from watermelon isolates and Hanada *et al.,* [33] purified an isolate of tomato spotted wilt infecting watermelon.

Initially many methods were tried for purification of TSWV [26,31,35,26]. In the present study, the sunflower necrosis tospo virus was purified according to the procedure of Satynarayana *et al.,* [10] with some modifications. The purified virus seen as single light scattering zone between 4 cm to 4.3 cm from bottom of the tube after sucrose gradient centrifugation. The UV absorption spectrum, of purified virus showed maximum and minimum absorption at 258.2nm and 240nm respectively. The 260/280 ratio of purified virus was between 1.12 to 1.224 which indicates that the purified virus contained about 12% nucleic acid. The A_{max} / $_{A\ min}$ was between 1.547 and 1.419. The purified virus found to be infectious when inoculated immediately after purification.

The structural proteins of different tospoviruses differ slightly in size. For the N protein reported sizes ranges between 28.7 and 32 KD. Purified particles, as well as ribonucleocapsids (but not the RNA) is infective when inoculated onto healthy plants. Only enveloped particles are transmitted by thrips [37]. Particles contain about 65% protein, 20% lipid, 7% carbohydrates and 5% RNA. In present study the purified virus preparations obtained by modified procedure resolved as 4 major bands in 12% SDS-PAGE. The four virus-associated proteins of estimated molecular weight 331 KD, 58 KD, 52 KD and 31 KD were consistently detected in PAGE in infected samples but not in healthy extracts. 31 KD proteins correspond to N-protein and 52 KD, 58 KD corresponds to G1 and G2 glycoproteins and 331 KD corresponds to L protein. Which is associated with L-RNA, G1 and G2 proteins are associated with M-RNA and 31 KD N-protein is associated with S-RNA of Tospo virus genome. The results obtained in PAGE are same as the reported tospovirus [35,36,38].

RNA extracted from the purified virus zone was analysed by electrophoresis on 1% agarose gel, the RNA resolved as three major bands corresponds to L-RNA, M-RNA and S-RNA. The size of L, M and S-RNA species was estimated to be 9kb, 5kb and 3.5kb nucleotides, respectively. The result obtained is same as the reported tospo virus. The genome of tospo virus consists of L, M and S-RNA segments. S and M RNA segments are ambience and L RNA is –ve sense.

At present detection and diagnosis of tospoviruses were mainly based on serological procedures. ELISA was first developed for the detection of TSWV in papaya [39]. Tospoviruses species are distinguished on the basis of N protein serology, N protein sequences and vector specificity [40]. Currently, eleven established species are recognized and clustered into four distinct groups, with the aid of polyclonal and monoclonal antisera directed to the viral nucleoproteins [40-45]. Only a few attempts have been made to use serology for the detection and identification of TSWV owing to difficulties in getting purified virus suitable for use as an immunogen. Earlier so many scientist performed agar gel diffusion test [36,46,47] in their results they mentioned that the title of antiserum used was 1/10, 1/28, 1/32 dilutions by which they were able to detect the virus and antigenic analysis of the virus. This indicates that, the results which we obtained for the sunflower tospo virus and same as above. In agar gel diffusion test the one harmonious antiserum used was 1/10 dilution, by which precipitin line was observed along with PBNV antiserum.

Another serological technique called Electro blot immunobinding assay (EBIA) has been developed for the detection of sunflower tospovirus which is more efficient than ELISA. This technique also earlier reported for the detection of tospoviruses [44,45]. In the present study western blot technique was used for analysing serological relationship among the N protein of the sunflower tospovirus.

Serological experiments to know the serological relationship of sunflower tospovirus with other tospoviruses indicated that sunflower tospo virus serologically closely related to PBNV when tested with the polyclonal antibodies of TSWV-T, INSV, IYSV and PBNV. The non reactions of TSWV, INSV and IYSV polyclonal antibodies to sunflower tospo virus clearly indicated that sunflower tospovirus is serologically unrelated to TSWV, INSV and IYSV. Further, Asian tospoviruses has N protein (32 Kd) which is larger than the N protein of 29 Kd of TSWV and INSV[71, 31]. The Asian tospoviruses are found to be serologically closely related and belongs to tospovirus serogroup IV [34, 44, 45, 49-51].

CONCLUSION

Hence in the present study, based on symptoms on sunflower, host range, purification, SDS-PAGE analysis, RNA by agarose gel electrophoresis and Electroblot immunoassay confirms that the virus infecting sunflower is identified as tospovirus serologically related to PBNV of serogroup IV.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. P. Sreenivasulu for providing different antisera of CMV, TSWV-T and PBNV, Prof. D.V.R. Reddy for PSNV antisera (ICRISAT, Hyderabad), Dr. M.

K. Reddy for INSV antisera (IIHR, Bangalore) and Prof. Kromenelink for IYSV antisera (Netherlands).

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