Biological and Molecular detection of leaf curl Begomovirus disease of sunflower (*Helianthus annuus* L.) in Tobacco (*Nicotiana benthamiana*)

M. Vindyashree[*], S. Maheswari[**] and M. R. Govindappa[1]

Present Address: *School of Agriculture Sciences and Forestry, Rai Technology University, Bangalore-561204, Karnataka, India.
* * School of Agriculture Sciences and Forestry, Rai Technology University, Bangalore-561204, Karnataka, India.
1Department of Plant Pathology, University of Agricultural Sciences, Raichur-584104 (Karnataka), India.

Abstract: The present study was conducted for detection of leaf curl begomovirus disease of sunflower (*Helianthus annuus* L.) in Tobacco (*Nicotiana benthamiana)*. In the present study tobacco seedlings were inoculated with leaf curl virus of sunflower under laboratory through insect vector whitefly (*Bemisia tabaci*), The characteristic symptoms were observed 10-15 days after inoculation, further all inoculated samples were retested (3-4 weeks after inoculation) by molecular based Polymerse 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*) and on Tobacco (*Nicotiana tabacum* L) in a short incubation period and also confirmed the presence of coat protein (CP) of leaf curl begomovirus in virus inoculated hosts of Tobacco.Thus, findings substantiate that *Nicotiana benthamiana* is also a major source of the virus inoculum and served as potential alternate host of the diseaseduring the off season.

Index Terms-Begomovirus, Polymerase chain reaction, Sunflower, Whitefly

I. 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 crop has been found suffering from many diseases like *Alternaria* leaf spot, downy mildew, powdery mildew, charcoal rot, scleorotium rot or wilt, rhizopus head rot, sunflower necrosis virus, and cucumber mosaic virus (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 from Main Agricultural Research Station (MARS), University of Agricultural Sciences (UAS) campus, Raichur, Karnataka, India. Causal agent of the disease was confirmed as ss 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 sequence 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, 2012). 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.

II. Materials and Method

2.1. Maintenance of 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(Plate 1).

Plate 1: Maintenance of SuLCV culture on sunflower hybrid KBSH-44 at MARS, Raichur

2.2. Maintenance of whitefly (*B. tabaci*)culture: Initially, whiteflies (*B. tabaci*) were collected from sunflower plants at MARS, Raichur and the colony was established on freshly grown cotton, *Gossypium hirsutum* plants kept in insect proof net house. There after a generation, freshly emerged whiteflies were collected using an aspirator and were transferred onto freshly grown cotton plants kept in an insect proof net house. The colony so developed was referred to be pure (a-viruliferous) and further periodically maintained by frequently introducing healthy cotton plants grown in pots (6 x 10 cm) into the insect proof net house which was maintained at temperature of 28 to 30°C in an insect proof polyhouse (Plate 2).

Plate 2: Maintenance of whitefly (*B. tabaci*) colony on cotton (*Gossypium hirsutum*) at MARS, Raichur

2.3. Raising of healthy seedlings: A healthy seedling of Tobacco crop plants were raised in polythene bags containing soil mixture in insect proof glashouse. A seedling at two leaf stage was inoculated with ten whiteflies after 24 h acquisition access feeding on SuLCV infected plant. The inoculated seedlings were kept in insect proof glasshouse for symptom development. Observation was made on time for initial and final symptoms expression and type of symptoms on each plant for biological confirmation. At the same time un inoculated healthy plants were maintained for comparison in insect proof cages.

2.4. Biological confirmation of alternate hosts of the virus: Seedlings were cross inoculated by using prepared cages and aspirator (Plate 3) with virus using *B. tabaci* at two leaf stage. For inoculation study, healthy whiteflies were collected from culture house and allowed to feed on sunflowerleaf curl diseased plant for 24 hrs as an acquisitionaccess period (AAP), Plate 4a. Such whiteflies were collectedand inoculated onto test seedlings which were furtherallowed to feed on healthy host for 24 hrs of inoculationaccess period (IAP), Plate 4b. The virus inoculatedseedlings were kept in insectproof glasshouse for symptom expression. Observationwas made on per cent transmission, time to initial andfinal symptoms expression and type of symptoms oneach seedlings. Healthy seedlings weremaintained in an insect proof cage without inoculationof the virus for comparison studies.

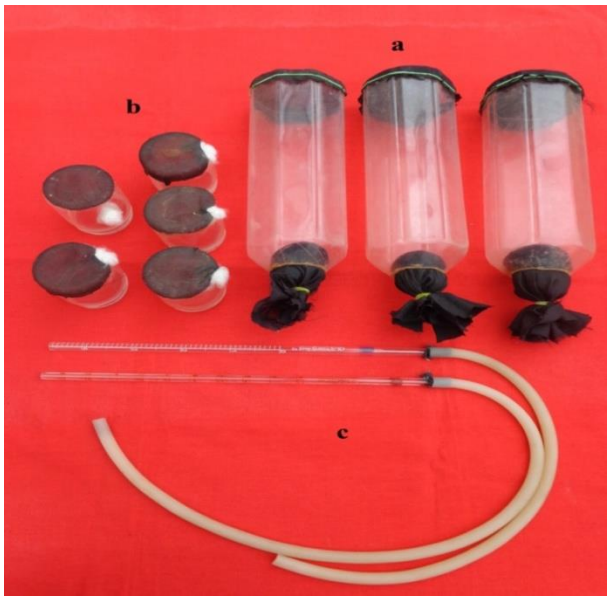

Plate 3: Different sized tubes used for whitefly mediated virus transmission studies
(a). Acquisition access feeding bottle, (b). Inoculation bottle
(c). Aspirator

Plate 4: Method of acquisition and inoculation
a. Aquistion of SuLCV by whiteflies
b. Inoculation of SuLCV to tobacco seedlings using whitefly B.tabaci

2.5. Molecular confirmation of leaf curl virus in tobacco: Further3-4 weeks after virus inoculation, all virus cross inoculated seedlings 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 *etal.*, 2005) and hence in the present study similar primers were used for detection of virus in inoculated and un icnoculated 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 therocycler (Eppendorf, Camridge, UK) according to Wyatt and Brown (1996). The PCR protocol consisted of 2 min- 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.

III. RESULT 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 *Nicotiana benthamiana*. The characteristic symptoms were observed 10-15 days after inoculation (Table. 1) The initial symptom such as curling of leaves with clearing and thickening of veins, entire plant remains very much dwarfed and after 25-30 days the final symptom were obtained with twisting of petioles; puckering of leaves and brittle and development of enations with 80 per cent transmission (Plate 5). 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 *etal*., 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,Conyza 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*, *Crotonbonplandianum* and *Euphorbia geniculata*) and tobacco species such as *N. benthamiana, N. glutinosa 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. hysterophorus* by molecular diagnostic polymerase chain reaction technique using virus coat protein (CP) primers with the amplified PCR product of size ~575 bp (Fig. 1). 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 *etal.*, 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 *etal.*, 2005) and mosaic disease of jatropha (Narayana *etal.*, 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. hysterophorus* are major potential alternate hosts of the virus inoculum. A band of approximately ~570 bp was consistently amplified from total DNA extracted from virus inoculated sunflower and tobacco samples, while it was absent in healthy samples (Fig 1).

Leaf curling with vein clearing

Twisting of petioles,thickening of veins

Puckering of leaves with brittleness

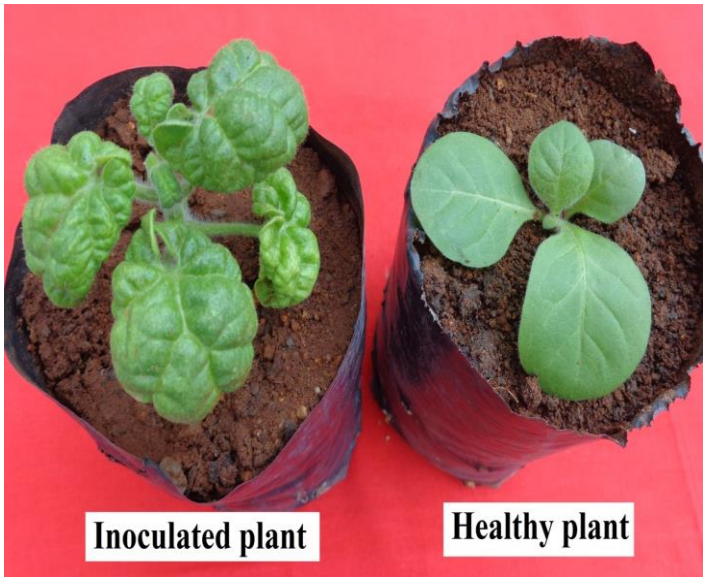

Plate 5: Symptoms of SuLCV on tobacco plants inoculated through whiteflies

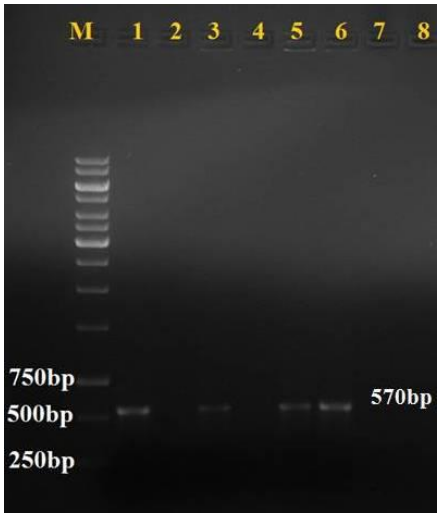

Fig 1: Agrose gel showing PCR products of SuLCV obtained with CP primers in Tobacco.
M: 1kb Ladder, 1. Infected Sunflower 2. Healthy sunflower
3. Infected Tobacco 4. Healthy Tobacco 5.Infected sunflower 6.Infected Tobacco

CONCLUSION

Host range studies of the virus inoculum of leaf curl virus disease of sunflower through biological and molecular approach revealed that tobacco acted as potential reservoir hosts.

ACKNOWLEDGMENT

This research is part of M.Sc (Agri.) dissertation and authors are very much thankful to Dr. M. R. Govindappa Scientist, All India Co-ordinate Research Project on Sunflower, Department of Plant Pathology, University of Agricultural Sciences, Raichur and also thankful to Dr. Chithiraichelvan PhD, Dean school of Agricultural Science and Forestry, Rai Technology University for his moral support in publishing the researchpaper.

REFERENCE

Banks, G. K., Colvin, J., Chowda Reddy, R. V., Maruthi, M. N., Muniyappa, V., Venkatesh, H. M., Kiran Kumar, M., Padmaja, A. S., Beitia, F. J. and Seal, S. E., 2001, First report of the *Bemisiatabaci* B-biotype in India and an associated *Tomato leaf curl virus* disease epidemic. *Plant Disease*, **85**: 231.

Bedford, I. D., Briddon, R. W., Jones, P., Alkaff, N. and Markham, P. G., 1994, Differentiation of three whitefly transmitted geminiviruses from the Republic of Yemen. *European J. Pl. Pathol.*, **100:** 243-257.

Brown, J. K., 1994, Current status of *Bemisia tabaci* as a plant pest and virus vector in agro-ecosystems world-wide. FAO *Plant Prot. Bull*., **42**: 3-32.

Chowda Reddy, R. V., Colvin, J., Muniyappa, V. and Seal, S. (2005). Diversity and distribution of begomoviruses infecting tomato in India. *Arch. Virol*.150: 845-867.

Colvin, J., Omango, C. A., Govindappa., M. R., Stevenson, P. C., Maruthi, M. N., Gibson, G., Seal, S.E., and Muniyappa, V., 2006, Host plant Viral infection effects on arthropod vector population growth, development and behavior: Management and epidemiological implications, *Advances in Virus Research*, Vol (67):420-453.

Deepa, 2013, Investgations on leaf curl disease of sunflower (*Helianthus annuus* L.) caused by begomovirus. *M.Sc. (Agri.)Thesis*, Univ. Agri. Sci., Raichur, 77pp.

Govindappa, M. R., Shankergoud, I., Shankarappa, K. S., Wickramaarachchi, W. A. R. T., Anjeneya Reddy, B. and Rangaswamy, K. T., 2011, Molecular detection and partial characterization of begomovirus associated with leaf curl disease of sunflower (*Helianthus annuus*) in Southren India. *Plant Pathol. J.*, **10**(1): 29-35.

Lodhi, M. A., Ye, G. N., Weeden, N. F. and Reisch, B., 1994, A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. *Pl. Mol. Biol. Rep.*, 12: 6-13.

Maruthi, M. N., Colvin, J., Seal, S. E., Gibson, G. and Cooper, J., 2002, Co-adaptation between cassava mosaic geminiviruses and their local vector populations. *Virus Res*., 86: 71-85.

Muhammad, W. A., Muhammad S. A. and Hammad, N. T, (2012). Combining ability analysis for achene yield and related traits in sunflower (*Helianthus annuus* L.). *ChileanJournal of Agricultural Research.*, 72(1): 21-26.

Muniyappa, V. and Veeresh, G. K., 1984, Plant virus diseases transmitted by whiteflies in Karnataka. *Indian Acad. Sci.* (Anim. Sci.), **93**: 397-406.

Rajeshwari, R., Reddy, R. V. C., Maruthi, M. N., Colvin, J., Seal, S. E. and Muniyappa, V., 2005, Host range, vector relationships and sequence comparison of a begomovirus infecting hibiscus in India. *Ann. Appl. Biol.*, **147**: 15-25.

Ramappa, H. K., Muniyappa, V and Colvin, J. 1998, The contribution of tomato and alternative host plants to tomato leaf curl virus inoculums pressure in different areas of south India. *Annals of Applied Biology*, **88**: 369-387.

Saharan, G. S., Naresh Mehta and Sangwan, M. S., 2005, *Diseases of oilseed crops*, ISBN-81-7387-176-0, Indus publishing company, New Delhi, pp.38.

Sharma, R. K., Gupta, V. K., Jindal. J and Dilawari, V. K., 2008, Host associated genetic variations in whitefly *Bemisia tabaci* (Genn.), *Indian J Biotechnol*, 7: 366-370.

Varma, A. and Malathi, V. G., 2003. Emerging geminivirus problems: A serious threat to crop production. *Ann. Appl. Biol*. **142**: 145-164.

Vindyashree, M., Govindappa, M. R., Ghante, V.N., Aswathanarayana, D.S., and Shankergoud I., (2016). Biological and molecular evidences on host range of leaf curl begomovirus disease of sunflower (*Helianthus annuus* L.)*Journal of Appl. and Nat. Sci*.7 (1).

Wyatt, S. D. and Brown, J. K., 1996, Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathology*, 86: 1288-1293.

AUTHORS

First Author – Ms. M. Vindyashree is presently working as Assistant Professor (Plant Pathology) at Rai Technology University, Bangalore, Karnataka.
E-mail ID: vindyashree4068@gmail.com
Second Author – Dr. S. Maheswari ispresently working as Assistant Professor (Microbiology) at Rai Technology University, Bangalore, Karnataka.
E-mail ID: mahehrp@gmail.com

Third Author – Dr. M. R. Govindappa Assistant Professor, UAS, Raichur. Karnataka. Email ID: mrgpathology1@gmail.com
Correspondence Author: Vindyashree M, Email ID: vindyashree4068@gmail.com Mob No: 9538532890

Table 1: Biological detection of SuLCV in Tobacco plants

Crops	Total No. of Plants tested	No. of plant infected	Per cent transmission	Per cent whitefly survival (%)	Days to first symptom	Days to final symptom	Early symptom appearance	Final symptom appearance	Cross inoculation to sunflower	PCR Test
Sunflower (*Helianthus annuus* L)	10	10	100	100	10-11	20-25	Vein clearing and thickening	Upward curling of leaves, reduction in the leaf size, enations on the lower surface of the leaves	+	+
Tobacco (*Nicotiana tabacum)*	10	8	80	70	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	+	+