http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (23): 2624-2628, 2008
ISSN 1028-8880

Detection and Some Properties of *Cowpea mild mottle virus* Isolated from Soybean in Iran

[1]M. Tavassoli, [2]N. Shahraeen and [1]S. Ghorbani
[1]Division of Microbiology, College of Science, Alzahra University, Tehran
[2]Department of Plant Virus Research, Iranian Research Institute for Plant Protection,
P.O. Box 19395-1454, Tehran, Iran

Abstract: During 2006-2007 growing seasons, survey were carried to identify a virus disease causing mosaic of soybean in the field in Southern region (Khozestan Province) of Iran. To detect the viral infection, diseased leaf samples showing mild mosaic and leaf malformation were collected from soybean fields in Dezful, located in Khozestan Province. Infected samples were carried to the lab in a proper condition on ice packages. TPIA and DAS-ELISA serological tests were applied to identify the viral agent. To investigate the host-range, several indicator plants were mechanically inoculated under green-house condition. Seed transmission of CPMMV was examined using the seeds obtained from infected plants. The virus isolate was not found to be seed-borne in Clark variety of soybean. Different steps of ultracentrifugation including sucrose density gradient (10-40%) were carried out in order to obtain partial purified virus. On the basis of biological, serological and EM results, CPMMV-Carla virus was identified in the infected soybean samples. This is the first report of CPMMV infection of soybean in Iran.

Key words: *Cowpea mild mottle virus*, soybean, DAS-ELISA, purification, electron microscopy

INTRODUCTION

Cowpea mild mottle virus (CPMMV) was first reported on cowpea in Ghana (Brunt and Kenten, 1973). Subsequently, it was reported from several tropical regions of Africa (Anno-Nyako, 1986; Brunt and Philips, 1981; Mink and Keswani, 1987) and Asia in a diverse range of plant species that includes leguminous and solanaceous food crops (Rodriguez and Arneodo, 2004). CPMMV is reported to be transmitted by the whitefly, *Bemisia tabaci*, in a non-persistent manner and through seed in cowpea, soybean and French bean. Naturally, infected cowpea plants exhibit a mild systemic mottle or symptomless. Differences in symptom severity on cowpea and on soybean plants make economic assessment of the disease difficult, but it is nevertheless considered a definite threat to successful production of leguminous crops such as soybeans, peanuts, beans and mung beans, all of which are intercropped with one another in many tropical countries in Africa and Asia (Brunt and Kenten, 1973; Mumiyappa and Reddy, 1983; Naidu *et al*., 1998). CPMMV has filamentous particles c. 650×15 nm in size with a coat protein of 32-36 kDa (Hartman *et al*., 1999; Irma *et al*., 2006).*Cowpea mild mottle virus* is located in Carla virus group that itself is belonged to plant virus family of Flexiviridae (Giovanni *et al*., 2007). *Cowpea mild mottle virus* CPMMV causes mosaic, chlorosis, necrosis and distortion on host plants leaves. Few virus host range reported consists of *Glycine max* L., *Arachis hypogaea* L., *Vigna unguiculata* L., *Lycopersicon sculentum* L., *Vicia faba* L. and *Nicotiana clevelandii* L. (Nolt and Rajeshwari,1987; Reddy,1991). Two strains of whitefly-transmitted *Cowpea mild mottle virus* (CPMMV) causing severe (CPMMV-S) and mild (CPMMV-M) disease symptoms in peanut host have been reported. Where CPMMV-S showed 70% sequence identity with CPMMV-M at the nucleotide level (Pappu *et al*., 1993; Naidu *et al*.,1998). In a biological way, they can differ from each other by causing unlike symptoms in some host plants. Disease survey and preliminary studies (Ghorbani *et al*., 2008) have shown that CPMMV is present on cowpea crops in Northern Iran. Evidence have shown that the virus is present in low percent in soybean field in some cultivation areas in different parts of Iran (Golnaraghi *et al*., 2004). Recently, Ghorbani *et al*. (2007) have reported CPMMV naturally infecting bean crops in Iran. The aim of this study was to investigate and identify the virus disease infecting soybean in Southern part of Iran using routine biological and serological methods.

MATERIALS AND METHODS

During the period 2006-2007, surveys were conducted in soybean fields from Southern part of Iran. Samples of

Corresponding Author: Marjan Tavassoli, Division of Microbiology, College of Science, Alzahra University, Tehran, Iran

soybean (*Glyscine max* L.) leaves with mild mosaic symptoms were collected from Dezful territory (Khozestan Province). For advanced virus detection and host-range determination, virus samples were inoculated by using phosphate buffer 0.1 M (pH = 7.2) containing 2-mercaptoethanol 1% to several plants in cotyledon stage as *Glycine Max* L., *Vigna unguiculata* L., *Arachis hypngaea* L., *Phaseolus vulgaris* L., *Vicia faba* L., *Lycopersicon sculentum, Chenopodium amaranticolor, Chenopodium quinoa, Datura stramonium, Gopherena globosa, Vigna radiate* L., *Vigna aconitifolia* L. and *Nicotiana tabacum* (Rustica type). Symptoms were appeared during 14 days post inoculation in local or systemic expression. DAS-ELISA serological test was performed in order to detect CPMMV in the samples (Clark and Adams, 1977).

Seed test: To clarify CPMMV seed-born characteristic, 400 seeds collected from infected soybean (Clark var.) plants with sharp mosaic symptoms were sown again. After three weeks the young seedlings leaves doubtful to be CPMMV transmitted were collected and checked using DAS-ELISA serological test. This procedure (El-Hammady *et al*., 2004) was repeated for several times and near 50 seeds were planted in each series in order to observe any symptoms and serological reaction, but there was no positive result.

DAS-ELISA testing: In this study we used polyclonal CPMMV antibody gifted from DSMZ Company, Germany. Buffer preparation and different antiserum dilutions were provided according to company's description and general protocol of Clark and Adams (1977) and Lister (1978). Absorbance at 405 nm was measured with Labsystem multiscan ELISA microplate reader (Denmark Co). Healthy soybean leaves extracted in general extraction buffer was used as negative control. A reaction was considered positive only if the absorbance was more than three times the background mean of negative control. The serological reagents used in ELISA did not reveal any considerable cross reaction with other virus species of the genus, hence permitted us accurate species identification.

Electron microscopy: To study virus particle morphology, electron microscopic 400 mesh copper grid was prepared according to Milne and Lesemann (1984) leaf-dip protocol. After carbon coating of copper grids 200-400 mesh using carbon-coater, they were covered with Formvar 0.35% in Ethylen D-Chloride or Chloroform and floated on drop of purified virus or infected crud leaf extract for 5-10 min. Then grids were washed by 40 drops of double-distilled water and stained using 10 drops of 2% uranyl acetate solution, excessive color drop lets was removed by clean tissue paper, TEM grids were kept in a sterile Petri dish to dry. Virus observation was performed using TEM electron microscopy (Zeiss, TEM 900- Model) in Medical College of Shahid Beheshti University, Tehran.

Tissue print immunoassay (TPIA): Parts of a fresh and thin stem of soybean or bean samples were cut using a sharp blade to create spots by pressing the fresh side of stem on nitro cellulose membrane (S and S 045 μ, pore size, Germany), the paper membrane was washed immediately three times for 5 min with Phosphate Buffer Saline-Tween (PBS-T), after the last wash, paper was floated with CPMMV polyclonal antibody (produced by DSMZ company, Germany) in 1:5 dilution by PBS, then kept for 1 h in room temperature. Washing procedure was repeated and samples were floated in 1:2000 dilution of second antibody goat anti-rabbit conjugate (GAR) prepared in conjugate buffer for 1 h. Then samples were rested in substrate buffer containing p-Nitro Blue Tetrazolium (NBT) and 5-Bromo-4-Chloro-3-Indol Phosphate (BCIP) in Triss buffer 0.1 M (pH = 9.5). Test results were observed by color changing of pressed or dot samples on membrane to violet color in infected samples. Healthy samples did not show any color changing.

Virus purification: Partial purification of the virus was performed according to Brunt and Kenten (1973); Antignus and Cohen (1987). In this method, 100 g of infected soybean (or Tender green bean) fresh leaves were extracted by borate buffer 0.02 M (pH = 9.5) with 1:2 ratio at 4-5°C cool condition, then the extract was filtrated through muslin cloth, then the sample was centrifuged in 11000 rpm for 15 min. Supernatant was separated, mixed with chloroform in volume of 1.2 (w/v) after 1 h, we centrifuged sample in 11000 rpm for 15 min. The pellet was resolved in borate buffer 0.01 M, kept in -20°C for 18 h. The virus sample was resuspended in different concentration of sucrose density gradient (10-40%) and was centrifuged in 16000 rpm for 2.5 h. The formation of virus band could be observed between the layers of sucrose solution in a dark room under a dim light (Fig. 1g, h). The virus band was removed using Pasteur pipette, solved in borate buffer 0.01 M and centrifuged in 24000 rpm for 1 h. Finally, the virus was resolved in borate buffer 0.01 M and kept in -20°C condition for further use.

Spectrophotometry: The purified virus in borate buffer 0.01 M was further studied using Spectrophotometry (Ultrospec-III, UK) in order to determine the presence of nucleic acids as a result of virus infection. So, we diluted our sample with borate buffer 0.01 M in 1:4 ratio and registered every obtained OD related to wavelengths of

210-300 nm. In this study, we used borate buffer as a negative control and the procedure was based on samples OD in 265-270 nm that indicates the presence of virus nucleic acids.

RESULTS

ELISA test and host range study: The Infected soybean leaves were collected from fields and were mechanically inoculated to series of indicator host plants in greenhouse condition to establish the source and propagate the virus. DAS-ELISA test using CPMMV antiserum was performed using original soybean samples and the inoculated hosts. The symptom reaction in inoculated host plants were included local chlorotic lesion in *Vigna unguiculata*, *Chenopodium amaranticolor* and *Ch. quinoa* (Fig. 1a, b), Necrotic local lesion and systemic down ward rolling in *Arachis hypngaea* (NC-2 cultivar) (Fig. 1c), mild mosaic/mottling in *Glycine max* and systemic mosaic and leaf distortion in *Phaseolus vulgaris* (Tender green and\or top crop cultivar of bean) (Fig. 1d, e). These test plants were reacted positive to CPMMV When rechecked by DAS-ELISA test. *Lycopersicon sculentum*, *Vigna radiate* and *V. aconitifolia* were positive in DAS-ELISA test but did not show any symptoms. *Datura stramonium*, *Gomphera globosa*, *Vicia faba* and *Nicotiana tabacum* (Rustica type) were reported negative in DAS-ELISA test.

Seed-born test: Among 400 collected seeds from infected soybean plants (Clark var.) that had been planted in a

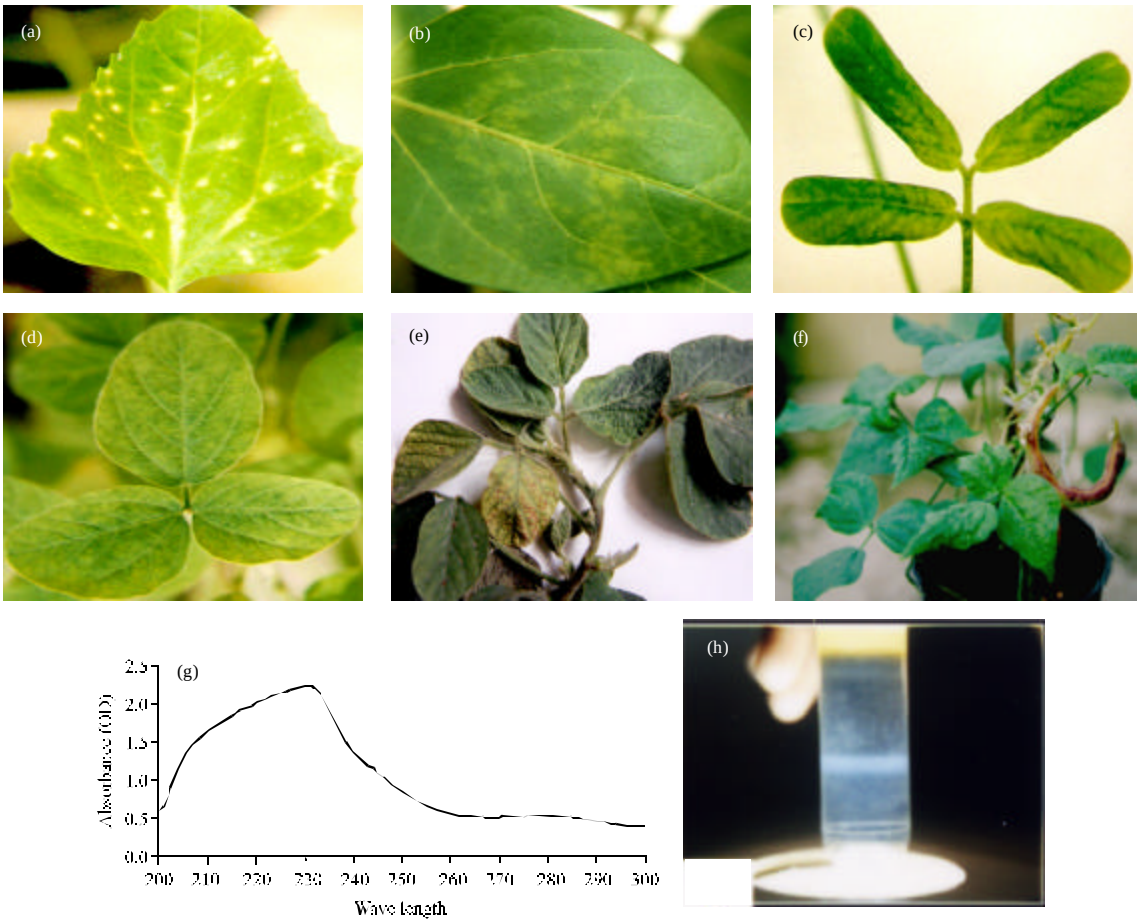

Fig. 1: (a) Local chlorotic lesion in *Chenopodium amaranticolor*, (b) local chlorotic lesions on cowpea, (c) systemic mosaic and downward rolling in *Arachis hypngaea* (NC-2 cultivar), (d) CPMMV field symptom of severe mosaic and leaf distortion in *Glycine max* (Clarck var.), (e) mild mosaic/mottling in *Glycine max* (Clarck var.) inoculated by CPMMV, (f) *Phaseolus vulgaris* (Tender Green) showing severe mosaic and leaf distortion symptoms by CPMMV, (g) spectrophotometric graph of purified virus in OD = 0.5 pick of 268 nm wavelength and (h) formation of CPMMV band in density sucrose gradient

proper condition, we could not prove the CPMMV infection using DAS-ELISA and Tissue Print Immunoassay. It seems that soybean Clark variety did not support seed-borne transmission. The CPMMV seed-born controversy is also supported by some other reports from different researchers elsewhere (El-Hammady *et al*., 2004).

Virus purification: Registered ODs of purified virus by spectrophotometry set in 220-200 nm range of wavelength proved that the maximum OD was in 230 nm and the minimum OD was in 268 nm (Fig. 1g, h). Our sample showed OD = 0.5 in 268 nm of wavelength that was indicative of virus infection.

Electron microscopy: Viral particles of approximately 650 nm resembling those reported for CPMMV (Brunt and Kenten, 1983; Pappu *et al*., 1993) were observed under TEM studies. For this study the sap of infected symptomatic Tender green bean leaves was prepared by Leaf dip method of Milne and Lesemann (1984) elongated like viral particles were observed using TEM electron microscopy in magnification of 30000x (Figure is not shown).

DISCUSSION

Cowpea mild mottle virus (CPMMV) was first reported on cowpea in Ghana (Brunt and Kenten, 1973). Subsequently, it was reported from several tropical regions of Africa and Asia in diverse range of plant species that include leguminous and solanaceous crops (Rodriguez and Arneodo, 2004). On the basis of biological, serological properties and particle morphology, the virus involved in mild mottle disease of soybean in Dezful Province, Southern Iran, was identified as CPMMV. Symptoms appearance on indicator hosts were comparatively similar to those reported elsewhere for CPMMV-S (Severe strain) (Iwaki *et al*., 1982). Serological reaction using DAS-ELISA with CPMMV polyclonal antibody (DSMZ- Co., Germany) ascertain that soybean samples and the inoculated indicator hosts were reacted positive with CPMMV isolate. There are various controversial reports on seed born nature of CPMMV in soybean plants. Some researchers have introduced CPMMV as 2-90% seed-born in some soybean cultivars. But in this study, Clark variety of soybean did not support Dezful CPMMV isolate as a seed transmitting virus. Grown seeds from infected CPMMV soybean plants did not show any positive reaction when tested by DAS ELISA. Nevertheless, depending on certain conditions the virus was reported transmittable by insect vector *B. tabaci* in non-persistent manner (Jeyanandarajah and Brunt, 1993; Muniyappa and Reddy, 1983). In this study we were not successful in transmitting CPMMV-S from infected to healthy soybean plants under greenhouse condition. As it has been discussed by other researchers, the biological transmission of the virus requires specific condition with efficient population of the vector *B. tabaci* as present in natural conditions (Hartman *et al*., 1999; Jeyanandarajah and Brunt, 1993). The virus was partially purified by applying different steps of centrifugation including sucrose density gradient (10-40%) (Brunt and Kenten, 1973). Viral band were observed under the scattering light indicating the partially purified virus from the host plant. EM studies revealed elongated like viral particle of the CPMMV with approximately 650 nm in length (Fig. not shown) similar to those reported elsewhere (Hartman *et al*., 1999; Irma *et al*., 2006). Among indicator hosts, Tender Green bean was found to be more susceptible to CPMMV infection reacting with ELISA shown severe systemic symptoms (Fig 1, f) which was also found suitable for virus propagation, purification, besides leaf dip electron microscopy for virus particle investigation. CPMMV can infect diverse range of plant species that includes leguminous and solanaceous food crops (Reddey, 1991; Rodriguez and Arneodo, 2004; Brunt and Kenten, 1983). Differences in symptom severity in different variety of soybean plants in the field make economic assessment of the disease difficult, but it is nonetheless considered a definite threat to successful production of such leguminous crops as soybeans, peanuts, beans and mungbeans, all of which are intercropped with one another in many tropical countries in Africa and Asia (Naidu *et al*., 1998; Ghorbani *et al*., 2007). Since the infected plants and located field may act as a locus of infection , proper identification of the virus and related strain can help in devising correct integrated control measures to avoid virus spreading in the fields and prevent losses of soybean crops.

ACKNOWLEDGMENTS

The study was part of the research of the first author for partial fulfillment of the M.Sc Thesis submitted to Alzahra University, Tehran. We thank Dr. S. Winter (DSMZ, Germany) for gifting CPMMV test Kit. Mr. Mahdi Afzali for help in screen house work and indicator host plant propagation and Miss S.V. Hosseinnia for lab. assistance.

REFERENCES

Anno-Nyako, F.O., 1986. Semipersistent of an extra mild isolate of *Cowpea mild mottle virus* on soybean by the whitefly *Bemisia tabaci* in Nigeria. Trop. Agric., 63: 193-194.

Antignus, Y. and S. Cohen, 1987. Purification and some properties of a new strain of *Cowpea mild mottle virus* in Israel. Ann. Applied Biol., 110: 563-569.

Brunt, A.A. and R.H. Kenten, 1973. *Cowpea mild mottle*, a newly recognized virus infecting cowpeas (*Vigna unguiculata*) in Ghana. Ann. Applied Biol., 74: 67-74.

Brunt, A.A. and S. Philips, 1981. Fuzzy vein, a disease of tomato (*Lycopersicon esculentum*) in Western Nigeria induced by *Cowpea mild mottle virus*. Trop. Agric., 58: 177-180.

Clark, M.F. and A.N. Adams, 1977. Characteristic of microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol., 34: 475-483.

El-Hammady, M., S.E. Albrechtsen, A.M. Abdelmonem, F.M. Abo El-Abbas and W. Gazalla, 2004. Seed-Borne *Cowpea mild mottle virus* on soybean in Egypt. Arab Univ. J. Agric. Sci. Ain. Shams Univ. Cairo, 12: 839-850.

Ghorbani, S.G.M., N. Shahraeen and A. Elahinia, 2008. Serodiagnosis of cowpea (*Vigna unguiculata*) viruses in Guilan province, Iran. Iranian J. Virol., 1: 28-32.

Ghorbani, S.G.M., N. Shahraeen, A. Elahinia and K. Bananej, 2007. Serodiagnosis of viruses infecting bean (*Phaseolus vulgaris*) in Guilan Province. Proceeding of 4th Iranian Congress of Virology, May 1-3, Tehran, pp: 1-1.

Giovanni, P., P. Martelli, M.J. Adams, J.F. Kreuze and V.V. Dolja, 2007. Family Flexiviridae: A case study in virion and genome plasticity. Ann. Rev. Phytopathol., 45: 73-100.

Golnaraghi, A.R., N. Shahraeen, R. Pourrahim, S. Farzadfar and A. Ghasemi, 2004. Occurrence and relative incidence of viruses infecting soybeans in Iran. Plant Dis., 88: 1069-1074.

Hartman, G.L., J.B. Sinclair and J.C. Rupe, 1999. Compendium of Soybean Disease. 4th Edn., American Phytopathological Society, St. Paul, MN., ISBN: 08-905-40934.

Irma, G., J. Laguna, D. Arneodo, P. Rodriguez and M. Fiorona, 2006. *Cowpea mild mottle virus* infecting soybean crops in Northwestern Argentina. Fitopatol. Bras., 31: 39-42.

Iwaki, M., P. Thongmeearkom, Y. Honda, M. Prommin, N. Deema and T. Hibi, 1982. Whitefly transmission and some properties of *Cowpea mild mottle virus* on soybean in Thailand. Pl. Dis., 66: 365-368.

Jeyanandarajah, P. and A.A. Brunt, 1993. The natural occurrence, transmission, properties and possible affinities of *Cowpea mild mottle virus*. J. Phytopathol., 137: 148-156.

Lister, R.M., 1978. Application of the enzyme-linked immunosorbent assay for detecting viruses in soybean seed and plants. Phytopat., 68: 1393-1400.

Milne, R.G. and D.E. Lesemann, 1984. Immunosorbent Electron Microscopy in Plant Virus Studies. In: Methods in Virology, Maramorosch, K. and H. Koprowski (Eds.). Academic press, USA.

Mink, G.I and C.L. Keswani, 1987. First report of *Cowpea mild mottle virus* on bean and mung bean in Tanzania. Plant Dis., 71: 557-557.

Muniyappa, V. and D.V.R. Reddy, 1983. Transmission of *Cowpea mild mottle virus* by *Bemisia tabaci* in a non-pesistent manner. Plant Dis., 67: 391-393.

Naidu, R.A., S. Gowda, T. Satyanarayana, V. Boyko, A.S. Reddy, W.O. Dawson and D.V.R. Reddy, 1998. Evidence that whitefly-transmitted *Cowpea mild mottle virus* belong to the genus *Carla virus*. Arch. Virol., 143: 769-780.

Nolt, B.L. and R. Rajeshwari, 1987. Properties of a *Cowpea mild mottle virus* isolate from groundnut. Indian Phytopathol., 40: 22-26.

Pappu, S.S., R. Brand, H.R. Pappu, E.P. Rybicki, K.H. Gough, M.J. Frenkel and C.L. Niblett, 1993. A polymerase chain reaction method adapted for selected amplification and cloning of 3' sequences of potyviral genomes: Application to *Dasheen mosaic virus*. J. Virol. Methods, 41: 9-20.

Reddy, D.V.R., 1991. Crop profile; Groundnut viruses and virus diseases: Distribution, identification and control. Rev. Plant Pathol., 70: 665-678.

Rodriguez, P.E. and J.D. Arneodo, 2004. First record of *Cowpea mild mottle virus* in bean crops in Argentina. Aust. Plant Pathol., 33: 129-130.