Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 23, No. (4) : 2021 : 682-689

ISSN-0972-3005

MOLECULAR IDENTIFICATION OF COAT PROTEIN GENE OF MUNGBEAN YELLOW MOSAIC VIRUS (MYMV) IN *MACROTYLOMA UNIFLORUM* LAM. (VERDC.) WITH METEOROLOGICAL ASPECTS FROM NORTHERN INDIA

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Keywords: *Horsegram, Bemisia tabaci, Mung bean yellow mosaic virus, GenBank*

Abstract – The most destructive yellow mosaic disease is mainly caused by begomovirus and transmitted by whitefly (*Bemisia tabaci)* affecting legumes production in India. The present investigation was carried out to identify coat protein gene of Mungbean Yellow Mosaic Virus (MYMV) in horsegram accessions showing yellow mosaic symptoms. Disease causing virus was detected by using Polymerase Chain Reaction (PCR) with begomovirus specific degenerate primers. The amplified region of 951bp was sequenced and submitted to GenBank (Accession number MT345791.1). Sequence analysis revealed the highest similarity of 99.16% with Mungbean yellow mosaic virus (Urdbean: New Delhi:2011). A total of 48 accessions of horsegram were screened for average disease incidence and well correlated with meterological parameters. The maximum average disease incidence of yellow mosaic disease was found to be 46.66% (L9), whereas L4, L14, L29 and L30 accessions showed 100% germination of seeds. The average maximum temperature (34.37°C), minimum temperature (24.80°C) and relative humidity (69.72%) were recorded whereas during the experiment, the total rainfall was found to be 111.20mm. It was concluded that the abrupt changes in meteorological parameters cause the progression of MYMV disease incidence under natural field conditions.

INTRODUCTION

Horsegram(*Macrotyloma uniflorum*) is considered as an important medicinal pulse in Ayurveda with various pharmaceutical use (Bhardwaj and Yadav 2015; Prasad and Singh, 2015; Rlds and Erhss, 2017; Kaundal *et al*., 2019). It is highly nutritious food source among well-known legumes (Kaundal and Kumar, 2020). Yellow mosaic virus acts as the major barrier for the cultivation of pulses in peninsular India and was first detected in southern districs of Karnataka. Yellow mosaic disease transmitted by white fly, *Bemisia tabaci* (Gennadius) was widespread in most parts of South India (Muniyappa and Reddy, 1976; Muniyappa *et al*., 1975; Williams *et al*., 1968; Prema, 2013). The disease incidence ranged from 50 to 100 per cent in both summer and early rainy season crops causing substantial loss in grain yield (Muniyappa *et al*., 1976). The disease caused yellow discoloration on the leaves that led to irregular, small, greenish yellow mosaic symptoms. Severe infection led to stunted growth of the plant and reduction in the leaf size (Muniyappa *et al*., 1987, Prema and Rangaswamy, 2017). The viruses causing yellow mosaic diseases of legumes across Southern Asia are identified as four distinct bipartite Begomoviruses namely Mungbean yellow mosaic India virus, Mung bean yellow mosaic virus, Horsegram yellow mosaic virus and Dolichos yellow mosaic virus. India is a country with diverse agro-climatic conditions expressing tropical, subtropical and temperate climates having diversity in pattern of temperature, rainfall and humidity. Such type of climatic condition is ideal for growth and development of pest/vectors which affect majority of the pulses (Narayan, 2009). Among the various diseases, viral diseases like mungbean yellow mosaic virus (MYMV) lead to tremendous yield losses in many legumes including mungbean, urdbean and soybean throughout the South-Asian countries. The viral diseases of pulses account up to 80 per cent yield losses with poor quality of seed, while the MYMV alone is capable to cause losses in the tune of 80 to 100 per cent in mungbean (Naimuddin, 2001). YMV disease is not seed, soil

borne or sap transmissible rather it is caused by begomoviruses with bipartite genomes transmitted through whitefly (Nariani, 1960; Nene, 1973). It is well known that weather parameters play a vital role in survival and multiplication of white fly (*B. tabaci*, Genn) and contribute to severity of MYMV disease incidence (Srivastava and Prajapati, 2012; Ali *et al*., 2015). This present research work is useful to identify and screen the percent disease incidence of YMV disease in the procured accessions sown under natural field conditions.

MATERIALS AND METHODS

Sample collection

Fourty eight horsegram accessions were procured from different geographical regions of India (Table 1). The leaf samples of horsegram plants showing severe yellow mosaic and mottling symptoms were collected from Agriculture field farm, DAV University, Jalandhar, Punjab. Leaf samples from healthy plants were also collected as controls. These asymptomatic and symptomatic leaves were stored at -80 ^{o}C deep freezer (Eppendorf) for further studies.

DNA Isolation

The total genomic DNA was extracted from leaf tissues of YMV infected horsegram plants by using CTAB method (Doyle and Doyle, 1990) along with healthy plants as control.

Primers used, PCR amplification and gel electrophoresis

In order to identify the YMV disease causing virus/ pathogen in horsegram, specific primers for this disease were used in this study. Both the forward and reverse primers namely HgYMVAF, HgYMVAR and HYMV-A1500F,

HYMV-A1500R were used in the identification of the DNA-A component of Horsegram yellow mosaic virus whereas HYMV-CP-F, HYMV-CP-R and MYMV-CP-F, MYMV-CP-R were used for the identification of coat protein of Horsegram yellow mosaic virus and Mungbean yellow mosaic virus (Table 2).

Polymerase chain reaction was performed on DNA samples extracted from infected leaves horsegram plants and the amplification of begomovirus genome was carried out using a pair of degenerate primers designed for the amplification of genomic components and coat protein. The

Table 1. Details of horsegram accessions from different geographical regions of India

Sr. No.	Accessions No.	Location	Sr. No.	Accessions No.	Location
1.	L4	Bakarti, Hamirpur-1	25.	L1	Usnar kalan, Hamirpur
2.	L7	Nerchownk, Mandi-1	26.	L3	Bakarti, Hamirpur-2
3.	L9	Rampur, Shimla-1	27.	L5	Dharmpur, Solan
4.	L11	Rampur, Shimla-2	28.	L6	Roru, Shimla
5.	L12	Sarahan, Sirmour	29.	L8	Baruhi, Una
6.	L13	Pehrwin, Bilaspur	30.	L15	Amb, Una
7.	L14	Bharmour, Chamba	31.	L18	Dalhousi, Chamba-2
8.	L18	Dalhousi, Chamba-1	32.	L19	Baldwara, Mandi
9.	L23(A)	Gondpur, Una-1	33.	L30	Sarahan, Sirmour-3
10.	L20	Rampur, Shimla-3	34.	L31	Patiala-5
11.	L22	Shoali, Shimla	35.	L35	Patiala-6
12.	L23(B)	Gondpur, Una-2	36.	L39	Patiala-7
13.	L24	Shrog, Shimla	37.	L41	Jammu
14.	L25	Nauni, Solan	38.	L42	Rajasthan-1
15.	L26	Sarahan, Sirmour-1	39.	L43	Rajasthan-2
16.	L27	Sarahan, Sirmour-2	40.	L45	Korba, Chhatisgarh
17.	L28	Nerchownk, Mandi-2	41.	L46	Chhatisgarh-2
18.	L29	Pandoga, Una	42.	L48	Chhatisgarh-3
19.	L36	Patiala-1	43.	L50	Chhatisgarh-4
20.	L37	Patiala-2	44.	L2	Kiyar, Solan
21.	L38	Patiala-3	45.	L10	Ghandhir, Bilaspur
22.	L40	Patiala-4	46.	L17	Gwalpathar, Hamirpur
23.	L44	Utrakhand	47.	L21	Sandal, Shimla
24.	L49	Chhatisgarh-1	48.	L34	Patiala-8

specific degenerate primers were used for the detection of yellow mosaic disease causing virus in horsegram (Table 2).

A master mix for final volume of 25 µl was prepared in 1.5 ml eppendorf tube by adding all PCR components. Then DNA template (1 µl) and 24 µl master mix were dispensed into PCR tubes. The PCR tubes were transferred in Veriti Thermal Cycler (Applied Biosystems, USA) as follows: Initially, the initial denaturation was done on 94 °C for 5 min followed by 30 cycles: 94 °C for 2 min (Denaturation), 55 °C for 1 min (Annealing) and 72 °C for 1 min (Extension) and then final extension with 72 °C for 10 min. PCR hold was done at 4 °C for infinite time. After the PCR amplification, the amplified PCR products were electrophoresed in agarose gel, eluted using Qiagen kit and PCR Clean-Up System (Promega, USA). Sequencing of the eluted product was done using 3730xl DNA Analyzer (Applied Biosystems, USA).

In silico analysis

The amplified sequence obtained after sequencing was first analyzed (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using Basic local alignment search tool (Altschul *et al.,* 1990). Among top 1000 BLASTn hits, the sequences of ICTV recognized begomovirus species were shortlisted based on the highest sequence identities. These sequences along with the outgroup sequence were used for multiple alignment and phylogenetic analysis using ClustalW programme (Thompson *et al.,* 1994). Mega X programme (https://www.megasoftware.net) (Kumar *et al.,* 2018) was used to construct phylogenetic tree using neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates (Felsenstein, 1985) and the evolutionary distances were computed using the Maximum Composite Likelihood Method (Tamura *et al.,* 2004). The partial and complete CP gene sequence of the present isolate was deposited in NCBI GenBank database.

Screening of horsegram accessions for disease incidence

The screening of 48 procured accessions of horsegram was carried out during August, 2019 in the agricultural field of DAV, University. All the selected accessions of horsegram were secreened against yellow mosaic disease. The screening of YMV disease was laid out in a randomized complete block design (RCBD) with three replication checks. The seeds were gown in five rows with spacing of 60

Table 2. List of specific primers used for molecular identification of yellow mosaic disease infecting horsegram

Primer Name	Nucleotide sequence (5'!3')	Target molecule	Product size	Annealing temperature	References
HgYMVAF	ATCATACTGAGAACGCTTTG	DNA-A	2.7 kb	55	Barnabas et al., 2010
HgYMVAR	TGTCATACTTCGCAGCTTC				
HYMV-A1500F	CTGCAGTGATGTTGTCCCCKG	DNA-A	2.7 kb	55	Maruthi et al., 2006
HYMV-A1500R	CTGCAGCTCAACTCAGGARTGG				
HYMV-CP-FHYMV-CP-F	ATG CTT GCA ATT AAG TAC TTG CA	Coatprotein	1000 bp	55	Akram and Singh, 2016
HYMV-CP-FHYMV-CP-R	TAG GCG TCA TTA GCA TAG GCA				
MYMV-CP-FMYMV-CP-F	ATG GG (T/G) TCC GTT GTA TGC TTG	Coatprotein	1000 bp	55	Akram and Singh, 2016
MYMV-CP-FMYMV-CP-R	GGC GTC ATT AGC ATA GGC AAT				

× 30 cm whereas 1 meter space was kept in between each block of RCBD to maintain and perform other agronomic practices like for weeding, for easy path and time to time irrigation. First twenty four accessions were germinated in A,B,C blocks and others were germinated in D,E,F blocks with three replications. The first two rows of each block were kept to prevent from bordering effect. The disease incidence was observed and calculated on 60th day after sowing of seeds to YMV disease to different accessions of horsegram based upon the following formula.

Disease incidence = (Total infected plant/Total healthy plant) × 100 (Khan *et al*., 2018).

Effect of Meteorological Parameters in MYMV Disease Progression

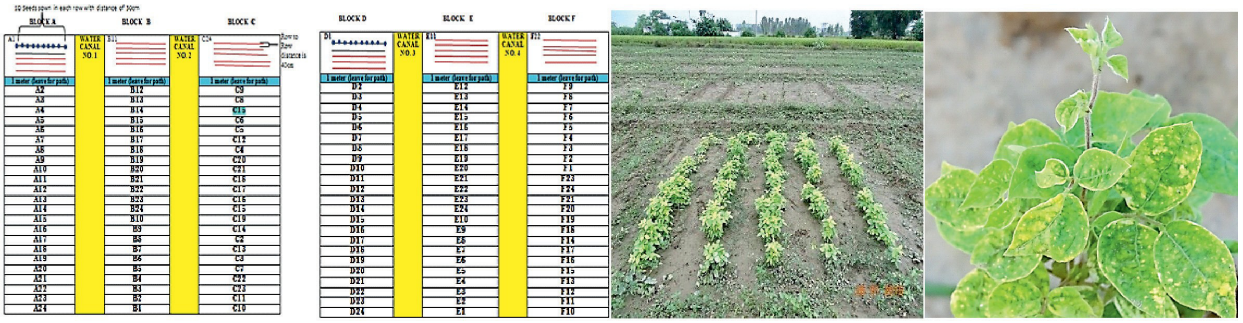
Data of meteorological parameters i.e. maximum and minimum temperatures, relative humidity and rainfall of Jalandhar region were collected from world weather online from the date of sowing (27/08/2019) to 60th day after sowing (25/10/2019) of procured 48 accessions under natural field conditions.

RESULTS AND DISCUSSION

During field trial, yellow discoloration on the leaves that led to irregular, small, greenish yellow mosaic symptoms were recorded in horsegram growing in the agricultural farms of DAV University, Jalandhar. These were quite similar to the begomoviral symptoms (Fig. 1C). The screened samples showing the yellow mosaic disease were transmitted by whitefly (*Bemisia tabaci*) in various accessions of horsegram in the field. The schematic representation of RCBD layout for 48 genotypes of horsegram collected from different regions of India is shown in Figure 1A.

A total of 48 accessions were collected from different geographical regions of India (Table 1) and used to screen for the begomoviral associated diseases in the field through randomised block design with three replication checks. The screening of disease incidence was taken on 60th days after sowing of seeds of horsegram. The viral genomic DNA was isolated from the highly resistant asymptomatic leaves (as control) and symptomatic infected leaves of different accessions of horsegram. The complete CDS of coat protein region of *Mung bean yellow mosaic virus* (accession number (JQ398669.1) have sequence identity of 99.16% was found after BLASTn. The sequencing result from the eluted PCR amplified product was submitted in Genbank with accession number MT345791.1 and protein ID QJX19345.1. A total of 257 amino acids coded for complete CDS of coat protein from 951bp of submitted nucleotide sequences in Genbank (Fig. 2A). On the other hand, the phylogenetic analysis with the coat protein of *Mung bean yellow mosaic virus* infecting horsegram with various other selected and well described begomoviruses was done using MEGA X (64-bit) software by Neighbour-joining method with bootstrap value 1000 (Fig. 2B).

The known begomoviruses infecting horsegram were clearly out-grouped. All these results confirm that the present isolate belongs to the genus *Begomovirus*. Cloning and sequencing of complete genome of the present isolates is being attempted in order to reveal its exact identity. In field screening, the accessions namely L4, L7, L14, L28 were found to be highly resistant against begomoviral diseases. These accessions were free from any diseases. L4,



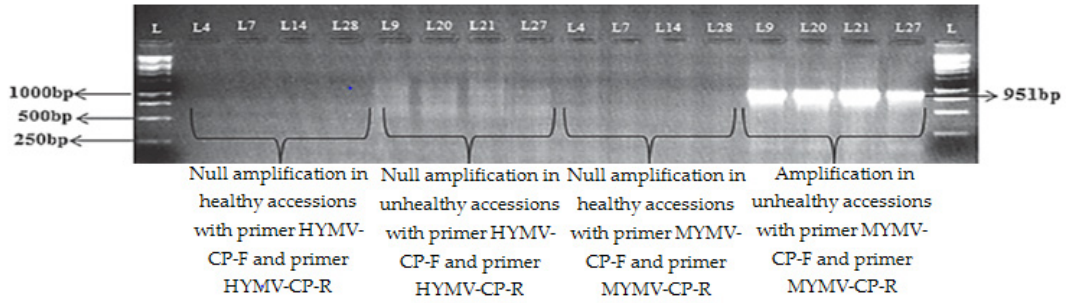
(1A) (1B) (1C)

Fig. 1. (1A) The schematic representation of RCBD layout for 48 genotypes of horsegram in field (1B) The asymptomatic plants of horsegram in field (1C) The symptomatic plant of horsegram with yellow mosaic disease

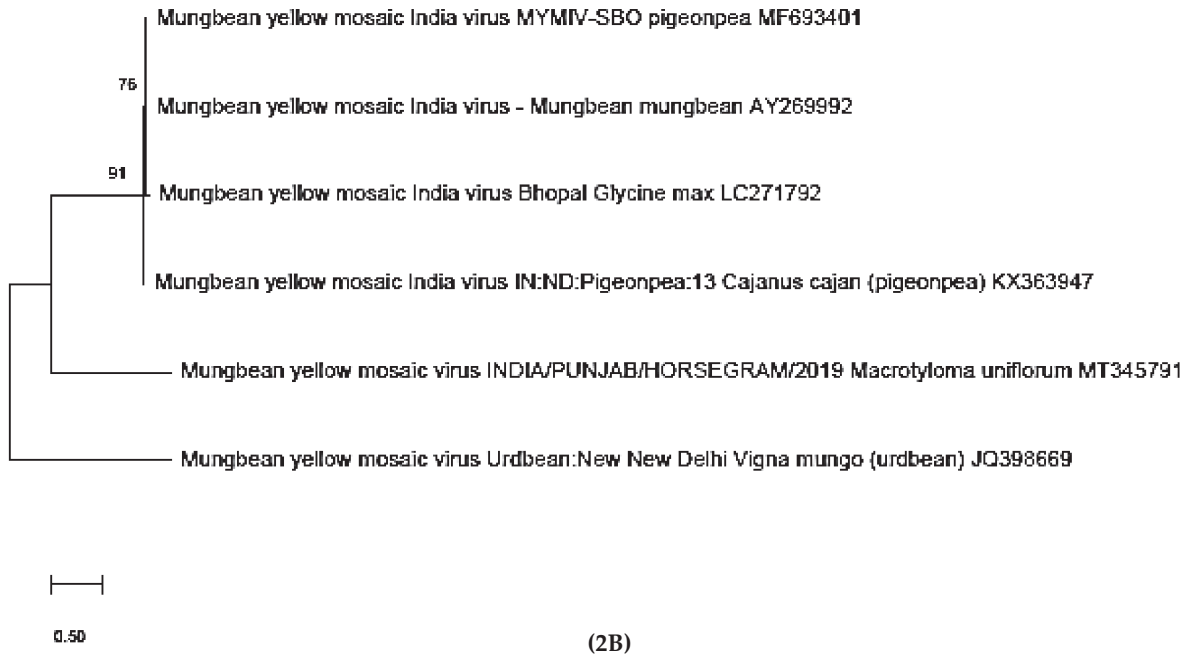
L14, L29 and L30 were the accessions which showed the 100% germination (Fig. 3).

It is clearly shown that L9, L21, L20 and L27 accessions showed the maximum disease incidence whereas L3, L18, L25 and L38 showed the least disease incidence with lesser rate of germination. The germination data and screening of disease incidence were taken after sowing of seeds of horsegram against yellow mosaic disease. The screening of these procured accessions to percent disease incidence was well correlated with the meteorological parameters i.e. maximum and minimum temperatures, relative humidity and rainfall of Jalandhar region. The average maximum temperature (34.37 °C), minimum temperature (24.80 °C) and relative humidity (69.72%) were recorded. The total rainfall was found to be 111.20mm. It was noticed that abrupt changes in meteorological parameters cause the progression of YMV disease incidence under natural field conditions (Fig 4).

Initially, it was observed that there was no disease incidence to YMV disease to the accessions of horsegram but later on due to the abrupt changes and fluctuation in the relative humidity, temperature and rainfall causes the progression of YMV disease among the accessions. After the heavy rainfall, decrease in maximum temperature and decrease in relative humidity, the first symptoms of the YMV disease appeared in the field and rapidly increased with the time. The presence of white flies was also increased during this period.



(2A)



(2B)

Fig. 2(A). PCR gel purified eluted amplified region of 951 bp with degenerate primers (MYMV-CP-F and MYMV-CP-R Primers) from infected leaves of susceptible accessions (L9, L20, L21 and L27) and no amplification with highly resistant accessions (L4, L7, L14 and L28) of horsegram, whereas L stands for Ladder (1kb, Promega) (B) Phylogenetic tree of amplified sequences of Mung bean yellow mosaic virus infecting Horsegram with the most identical sequences of various viruses.

Therefore, the intensity of disease was found to increase with the passage of time. Disease pressure, Location specific weather factors and virus strain are the important factors responsible for inconsistency in response pattern of the genotypes. It is well-known that initial period of 2-3 weeks is highly critical due to early landing of whitefly for the development and spread of MYMV. Increasing incidence and epiphytotic conditions of MYMV depend upon a combination of factors such as high population of whitefly, buildup of inoculums potential in some hosts and wide range of favourable weather conditions (Shad *et al*., 2006). Besides, temperature has a deep effect on distribution and prevalence of whitefly (James *et al*., 2002 and Hoffmann *et al*., 2003). The capacity to survive under thermal stress with other factors plays an important role in defining the distribution pattern of whitefly concurrently the incidence of MYMV (Bale *et al*., 2002). It was also assumed that relative humidity played a highly significant role in outbreak of MYMV disease. These observations are

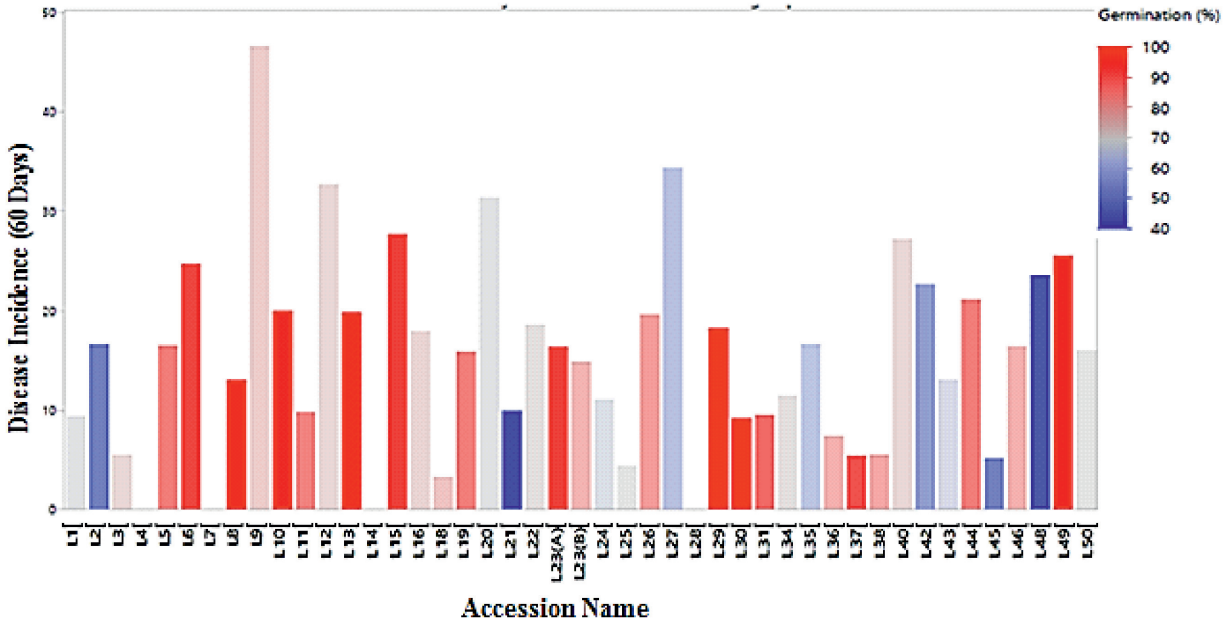


Fig. 3. Graphical representation of germination data and disease incidence by each procured accession of Horsegram

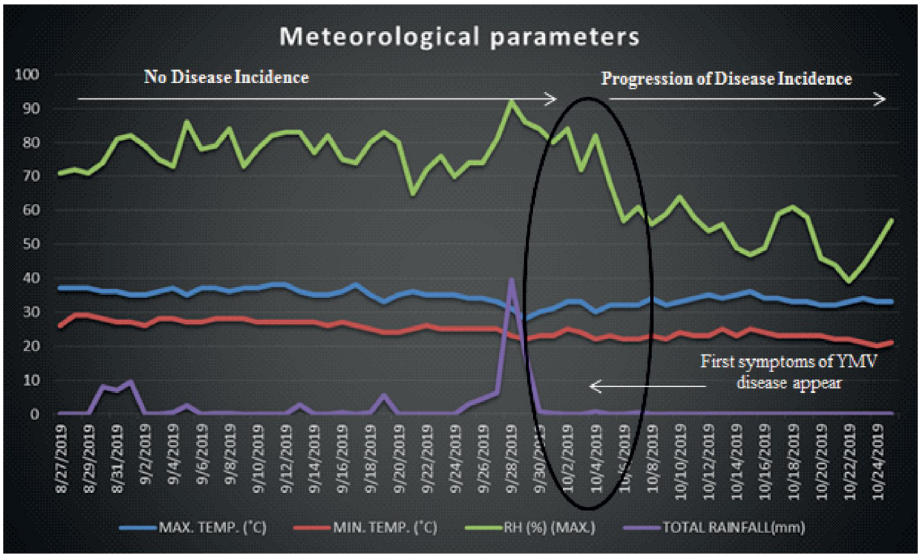


Fig. 4. Pattern of variability of meteorological parameters, i.e. Maximum temperature (°C), Minimum temperature (°C), Relative humidity (%) and Total rainfall (mm) during screening of horsegram accessions to YMV disease new generation of plant varieties in which the plant immune system is more resilient to environmental fluctuations.

further supported by the earlier report who also observed highly significant correlation of relative humidity with disease severity (Ali *et al*., 2005). It was also observed that the whitefly population being of high magnitude and directly related to disease spread. Corroboratively due to change in climatic conditions and gradual warming, there has been an increase in average temperature and relative humidity (Bakr *et al*., 2010).

CONCLUSION

In this study, *Mungbean yellow mosaic virus* (Urdbean: New Delhi: 2011) from Northern region of India was isolated, identified and screened for percent disease incidence under natural field conditions. Yellow mosaic disease is commonly occurring and has also been previously reported by different researcher in southern region of India. The nucleotide uniqueness and arrangement of the genes of this virus revealed that it belong to the genus *Begomovirus* of Family *Germiniviridae*. It is further used to check the whole genome of this virus by modern molecular techniques. The present research work also concluded that the abrupt changes in meteorological parameters cause the progression of YMV disease incidence under natural field conditions. The abrupt change in relative humidity, maximum temperature and heavy rainfall cause the progression of disease incidence and whitefly population in the field. Environmental fluctuations reduce the strength of plant immunity against pathogen/viral attack. Therefore, it is a need to understand how environmental conditions influence plant immunity, pathogen virulence and disease development? Such knowledge will hopefully provide a foundation for developing a new generation of plant varieties in which the plant immune system is more resilient to environmental fluctuations.

ACKNOWLEDGEMENTS

Authors acknowledge the financial assistance received from SERB-DST, GOI New Delhi with Project No. (YSS/2015/00521). Infrastructure support provided by DAV University is also acknowledged.

Conflict of interest

Authors declare that there is no conflict of interest.

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