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Effect of Temperature on Symptoms Expression and Viral RNA Accumulation in Groundnut Bud Necrosis Virus Infected *Vigna unguiculata*

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ABSTRACT

Groundnut bud necrosis virus (GBNV) (Tospovirus genus, Bunyaviridae family) infects the major crops of solanaceae, leguminosae and cucurbitaceae in India. Temperature is an important factor which influences the plant growth and development under diseased conditions. Here, we investigated the effect of four temperatures (30°C, 25°C, 20°C and 15°C) on viral symptom expression and RNA accumulation in GBNV inoculated cowpea plants. The first visible symptom of GBNV infection at inoculated site was observed in the form of chlorotic spots which were converted into the necrotic spots as the infections succeed. Some yellow mosaic symptoms were also observed at the systemic site during viral infection cycle. Plants incubated at higher (30°C and 25°C) temperatures showed severe necrosis and higher viral RNA accumulation at inoculated site, and facilitated the viral spread at systemic site. However, viral RNA accumulation was less at systemic site than the inoculated site. In contrast, symptoms expression and viral RNA accumulation were decreased at inoculated site at low (20°C and 15°C) temperatures and no viral symptoms were observed at systemic site (15°C); viral RNA accumulation was also suppressed at this site. GBNV infection at the inoculated site induced the higher accumulation of H_2O_2 followed by the induction of cell death at higher temperatures (30°C and 25°C) than the lower (20°C and 15°C) temperatures. These results suggest that the accumulation of viral RNA accumulation parallel with the H_2O_2 production and induction of cell death by GBNV infection in cowpea plants is temperature dependent.

Key words: *H_2O_2, Chlorosis, Necrosis, Hypersensitive response (HR), Programmed Cell Death (PCD).*

INTRODUCTION

Temperature is one of the most important environmental factors that affects plant-pathogen interactions, and it can either increase or decrease disease resistance. This reflects the differential influence of the same temperature variation on different plants.

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Temperature affects the growth and populations of microorganisms living on plants such as viruses. Several reports give the contradictory data on the temperatures favoring viral replication, which depend on the virus species as well as the analyzed plant host. It has been reported that temperature affects the viral transmission, replication and translocation inside the plant, as well as susceptibility and symptom expression in the host. *Tomato spotted wilt virus* (TSWV) infection in tomato resulted higher replication at 20°C while symptoms were more severe at 36°C[1]. *Turnip crinkle virus* (TCV) is rigorously replicated under the high temperature in Arabidopsis plants[2]. Higher temperature facilitated the spread of *Tobacco mosaic virus* (TMV) or *Turnip mosaic virus* (TuMV) by weakening the plant defense responses[3]. The importance of temperature on virus infection and accumulation is under intense investigation[4,5,6]. *Groundnut bud necrosis virus* (GBNV), a member of the genus *Tospovirus* and family *Bunyavirdae*, is a devastating thrips-transmitted virus. GBNV induces chlorotic and necrotic spots, mosaic, mottling and yellowing on leaves. *Tospoviruses* are 80-120 nm diameter, quasi-spherical and defined by a membranous envelope contain two types of glycoproteins designated as G1 (78kDa) and G2 (50kDa). The envelope covered a tripartite RNA genome which is tightly packaged by numerous copies of nucleocapsid (N) protein subunits (29kDa) and 10-20 copies of a large (L) protein, which is the putative RNA dependent RNA polymerase. The L RNA (9kb) has a single ORF in the viral complementary sense and encodes the RNA polymerase of 331.5kDa[7]. The M RNA is also ambisense and approximately 4.8 kb, which contains two ORFs. The M RNA encodes for glycoproteins G1 and G2 in the viral complementary sense RNA (vcRNA) and a nonstructural movement protein (NSm) in viral sense RNA (vcRNA). The G1/G2 proteins are more highly conserved between tospoviruses than the N protein. The extra NSm gene, therefore may reflect an adaptation of tospovirus to plant hosts, i.e. it may act as the movement protein. The S RNA is ambisense and approximately 3 kb (7, 8), which contains two ORFs. The ORF nearer the 5' end of the RNA codes for a nonstructural protein in the viral sense designated NSs (52.4kDa) whose function has not been determined. The ORF nearer the 3' end is in the viral complementary sense and codes for the N protein (29kDa) which encapsidates the viral RNAs within the viral envelope. Virus infection in plants may result in physiological changes which are manifested by visible symptoms. At the molecular and cellular level, viral proteins are recognized by the host defense machinery which resulted the induction of plant defense responses like RNA silencing, hypersensitive response (HR), and stress responsive proteins[9,10,11,12]. TMV resistance in host plants is compromised at higher temperatures. During TMV infection tobacco plants carrying the N gene which do not generate a hypersensitive reaction in response and TMV rather spreads systemically at temperatures above 28°C [13]. Similarly, *Capsicum chinense* plants carrying the resistant *Tsw* gene develop systemic infections of TSWV at 32°C [14]. Several reports showed that during tospoviruses infection temperature is an important factor that affect the symptom expression and viral movement in pathogens such as *Cucumber green mottle mosaic virus* (CGMMV) on cucumber and melon, *Cucumber mosaic virus* (CMV) on melon, *Melon necrotic spot virus* (MNSV) on melon and TSWV on bonnet pepper (*Capsicum chinense*) and peanut (*Arachis hypogaea*) [15,16,17,18,19,20]. In the present study, we sought to predict that low temperature might limit the establishment of viral disease by lowering the accumulation of viral RNA, H_2O_2 and cell death in the host cell. Therefore, in the investigation of temperature effect on viral symptom expression of GBNV, we examined viral RNA accumulation in the GBNV inoculated leaves of cowpea (*Vigna unguiculata* L.). We employed the quantitative RT-PCR reaction to estimate the GBNV replication.

MATERIALS AND METHODS

Plant and virus inoculation

GBNV inoculum was maintained on cowpea cv. Pusa komal (*Vigna unguiculata* L.) grown under controlled conditions. Viral symptomatic leaves were harvested and macerated in the sterilized and chilled pestle and mortar adding 0.01 M phosphate buffer (pH 7.2, 1:1, w/v) containing 0.1% β-mercaptoethanol. The extracted sap was used as viral inoculum for inoculation. The healthy cowpea seedlings (2-3 leaf stage) were dusted with celite (as abrasive) and the extracted sap was applied directly by rubbing gently on the leaves with the chilled pestle to exert uniform pressure (20). The seedlings were gently washed with water and grown in a growth chamber at four different temperatures (30°C, 25°C, 20°C and 15°C) with 14-h light and 10-h dark for the symptom development.

GBNV symptom severity rating

The severity of symptoms expressions were categorized on the basis of phenotypic symptoms developed during viral infection. Symptom severity score was rated on a 1-point scale: 0 = no symptoms, 0.25 = appearance of mild chlorosis, 0.5 = severe chlorosis, 0.75 = yellow mosaic, 1.0 = necrotic spots. The infected plants were incubated at four different temperatures (30°C, 25°C, 20°C and 15°C) and observed for the development of GBNV symptoms. The expressions of symptoms were scored on the basis of above mentioned scale.

GBNV detection using enzyme linked immunosorbent assay (ELISA)

GBNV infection was detected and quantified serologically using the plate trapped-antigen enzyme linked immunosorbent assay (ELISA) method (21) in Maxisorb microtiter plates (Nunc, Roskilde, Denmark), using 50 mg fresh leaf sample of buffer (Mock) and GBNV inoculated cowpea plants at different stages of symptom development; in inoculated and systemic sites of four different temperatures (30°C, 25°C, 20°C and 15°C). Coating samples were prepared with leaf tissue in coating buffer (0.05 M sodium carbonate, pH 9.6, 1:10 w/v). Coating samples were poured in ELISA plate wells and incubated overnight at 4°C. All further incubations for complete ELISA procedure were performed at 37°C. At each step of procedure, the wells were washed thrice with phosphate buffer saline (PBS) containing 0.05% Tween-20 (PBS-T). Bound virus particles were detected by using GBNV antiserum (produced in-house), with a dilution of 1:1000 and goat anti-rabbit alkaline phosphatase conjugated secondary antibodies (Sigma, St Louis) a dilution of 1:30,000 in PBS. The interaction of enzyme conjugated on secondary antibody and substrate p-nitrophenyl phosphate (Sigma, 1 mg/ml) was detected; and quantification was carried out with Dynatech MR 7000 plate reader at an absorbance of 405 nm. Samples were considered positive when the absorbance exceeded twice the mean of the absorbance values for the mock-inoculated controls.

RNA isolation and RT-PCR analysis

Total plant RNA was isolated from 50 mg leaf sample from mock and virus inoculated cowpea plants maintained at four different temperatures (30°C, 25°C, 20°C and 15°C) with different stages of symptom development as described by (22) using TRIzol reagent (Invitrogen). Isolated RNA was treated with DNase I (Ambion) prior to RT-PCR analysis. For viral RNA (non-structural; NSs) transcript analysis, cDNA was synthesized from 0.5 µg total RNA by M-MuLV reverse transcriptase (NEB), using primer NSs-R 5'-CCCTCGAGGGTTACTCTGGCTTCACAATGAAAT-3'. cDNA was then subjected to conventional PCR. Amplification of NSs (1320 bp) was done using the oligonucleotide primers NSs-F 5'-CTGTCTAGAATGTCGACCGCAAGGAGT-3' and NSs-R 5'-CCCTCGAGGGTTACTCTGGCTTCACAATGAAAT-3'. Amplification was confirmed on 1 % agarose gel with electrophoresis.

Quantification of viral RNA in GBNV inoculated plants using Real-time qPCR

Total plant RNA was extracted from 500 mg leaf material from mock and virus inoculated cowpea plants maintained at four different temperatures (30°C, 25°C, 20°C and 15°C)

with different stages of symptom development as described previously using TRIzol reagent (Invitrogen). Isolated RNA was treated with DNase I (Ambion) prior to quantitative RT-PCR analysis to eliminate genomic DNA contamination. For the viral RNA accumulation analysis, cDNA was synthesized from 0.5 µg total RNA by M-MuLV reverse transcriptase (NEB), using random hexamer primers. cDNA was then subjected to conventional PCR and qPCR. Quantitative RT-PCR was performed using 2X SYBR Green PCR Master Mix (Roche) with 20 ng of synthesized cDNA and 5 µM of each primer in 25 µl reaction volume. Amplification was done using the oligonucleotide primers NSs-F 5'-ATCTGCATTCAGCATCAACG-3' and NSs-R 5'-CCTCACAACCAGGCTCATTT-3' for the GBNV NSs gene. The amplification conditions were 95°C for 2 min, then 40 cycles of 95°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec[22].

Spectrophotometric determination of H_2O_2

Endogenous H_2O_2 present in the extracts of GBNV infected cowpea leaf samples incubated at four different temperatures was estimated according to the procedure of Frew et al. (23).For the measurement H_2O_2 in the virus infected cowpea leaves, each sample was prepared by homogenizing the leaf in 0.1 M phosphate buffer, pH 7.2. The homogenate was centrifuged at 4°C at 10,000 rpm for 10 min. The supernatant was taken for estimating H_2O_2 and 3.0 ml of reagent solution (100 ml contains 0.234 g of phenol, 0.1 g of 4-aminoantipyrine, 1.0 ml of 0.1 M phosphate buffer, pH 7.2) was used to estimate the H_2O_2. Quantified H_2O_2 is expressed in µmol g^{-1} fresh weight. All spectrophotometric analyses were carried out on HITACHI, U-2900, spectrophotometer.

Cell Death detection

Virus infection induced cell death was analyzed as described by Turner and Novacky (24) with minor modifications. The leaves of GBNV infected cowpea plants were incubated in 0.25% Evans blue solution for 20 min at room temperature and washed with distilled water. The trapped Evans blue was released from the virus infected cowpea leaves by homogenizing with 1.0 ml of 80% ethanol. The homogenate was incubated at 50°C in a water bath for 20 min and centrifuged at 10,000 X g for 15 min. The absorbance of supernatant was measured at 600 nm and calculated on the basis of fresh weight.

RESULTS

Symptoms expression in GBNV infected cowpea plants at different temperatures

GBNV inoculated cowpea plants maintained at four different temperatures (30°C, 25°C, 20°C and 15°C) were observed for symptom development from the first day of viral inoculation to the complete collapse of the plant leaves. The first visible symptoms of GBNV on the inoculated leaves were observed in the form of chlorotic spots within the four days of post inoculation (dpi). These chlorotic spots were converted to necrotic spots after 8 dpi. Within these 8 days, some yellow mosaic symptoms were started to appear on the newly grown (systemic) leaves (during 30°C and 25°C incubation) (Fig. 1a i & ii). Whereas, at 20°C and 15°C plants showed the reduction in viral symptom development both on inoculated and systemic sites (Fig. 1a iii & iv). At the inoculated site these plants showed lesser chlorotic spots than the plants those were incubated at 30°C and 25°C, and surprisingly these inoculated leaves were not showed any necrotic spots up to 8 dpi. However, at systemic site, 20°C incubated plants showed mild yellow mosaic symptoms at 8 dpi, but plants incubated at 15°C did not showed any viral symptoms on the systemic sites (Fig. 1a iv).

Disease severity index

The severity of GBNV symptoms expressions were scored to plants on the basis of phenotypic symptoms developed during viral infection. The expressions of symptoms were different from the higher to lower temperature. Plants maintained at higher temperature (30°C and 25°C) induced higher severe symptoms rated 0.5 to 1.0 scales. While plants incubated at lower temperatures

(20°C and 15°C) showed reduced symptoms rating 0.25 to 0.5 scales (Fig. 1b).

Detection of GBNV in the viral inoculated cowpea plants

Viral titer was estimated in the GBNV inoculated leaves of cowpea plants maintained at different temperature (30°C, 25°C, 20°C and 15°C) and days (4 and 8) post inoculation (dpi) with ELISA assay at O.D.405 (A_{405}). Based on ELISA values (A_{405}) after GBNV inoculation at inoculated sites from 4 dpi to 8 dpi was continuously increased and high levels of virus accumulated in the inoculated leaves of cowpea at 8 dpi (at 30°C and 25°C; 0.880±0.238 and 0.826±0.324, respectively). In contrast, at systemic site during these temperatures (30°C and 25°C), ELISA values for GBNV inoculated plants at 8 dpi were 0.542±0.124 and 0.440±0.140, respectively. Whereas viral titer was not significantly increased in the cowpea plants at 20°C and 15°C, as compared to plants at 30°C and 25°C. While plants incubated at 20°C accumulated lower viral titer 0.240±0.024 at 8 dpi (inoculated site) and 0.084±0.022 at the systemic site. Interestingly, viral titer was highly decreased in the plants maintained at 15°C at inoculated site (8 dpi; 0.232±0.064), and at systemic site (8 dpi; 0.062±0.024). Virus titer level did not differ significantly with mock plants suggest that virus did not reached up to the systemic site in plant maintained at 15°C (Fig. 1c, Table).

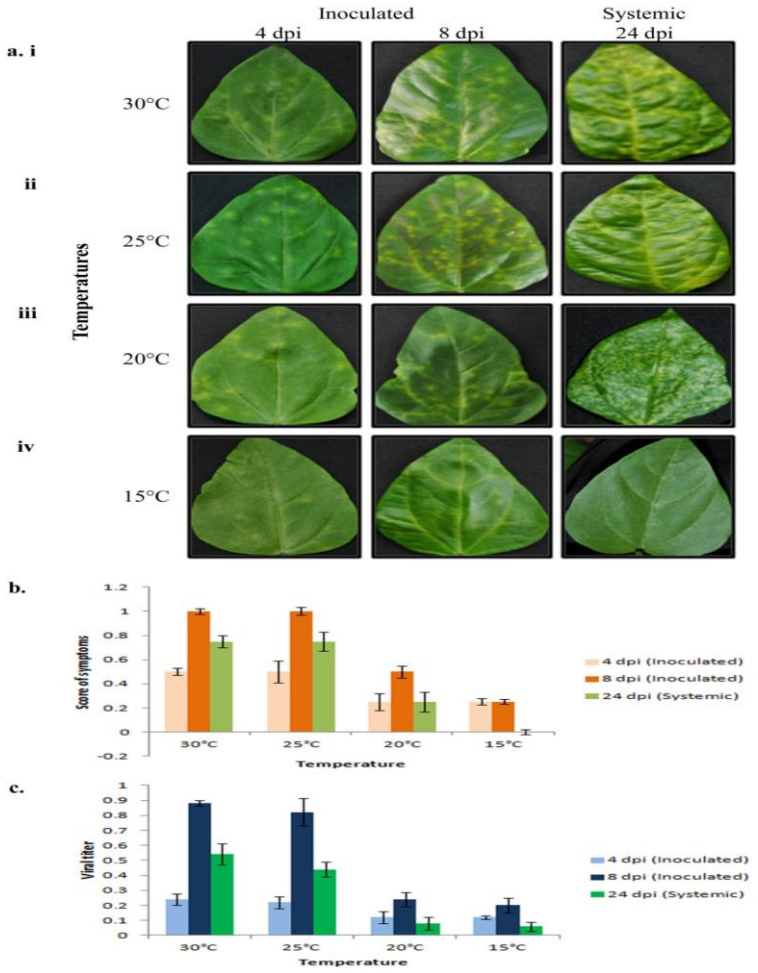

Fig. 1 Different stages of symptoms development in GBNV inoculated cowpea plants, maintained at different temperatures. (a) Symptoms produced by GBNV after mechanical inoculation on cowpea plants in comparison to different temperatures at inoculated and systemic sites (i-iv). (b) Represents symptoms severity kinetics in GBNV inoculated cowpea plants maintained at different temperatures. Symptom severity was scored by early appearance of mild chlorotic spots followed by severe chlorotic symptoms on leaves, which further resulted in severe necrosis. (c) Virus accumulation kinetics in leaves of cowpea plants. Virus titer was estimated at different stages of viral infection maintained at different temperatures of post viral inoculation by ELISA (O.D. A_{405}) using GBNV polyclonal antibodies (dilution 1:1000).

Table: Accumulation and detection of *Groundnut bud necrosis virus* (GBNV) in inoculated leaves of cowpea at different temperatures and different days post inoculation (dpi)

S. No.	Temperature	Dpi and Site	ELISA (A_{405})[a]	No of positives/total plants[b]	Symptoms Score
1.	30°C	4 dpi (Inoculated)	0.242±0.012	6/6	0.50±0.024
		8 dpi (Inoculated)	0.880±0.238	6/6	1.00±0.146
		8 dpi (Systemic)	0.542±0.124	6/6	7.50±0.248
		Mock	0.062±0.014	0/3	0
2.	25°C	4 dpi (Inoculated)	0.224±0.016	6/6	0.50±0.264
		8 dpi (Inoculated)	0.826±0.324	6/6	1.00±0.282
		8 dpi (Systemic)	0.440±0.140	6/6	7.50±0.286
		Mock	0.082±0.018	0/3	0
3.	20°C	4 dpi (Inoculated)	0.122±0.012	6/6	0.25±0.284
		8 dpi (Inoculated)	0.240±0.024	6/6	0.50±0.280
		8 dpi (Systemic)	0.084±0.022	3/6	0.25±0.260
		Mock	0.041±0.012	0/3	0
4.	15°C	4 dpi (Inoculated)	0.122±0.014	6/6	0.25±0.248
		8 dpi (Inoculated)	0.232±0.062	6/6	0.25±0.246
		8 dpi (Systemic)	0.062±0.024	0/6	0
		Mock	0.062±0.014	0/3	0

[a]Values are mean absorbance and standard deviation (±SD) at 405 nm in enzyme-linked immunosorbent assay (ELISA) analysis (n = 6). Means followed by different letters are significantly different by Scheffe's test (P\0.05)[b]Number of GBNV positive plants/total inoculated plants.

Accumulation of viral RNA in the GBNV in inoculated plants

Before the estimation of viral RNA, viral infection was conformed with conventional PCR by amplification of GBNV-NSs (1320 bp) gene in the GBNV inoculated cowpea plants maintained at four different temperatures (30°C, 25°C, 20°C and 15°C) (Fig. 2a).

Viral RNA accumulation analysis using RT-qPCR showed that viral accumulation was continuously increased (4.4-10.4 $x10^5$ copy no.) in plants maintained at 30°C and 25°C from 4 dpi to 8 dpi at inoculated site (Fig. 2b i). During this time period (4-8 dpi) virus also moved from inoculated to systemic site at 30°C and accumulates about 5.2 $x10^5$ viral copy, while at 25°C plant accumulates viral copy about 5.8 $x10^5$ at systemic site (Fig. 2b ii). Plants incubated at 20°C accumulate lower viral copy (2.8 $x10^5$; at 8 dpi) than the plants incubated at

30°C and 25°C (Fig. 2b i), and during this time (8 dpi) and temperature (20°C) very less viral copy (0.8 x10^5) was observed at systemic site (Fig. 2b ii). This showed that temperature reduction decreased the viral movement to systemic site. Interestingly, plants incubated at 15°C accumulate very less copy of viral RNA both at inoculated (1.0 x10^5) (Fig. 2b i) and systemic sites (0.2 x10^5) (Fig. 2b ii) at 8 dpi. This data suggest that low temperature reduced the viral movement as well its replication in the host plant.

Lower temperature incubation reduced the accumulation of H_2O_2 and cell death than the higher temperature

H_2O_2 accumulation was analyzed in the plants incubated at four different temperatures (30°C, 25°C, 20°C and 15°C). Higher H_2O_2 accumulation, was observed in the GBNV inoculated cowpea plants incubated at higher (30°C and 25°C) temperature both at inoculated and systemic sites than the plants incubated at lower temperature (20°C). Interestingly H_2O_2 accumulation was drastically reduced in plants incubated at 15°C temperature at both inoculated and systemic sites (Fig. 2c).

To identify the role of temperature in virus induced leaf cell death, virus infected cowpea plants, incubated at four different temperatures were analyzed for cell death by Evans blue staining. Plants incubated at higher temperatures (30°C and 25°C) were observed to induce more (40-90%) cell death both at inoculated as well as systemic sites. While plants incubated at 20°C showed lower (20-50%) cell death during virus infection than plants incubated at higher temperatures. Surprisingly those plants incubated at 15°C showed only 10% cell death at inoculated site and no cell death were observed at systemic site at this temperature (Fig.2d).

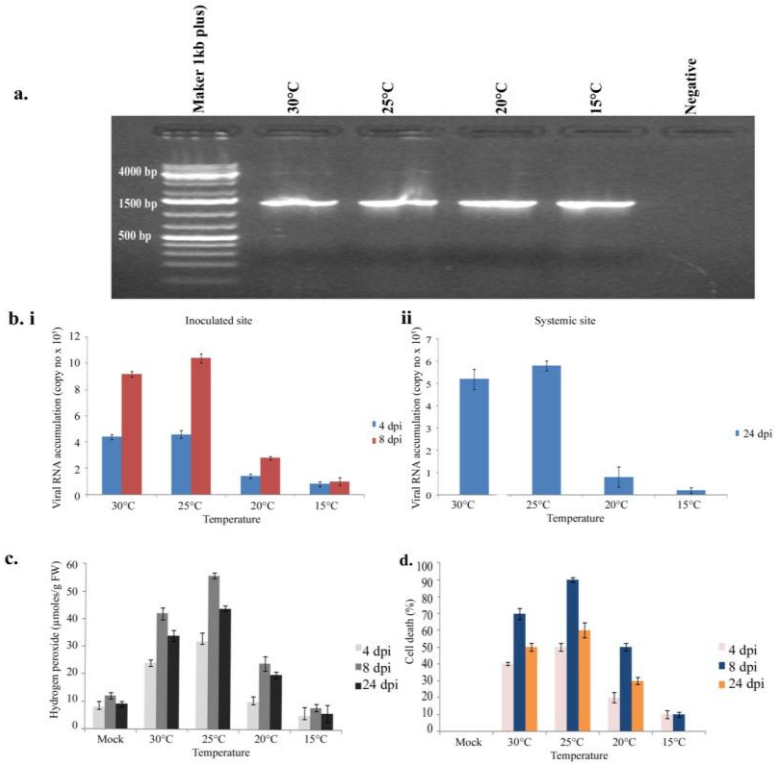

Fig. 2 Accumulation of viral RNA and H_2O_2, and induction of cell death kinetics in GBNV inoculated cowpea plants incubated at different temperatures. (a) RT-PCR gel for confirmation of viral infection in cowpea plants maintained at different temperatures (30°C, 25°C, 20°C and 15°C). (b) RT-qPCR quantification of GBNV RNA with amplification of the 5' end of the small segment (NSs) of the viral genome (nucleotides 146-297). The copy number of the transcript per ml of sample was plotted at different stages (i) inoculated and (ii) systemic sites of infection. (c) H_2O_2 accumulation and (d) induction of cell death in GBNV infected cowpea leafs samples at inoculated and systemic sites at four different temperatures. Error bar represent ± standard deviation (SD) of mean data in three independent experiments.

DISCUSSION

The present study showed that GBNV infection severity and viral RNA accumulation was affected by temperature and the high temperature (30°C and 25°C) facilitated symptom expression and viral spread in the cowpea plants. The patterns of symptom expression at the various temperatures differed according to the host–virus combination[15]. Temperature affects the viral accumulation and spread inside the plant, as well as host susceptibility and viral symptom expression[1]. Tomato infected with TSWV accumulated higher viral RNA at 20°C and severe symptoms at 36°C [1]. TSWV inoculated *C. chinense* induced more systemic infection at 30/18°C (day/night) than at 25/18°C [15]. Whereas TSWV inoculated peanut plants resulted higher resistance and reduced systemic infection at higher temperature (30–37°C) than the lower temperature (25–30°C)[16]. Llamas-Llamas et al. (1) reported that at higher temperature [29/24°C (day/night)], TSWV induced viral symptoms both at local and systemic site in *Nicotiana tabacum* and *Datura stramonium*. At low temperatures, TSWV infected tobacco resulted to only local lesions (chlorotic rings), while at high temperatures both local and systemic symptoms develop (25). In the case of GBNV, higher temperatures (30°C and 25°C) facilitate the induction of viral symptoms and spread from inoculated to systemic sites in cowpea.

As expected, at the higher temperature, higher replication of the GBNV RNA was observed. The onset of symptom development and severity were associated with the virus titer in host cells and clearly suggested major changes in host plants metabolism. RNA silencing mediated plant defenses was temperature-dependent and the levels of siRNAs increased gradually with rising temperatures[26]. TCV infection in *Arabidopsis thaliana* is facilitated more vigorously at higher temperature than the lower temperatures[2]. Our study also reprove that higher temperature favors the viral replication and symptoms severity.In the present study, GBNV infected plants showed less symptoms and reduced viral accumulation at the lower temperatures (20°C and 15°C). We hypothesize that reduction of viral symptom is evident when replication is reduced, viral movement slowed, viral suppressor levels decrease, or host plant resistance increases. A recent report[27] showed that the *Potato virus Y* and *Potato virus X* titers and viral RNA were increases at higher temperatures.

We previously reported that GBNV infection induced its typical symptoms (chlorosis and necrosis) within 4-8 dpi in mechanically inoculated cowpea plants and GBNV infection was spread systemically at 8 dpi which instigate the two types of cell death at inoculated (necrosis) and systemic site (premature senescence) at 25°C. The necrotic site of virus inoculation accumulates more H_2O_2 which play a central role in virus induced programmed cell death (PCD) than the non-inoculated (systemic) sites (28). In this study, we confirmed that viral symptoms (chlorosis, necrosis and yellow mosaic) and viral spread of GBNV is temperature dependent and lower temperature (20°C and 15°C) limits the viral infection in cowpea.

Several studies suggested that the presence of virus is generally diminishing photosynthetic capacity by decreasing the accumulation of photosynthetic proteins in infected plants[11,12]. This study has shown that the accumulation of virus is affected by the temperature regime to which the plants are exposed. Overall, the results show clearly that the viral response (infection and spread) is more active at the higher temperature than the lower temperature. Genetic analyses and further detailed studies are needed to clarify these mechanisms in cowpea plants.

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