



Biological and molecular evidences on host range of leaf curl begomovirus disease of sunflower (*Helianthus annuus* L.)

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Abstract: The present study was conducted to identify the alternate hosts of new leaf curl virus disease of sunflower. In the present study several crops and weed hosts were cross inoculated with leaf curl virus of sunflower under laboratory through insect vector whitefly (*Bemisia tabaci*), further all inoculated samples were retested (3-4 weeks after inoculation) by molecular based Polymerase chain reaction diagnosis for the presence of virus. The results revealed that the causal virus of the disease was successfully transmitted from sunflower to sunflower (*Helianthus annuus*), tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum* L) and weed hosts such as *Acanthospermum hispidum*, *Amaranthus viridis* and *Parthenium hysterophorus* in a short incubation period (2-3 weeks after inoculation), while on other hosts Chilli (*Capsicum annum* L) and *Datura stramonium*, infection occurs in delayed incubation period. Further molecular analysis through polymerase chain reaction (PCR) diagnostic technique using virus specific primers also confirmed the presence of coat protein (CP) of leaf curl begomovirus in virus inoculated hosts viz., chilli, sunflower, tomato, and tobacco and weed hosts such as *Acanthospermum hispidum*, *Amaranthus viridis*, *Datura stramonium* and *Parthenium hysterophorus*. Thus, findings substantiate that the above hosts are major sources of the virus inoculum and served as potential alternate hosts of the disease during the off season.

Keywords: Begomovirus, Host range, Polymerase chain reaction, Sunflower, Whitefly

INTRODUCTION

Sunflower is one of the important edible oilseed crops grown in the world after soybean and groundnut and crop has gained importance due to its short duration of maturity, excellent quality oil, and photo-insensitivity and drought tolerance (Muhammad *et al.*, 2012). In the world, sunflower being cultivated over an area of 20 million hectares and production around 30 million tonnes. In India, sunflower is being grown over an area of 0.72 million hectares with a production of 0.50 million tonnes. The major sunflower producing states in India are Karnataka (53.19%), Andhra Pradesh (20.83%) and Maharashtra (14.58%) and in recent years, its cultivation is taken up in non-traditional states like Punjab, Haryana, Uttar Pradesh, Gujarat, Orissa, Madhya Pradesh and Rajasthan. Presently, Karnataka is the leading state in the country contributing 53.19 and 38.61 per cent of total area and production, respectively. It is the second important oilseed crop after groundnut having an area of 0.38 million hectares with production of 0.19 million tonnes. However, productivity (503 kg ha⁻¹) is lesser than the national average of 692 kg/ha (Anonymous, 2013).

Although area under crop cultivation increased with the advent of new sunflower commercial genotypes and their wider adoptability to diverse agroclimatic conditions with low economic attention of farmers on plant protection measures, the crop has been found suffering from many diseases which included fungi and viral diseases (Ramaiah *et al.*, 2001; Saharan *et al.*, 2005). Among viral disease affecting sunflower, very recently leaf curl disease caused by begomovirus of the geminiviridae family was reported for the first time in India from Main Agricultural Research Station (MARS), University of Agricultural Sciences (UAS) campus, Raichur and the causal agent of the disease was confirmed as DNA begomovirus which is clustered next to tomato leaf curl Karnataka virus isolate Lucknow (ToLCKV-[Luc] (Accession no. EU604297.2) and Tomato leaf curl virus - Bangalore II (ToLCBV-[Ban2]) (Accession no. EU604297.2) and shared 97.5 per cent nucleotide identities (Govindappa *et al.*, 2011). Further, viral full genome was sequenced and the analysis of the study revealed that, leaf curl virus having DNA-A and the associated satellite beta DNA components of 2761 and 1375 (Nucleotides) in length respectively. The DNA-A molecule shared

maximum identity with tomato leaf curl Karnataka clone IKH12 (ToLCKV- IKH12) (Vanitha *et al.*, 2013).

Although several reports revealed that whitefly transmitted geminiviruses (Geminiviridae, Begomovirus) are economically important pathogens causing serious losses in food crops globally. The emergence of the *B. tabaci* geminivirus complex around the world depends on various factors, such as evolution of variants of the viruses, changes in the biology of vectors, movement of infected planting materials, sources of volunteer and weed hosts plants, introduction of new crops and host susceptibility genes through the exchange of germplasm, changes in cropping systems and climatic factors (Ramappa *et al.*, 1998; Varma and Malathi, 2003). Host range studies revealed by Muniyappa *et al.* (2000) with tomato leaf curl virus (ToLCV), pumpkin yellow vein mosaic in pumpkin (Maruthi *et al.*, 2007), hibiscus leaf curl virus in hibiscus (Rajeshwari *et al.*, 2005) and croton leaf curl virus in cotton (Mahesh *et al.*, 2010) indicated that begomoviruses have wide host compatibility, however their infection and further symptoms expression varied between the host plants. Although, leaf curl disease on sunflower being the first occurrence of begomovirus on sunflower crop from south India, meagre research efforts are made in relevance to identification of alternate hosts of the virus by biological and molecular means in relation to its survival is not been understood properly. Hence, the present study was conducted to find out the alternate hosts of the causal virus based on biological and molecular assay in relation to disease spread.

MATERIALS AND METHODS

Maintenance of sunflower leaf curl virus (SuLCV)

culture: Sunflower plants showing characteristic leaf curl virus symptoms of vein thickening, upward leaf curling, enation and stunted growth was brought to the laboratory from sunflower fields of Main Agricultural Research Station, University of Agricultural Sciences, Raichur, and virus culture was maintained by inoculating 8-10 days old healthy sunflower seedlings using whiteflies (*B. tabaci*). All process was carried out under nylon net (40 mesh) protected greenhouse.

Maintenance of whitefly culture: Initially, whiteflies (*B. tabaci*) were collected from sunflower plants at Main Agricultural Research Station (M.A.R.S), Raichur and the colony was established on freshly grown cotton (*Gossypium hirsutum*) plants kept in an insect proof net house. Four weeks after release, freshly emerged whiteflies were collected using an aspirator and were transferred to healthy cotton plants kept in an insect proof cages. The colony so developed was referred to be virus free (a-viruliferous) colony and further same colony was periodically maintained by frequently introducing healthy cotton plants grown in pots (6 × 10 cm) into the insect proof cages

maintained in an insect proof polyhouse of controlled temperature of 28 to 30°C.

Biological confirmation of alternate hosts of the virus: For this study, healthy seedlings of different cultivated crop plants *viz.*, bhendi (*Abelmoschus esculentus*), cotton (*Gossypium hirsutum* cv), chilli (*Capsicum annuum*), greengram (*Vigna radiata*), horsegram (*Dolichous biflorus*), tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*) and several weed hosts such as *Acanthospermum hispidum*, *Ageratum conyzoides*, *Amaranthus viridis*, *Cassia tora*, *Croton bonplandianum*, *Datura stramonium*, *Euphorbia geniculata*, *Euphorbia hirta*, *Parthenium hysterophorus* and *Phyllanthus niruri* were raised in polythene bags contained soil mixture of FYM and sand. Seedlings of respective hosts were cross inoculated with virus using *B. tabaci* at two leaf stage. For inoculation study, healthy whiteflies were collected from culture house and allowed to feed on sunflower leaf curl diseased plant for 24 hrs as an acquisition access period (AAP). Such whiteflies were collected and inoculated onto test seedlings which were further allowed to feed on healthy host for 24 hrs of inoculation access period (IAP). The virus inoculated seedlings of the respective hosts were kept in insect proof glasshouse for symptom expression. Observation was made on per cent transmission, time to initial and final symptoms expression and type of symptoms on each host. For each host, healthy seedlings were maintained in an insect proof cage without inoculation of the virus for comparison studies.

Molecular confirmation of leaf curl virus in weeds and crop plants:

Further, 3-4 weeks after virus inoculation, all viruses cross inoculated crop plants and weed hosts were subjected to polymerase chain reaction (PCR) analysis for leaf curl virus detection using begomovirus specific primers. For the PCR analysis, initially total genomic DNA of the inoculated and uninoculated test plants were extracted by following CTAB (Cetyl Trimethyl Ammonium Bromide) method of Lodhi *et al.* (1994). Set of begomovirus coat protein specific primers 5'GCC(C/T)AT(G/A)TA(T/C)AG(A/G)AAGCC(A/C)AG 3' (AV494) and 5' G(A/G)TT(A/G/T)GA(G/A)GCATG(T/A/C)GTACATG 3' (AC1048) (Wyatt and Brown, 1996) which were capable of amplifying the core coat protein (CP) region of many begomoviruses (Brown *et al.*, 2001; Briddon *et al.*, 2002; Chowda Reddy *et al.*, 2005; Rajeshwari *et al.*, 2005) and hence in the present study similar primers were used for detection of virus in inoculated and uninoculated test samples. PCR reaction was carried out in 25µl reaction mixture containing 6.0 mM Tris HCl, 2.5mM dNTPs, 20µM of each primer, 2.5 units of Tac DNA polymerase (Bangalore Genie Pvt. Ltd., Bangalore) and 10-15µg of DNA of the respective samples. PCR was performed in thermocycler (Eppendorf, Cambridge, UK) according to Wyatt and Brown (1996). The PCR protocol consisted of 2 min-

Table 1. Detection of host range of leaf curl virus of sunflower in crop plants through *B. tabaci* and PCR diagnosis.

Crops	Total No. of Plants tested	No. of plant infected	Per cent transmission	Days to first symptom	Days to final symptom	Early symptom appearance	Final symptom appearance	Cross inoculation to sunflower	PCR test
Sunflower (<i>H. annuus</i>)	10	10	100	10-11	20-25	Vein clearing and thickening	Upward curling of leaves, reduction in leaf size, enations on the lower surface of the leaves	+	+
Bhendi (<i>A. esculents</i>)	10	0	0	Nil	Nil	Nil	Nil	-	-
Chilli (<i>C. annuum</i>)	10	6	60	20-25	30-35	Marginal curling, vein clearing of the leaves	Thickening and swelling of veins, reduction of leaf size and puckering of leaves	+	+
Cotton (<i>G. hirsutum</i>)	10	0	0	Nil	Nil	Nil	Nil	-	-
Tobacco (<i>N. tabacum</i>)	10	8	80	10-15	25-30	Curling of leaves with clearing and thickening of veins, entire plant remains very much dwarfed	Twisting of petioles, puckering of leaves and brittle and development of enations	+	+
Tomato (<i>S. lycopersicum</i>)	10	10	100	10-12	20-25	Downward leaf curling, crumpling and interveinal and marginal yellowing	The internodes of infected plants become shortened and together with the stunted growth, plants often take bushy appearance	+	+
Horsegram (<i>D. biflorus</i>)	10	0	0	Nil	Nil	Nil	Nil	-	-
Green gram (<i>V. radiata</i>)	10	0	0	Nil	Nil	Nil	Nil	-	-

Number of whiteflies/seedlings: 5; Culture: Leaf curl virus (SuL.CV); Acquisition access period (AAP): 24 hrs; Inoculation access period (IAP): 24 hrs; +: Symptom expressed after cross inoculation from respective crop; -: No symptom expression.

Table 2. Detection of host range of leaf curl virus of sunflower in weeds through *B. tabaci* and PCR diagnosis.

Weeds	Total no. of plants	No. of plants infected	Per cent transmission	Days to first symptom	Days to final symptom	Early diagnostic symptoms	Final diagnostic symptoms	Cross inoculation on flower	PCR test
<i>A. hispidum</i>	10	5	50	20-22	25-29	Curling of leaves, vein clearing and thickening	Reduction in the leaf size, followed by stunting of plants	+	+
<i>A. conyzoides</i>	5	0	0	Nil	Nil	Nil	Nil	-	-
<i>A. viridis</i>	10	6	60	15-20	25-30	Vein clearing	Curling of leaves, Reduction of leaf size	+	+
<i>C. tora</i>	10	0	0	Nil	Nil	Nil	Nil	-	-
<i>C. bonplandianum</i>	10	0	0	Nil	Nil	Nil	Nil	-	-
<i>D. stramonium</i>	5	2	20	25-26	30-35	Vein clearing and thickening of veins, upward curling of leaves	Shortening of internodes; stunting	+	+
<i>E. geniculata</i>	10	0	0	Nil	Nil	Nil	Nil	-	-
<i>E. hirta</i>	10	0	0	Nil	Nil	Nil	Nil	-	-
<i>P. hysterophorus</i>	10	10	100	10-12	15-18	Vein clearing and crinkling of leaves	Reduction in plant growth, downward leaf curling	+	+
<i>P. niruri</i>	10	0	0	Nil	Nil	Nil	Nil	-	-

Number of whiteflies/seedlings: 5; Culture: Leaf curl virus (SuLcV); Acquisition access period (AAP): 24 hrs; Inoculation access period (IAP): 24 hrs; +: Symptom expressed after cross inoculation from respective crop; -: No symptom expression.

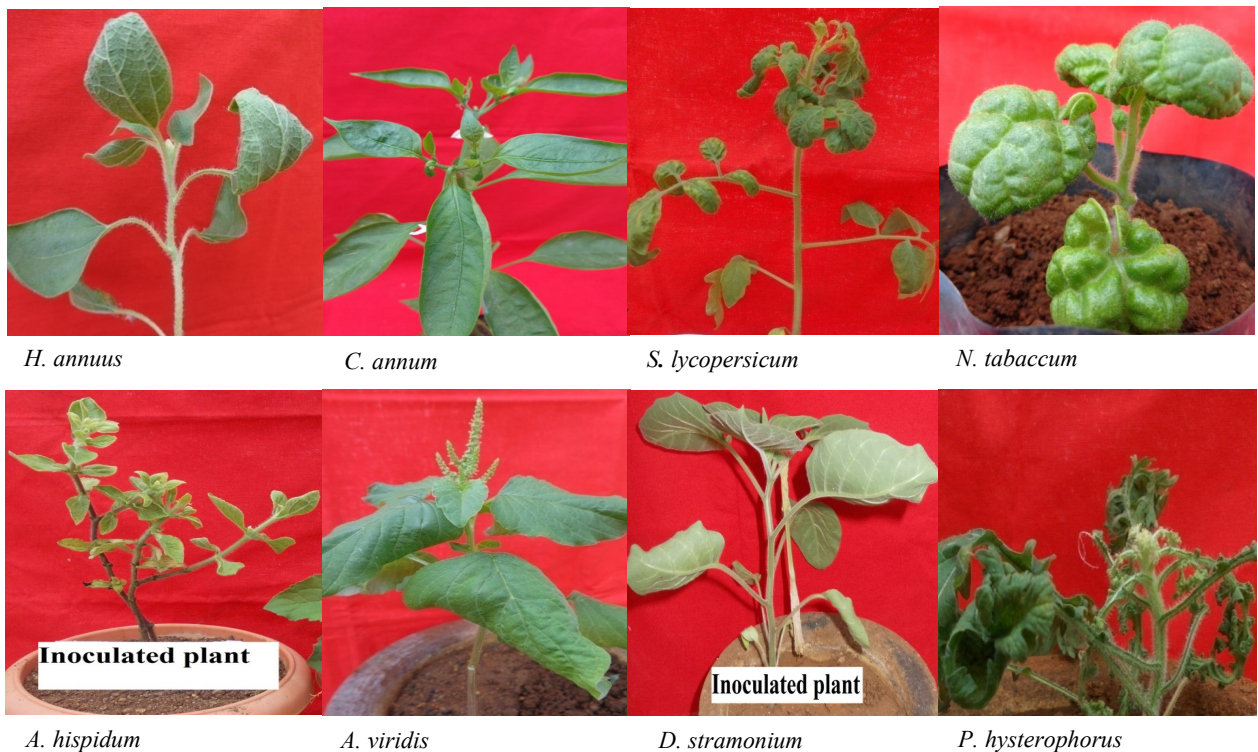


Fig. 1 . Expression of leaf curl virus symptoms on crops and weeds upon inoculation through whitefly *B. tabaci*

utes at 94°C (Initialisation step) followed by 30 cycles of 1 min at 94°C (Denaturation step), 1 min at 61°C (Annealing step), 2 min at 72°C (Extension step) and finally 10 min at 72°C (Final hold). Later PCR amplified products were separated by electrophoresis on 1% agarose gel and DNA fragments were visualised using ethidium bromide stain and analysed by alpha imager gel documentation system. DNA ladder set (1Kb, MBI Fermentas, Germany) was included as sized molecular marker.

RESULTS AND DISCUSSION

Host range studies of virus inoculum through biological approach by using insect vector whitefly *B. tabaci* revealed that the virus was successfully transmitted to only four crop species and four weeds (Tables 1 and 2). Among the cultivable crops tested, three crops *viz.*, sunflower (*H. annuus*), tomato (*L. solanacearum*) and tobacco sp (*N. benthamiana*) were found infected and exhibited leaf curl virus symptoms in a short incubation period of 2-3 weeks. While chilli (*C. annuum*) found delayed in expression of symptoms. Among the weed species tested, successful transmission of virus was obtained only with *Acanthospermum hispidum*, *Amaranthus viridis*, *Datura stramonium* and *Parthenium hysterophorus* (Fig. 1). However *A. hispidum*, *A. viridis* and *Parthenium hysterophorus* could induce early symptom expression as compared to other weeds tested. This difference in expression of virus symptoms could be associated with the insect preference and also due to host biochemical compositions interfere with

virus multiplications (Colvin *et al.*, 2006; Sharma *et al.*, 2008). Similarly tomato leaf curl begomovirus (ToLCV) from tomato was successfully transmitted to weeds by *B. tabaci* in a varied period of incubation with *A. hispidum*, *Ageratum conyzoides*, *Bidens biternata*, *Coryza stricta*, *Datura stramonium*, *Euphorbia geniculata*, *Oxalis corniculata*, *P. hysterophorus*, *Solanum nigrum*, *Sonchus brachyotis*, *Stachyterpicta indica* and *Synedrella nodiflora* and tobacco (*N. Benthamiana*) (Ramappa *et al.*, 1998). In addition, leaf curl begomovirus of Hibiscus plant found infecting weeds (*Ageratum conyzoides*, *Croton bonplandianum* and *Euphorbia geniculata*) and tobacco species such as *N. benthamiana*, *N. glutinosa*

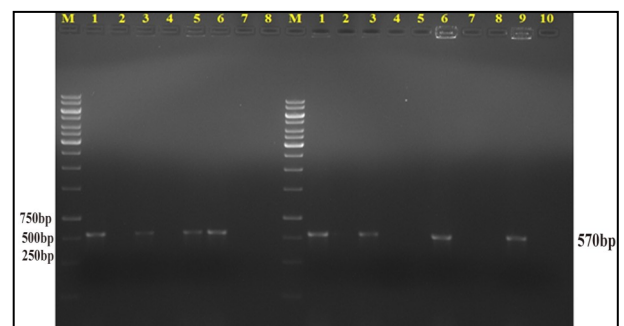


Fig. 2. Agarose gel showing the PCR amplified products (570 bp) obtained with the CP primer of begomovirus in selected crops and weeds. M: 1KB Ladder; 1: Sunflower, 3: Chilli; 5: tomato; 6: Tobacco; M: 1KB Ladder; 1: *A. viridis*; 3: *A. hispidum*; 6: *D. stramonium*; 9: *P. hysterothorus*.

a n d
N. tabacum (var. Samsun), cotton and tomato (Rajeshwari et al., 2005). The hosts confirmed with the tomato leaf curl begomovirus in tomato (ToLCV) (Ramappa et al., 1998) and begomovirus of Hibiscus were similar with the present findings as the virus infecting sunflower crop is belongs to begomovirus of the family geminiviridae. In the earlier molecular evidences, leaf curl virus on sunflower which has shared highest coat protein nucleotide identity (97%) with tomato leaf curl Karnataka clone IKH12 (ToLCKV- IKH12) (Govindappa et al., 2011 and Vanitha et al., 2013). Further in the present investigations, leaf curl virus infection was further confirmed in all virus infected hosts such as sunflower, chilli, tomato and tobacco and weed hosts such as *A. hispidum*, *A. viridis*, *D. stramonium* and *P. hystero-phorus* by molecular diagnostic polymerase chain reaction technique using virus coat protein (CP) primers with the amplified PCR product of size ~575 bp (Fig. 2). These primers were specially designed to amplify the conserved region of the CP gene of begomoviruses infection in several crops employed by many workers (Muniyappa et al., 2000; Brown et al., 2001; Briddon et al., 2002; Chowda Reddy et al., 2005; Maruthi et al., 2007) to confirm the association of begomoviruses in wide range of crop plants. Further, these primers used elsewhere for detection of begomovirus such as leaf curl disease in Hibiscus (HLCuD) (Rajeshwari et al., 2005) and mosaic disease of jatropa (Narayana et al., 2007), leaf curl virus (CrLCuV) disease in Croton (Mahesh et al., 2010). Hence, biological and molecular evidence proved that the crops such as chilli, sunflower, tomato, and tobacco and weed viz., *A. hispidum*, *A. viridis*, *D. stramonium* and *P. hystero-phorus* are major potential alternate hosts of the virus inoculum.

Conclusion

Host range studies of the virus inoculum of leaf curl virus disease of sunflower through biological and molecular approach revealed that among different plant species tested, crops such as Sunflower, chilli, tomato and tobacco acted as potential reservoir hosts. Apart from cultivated crops, weed species viz., *Acanthospermum hispidum*, *Amaranthus viridis*, *Datura stramonium* and *Parthenium hystero-phorus* also confirmed as potential sources of virus inoculum.

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