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Original scientific paper

GENE EXPRESSION IN RESPONSE TO COTTON LEAF CURL VIRUS INFECTION IN *Gossypium hirsutum* UNDER VARIABLE ENVIRONMENTAL CONDITIONS

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Cotton Leaf Curl Disease (CLCuD) is one of the threatening constrains of cotton production in Pakistan for which no adequate remedy is available until now. Local variety of *Gossypium hirsutum* (FH-142) was grown in field and infected naturally by CLCuV under variable range of temperature and humidity. Plants showed thickening of veins in lower leaf surface at 34°C and 60% relative humidity at 15days post infection (dpi) and curling of leaf margins at 33°C with 58% relative humidity at 30dpi. Remarkable leaf darkening was observed with reduced boll formation at 45dpi at 26°C and 41% relative humidity. Enation developed, severe thickening and curling of leaves intensified and plants showed dwarf growth at 60dpi at 24°C with 52% relative humidity. PCR amplification of Rep associated gene confirmed the presence of CLCuD-associated begomovirus in the infected samples. Quantitative RT-PCR confirmed the amplification and differential expression of a number of pathogen stress responsive genes at different levels of temperature and humidity. This observation

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predicts that Cotton Leaf Curl Virus (CLCuV) interacts with several host genes that are upregulated to make plants susceptible or suppress other genes to overcome host defense responses.

Keywords: Cotton leaf curl virus, gene expression, *Gossypium hirsutum*, molecular analysis, plant pathogen

INTRODUCTION

Cotton is the principal cash crop in the world that is primarily grown for fiber. Cotton crop contributes 1.0 percent share in GDP and 5.2 percent in agriculture value addition according to economic survey of Pakistan report 2016-17. CLCuD is one of the most devastating threats to cotton production in Pakistan. This is caused by the Cotton leaf curl virus (different Begomoviruses) which is transmitted by whitefly (*Bemisia tabaci*). Infection rate influence differentially, due to the intricate interaction of environmental conditions including temperature, humidity, rainfall and wind velocity with virus and its vector development (PERVEEN *et al.* 2010; ABBAS *et al.* 2016). The ideal temperature for cotton growth is 20ºC to 30ºC while 25-30°C is most favorable for development of eggs and nymphs of *B. tabaci* (ILHAI *et al.* 2013; ABBAS *et al.* 2015). Severity of CLCuD enhanced in maximum and minimum temperature such as 33-45°C and 25-30°C, respectively and relative humidity (RH) of 58-60% (FAROOQ *et al.* 2011; ABBAS *et al.* 2013). Progress has been made to control such drastic disease through traditional breeding techniques and relatively tolerant cultivars have been developed but it is still a challenge even after extensive research (NAZEER *et al.* 2014; PUSPITO *et al.* 2015).

Plants exhibit specialized mechanisms against infection through constitutive defense responses. It acts as a physical barrier in the form of cell wall, epicuticular wax or induced defense responses that showed activation of proteinase inhibitors, polyphenol oxidases, chitinases and other pathogen related (PR) proteins (ALI *et al.* 2014; KHAN *et al.* 2015). However, detailed genetic regulatory mechanisms that govern plant's interactions with pathogens are yet to be fully understood. In case of viral attack, vectors also influence changes in trancriptome of their hosts to promote their infestation. It is important to identify the certain genes families in *G. hirsutum* that show their involvement in defense related mechanisms to CLCuV (AHMAD *et al.* 2011; AZAM *et al.* 2013; FAROOQ *et al.* 2014; SHAHID *et al.* 2016). These genes either expressed or suppressed during pathogen infection and lead to activation of glucosinolates, reactive oxygen species, hypersensitive response, pathogenesis related genes and many other secondary metabolites pathways (AFTAB *et al.* 2014; ALAM *et al.* 2015).

Gossypium hirsutum exhibits high susceptibility towards CLCuV. Present study is proposed to identify the expression of certain genes in a local variety of *G. hirsutum* that particularly respond during CLCuV infection under different conditions of temperature and humidity. This will lead to study the functions and interaction of those genes that will provide information for understanding of mechanism(s) involved in response of such threatening disease.

MATERIAL AND METHODS

Seeds germination, plant materials and CLCuV infection

Seeds of local *G. hirsutum* var.FH-142 were sown for germination under natural environmental conditions with regular agronomic practices. Plants started typical CLCuD symptoms after six weeks of seed germination. At this stage morphological characteristics and severity of CLCuD were assessed through visual observation by following disease rating scale

system as described by Akhtar *et al* (AKHTAR *et al.* 2010; FAROOQ *et al.* 2014)**.** Data was collected fortnightly from August to November 2014. Data about temperature and humidity was collected following weather forecast in Lahore (www.accuweather.com/en/pk/lahore/260622/weather-forecast/260622).

Genomic DNA isolation and confirmation of CLCuV through PCR

Leaf samples were collected from CLCuV infected and control plants. Sampling was done five times in three replicas after every fifteen days starting from mid of August, 2014 (Table 1). DNA was isolated by CTAB method (DOYLE 1991) with some modifications. Replication associated protein (Rep) gene was amplified for the detection of CLCuV in the samples collected during all stages of infection. Rep gene specific primers i.e. 5'-AGCCCAGTACAGCAACTGTG-3' and 5'-CTTGACCAAAGCCTGTTCCT-3' were used. Reaction master mix of 25µl was prepared as following: Template DNA (2µl), 10X PCR Buffer (2.5µl), 50mg $MgCl_2$, dNPTs (1mM) (3µl), CLCuMV-F (10pmol/µl) (1µl), CLCuMV-R (10pmol/µl) (1µl), TaqDNA polymerase enzyme (5U/µl) and dH_2O (14µl). The thermocycler profile was set for 35 cycles following the initial denaturing at 95°C for 5min, denaturation at95°C for 30s, annealing at 58°C for 30s, extension at 72°C for 30s and final extension at 72°C for 10 min.

Disease stress responsive genes selection

Different gene families responsive to the infection caused by different pathogens in a number of plant species were selected from the literature. These gene families included WRKY family, NAC domain, Trehalose-Phosphatase, Oxidoreductase, NADPH Dehydrogenase, Pyrophosphatase, ADP-Ribose Pyrophosphohydrolase, Hydrolase and Threonine Aldolase http://www.ncbi.nlm.nih.gov/pubmed. Primers of these genes were designed by using the Primer3 Software.

Total RNA isolation and quantitative RT-PCR

Plant leaf samples were collected and total RNA was isolated following MUOKI *et al.* (2012) with some modifications (MUOKI *et al.* 2012). cDNA was prepared by using an anchored oligo-$(dT)_{18}$ primer RevertAid^{TM} H minus First Strand cDNA Synthesis Kit (invitrogen). Expression of selected genes was analyzed by quantitative RT-PCR performed in triplicates. The reaction was performed on an ABI 7500 Quantitative RT-PCR detection system (BioRad) by using Maxima SYBR Green PCR Master Mix (Thermoscientific, USA) according to manufacturer instruction. Expression of selected genes was normalized against an internal reference gene ubiquitin. The relative gene expression analysis was done by using SDS3.1 software provided by ABI. Level of Significance and standard error was determined by Statistics 8.1 software for Windows.

RESULTS

Effect of CLCuV infection on morphology of plants

CLCuV infection caused changes in morphological characteristics of cotton plants from seedlings to boll formation by inducing typical symptoms under variable temperature. Plants were growing normally in the field and there were no symptoms of CLCuD until the plants were 80 days old having temperature 35°C and RH 70% (Table 1). Morphology of plants was

disturbed after infection caused by CLCuV (Fig 1). The symptoms included: Minor thickening of veins on lower surface of leaves at 15 days post infection (dpi) was observed as in Fig.1 (B), when temperature was 34°C with RH 60%. At 30dpi, thickening of all veins with minor curling of leaves was observed under prevailing temperature of 33°C and 58% R.H. Severe vein thickening, moderate leaf curling, lesser internodal distance and reduction in boll setting was observed at 45dpi when temperature was 26°C and RH was 52%. In our experiment plants infected with CLCuV showed intense symptoms in the month of September to October when the temperature was in the range of 35-24°C and 70-51% RH. Severe vein thickening, leaf curling and enation, stunted growth and reduced boll setting were noted at 60dpi in extreme conditions of infection at 24°C temperature and 41% RH (Table 1).

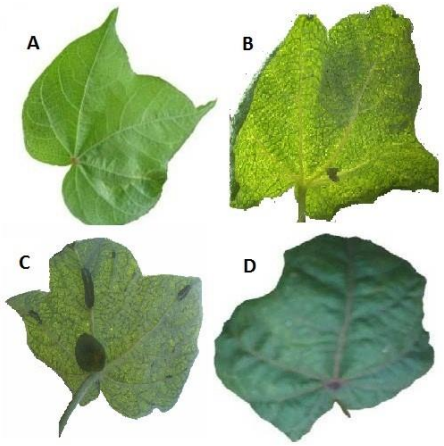

Fig 1. (A) Non-infected (B) Veins Thickening (C) Enations (D) Downward Leaf Curling

Table 1. CLCuD Response of G. hirsutum at different levels of Temperature and Humidity

Plant age (days)	Sampling time	Temp (°C)	Humidity (%)	Symptoms of viral infection on plants	Disease rating response*
80	19 Aug control	35	70	No symptoms of infection	Immune
95	5 Sep 15dpi	34	60	Minor thickening of scattered veins	Resistant
110	20 Sep 30dpi	33	58	Thickening of all veins, minor downward curling of leaves	Moderately resistant
125	5 Oct 45dpi	26	52	Severe vein thickening, moderate leaf curling, reduction in boll setting	Moderately susceptible
140	21 Oct 60dpi	24	41	Severe vein thickening and leaf curling, stunted growth, boll setting reduced	Susceptible

*Proposed by AKHTAR *et al*. (2010)

Confirmation of CLCuV infection through PCR

CLCuV infection in cotton plants was confirmed by PCR amplification using Rep gene at different stages of infection under variable range of temperature and humidity. Amplification of Rep gene of CLCuV in infected samples confirmed the presence of the virus while the non-infected samples did not show amplification of the Rep gene as shown in Fig 2.

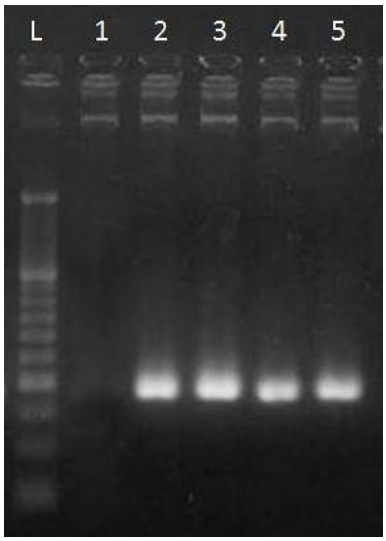

Fig 2. PCR amplification of Rep gene in *G. hirsutum* var. CLCuV FH-142 infected with at different time intervals with variable temperature and humidity levels.
Lane L: DNA ladder 50 bp
Lane 1: Non-infected/control sample
Lane 2-5 Amplification of Rep gene in CLCuV infected samples at 15,30,45,60 dpi respectively

Quantitative RT-PCR analysis of drought stress responsive genes

To explore the response of *G. hirsutum* towards CLCuD, the differentially expressed transcripts were analyzed through quantitative RT-assay at different stages of infection under variable temperature and humidity levels in figure 3.

It was persuaded form Table 2 that there was significant differences found among all the studied treatments for different quantitative gene expression analysis. The relative expression of WRKY gene was enhanced 6.2 folds on onset of CLCuV infection at 15dpi under 34°C with RH 60%. Its expression was gradually lowered down to 3.9 folds as temperature was decreased to 33°C with 58% RH. As the temperature fell down to 26 and 24°C at 45dpi and 60dpi with 52% and 41% RH respectively, infection was increased as observed in the morphological data and gene expression was further reduced.

Relative expression of NAC gene was enhanced to 3 fold on onset of infection when temperature was 34°C with 60% RH. Gene expression remained constant after 15 days at temperature of 33°C and 58% RH. During intense symptoms, expression of NAC was reduced as 2.1 fold to 1.5 fold as temperature was reduced 26°C-24°C with 52-41% RH.

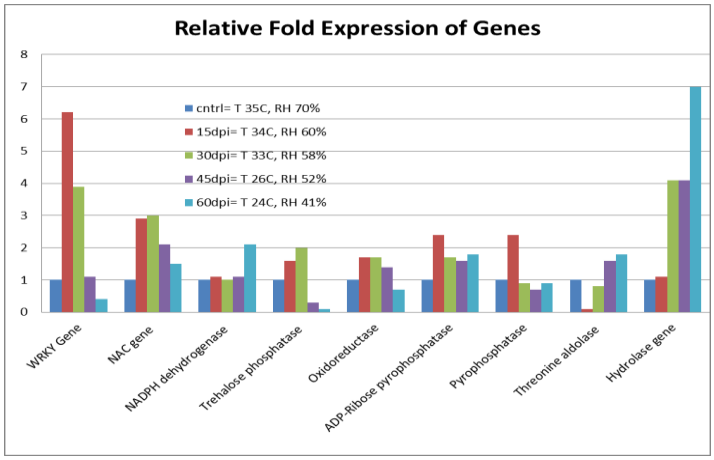

Fig. 3. Quantitative RT-PCR of genes in *G. hirsutum* var. FH-142 infected with CLCuV at different time intervals with variable temperature and humidity levels. Data are mean of biological replicates (n=3). T=Temperature, RH=Relative humidity, dpi=Days post infection.

Table 2. ANOVA for Quantitative RT-PCR of genes

Source	ADP	HG	NAC	NADPH	O	P	TP	TA	WRKY	T	RH%
treat	0.2583*	11.6067*	1.005*	0.6183*	0.4450*	1.2450*	1.440*	1.773*	15.285*	23.546*	76.420*
Error	0.0072	0.0132	0.0212	0.0672	0.0023	0.0541	0.0853	0.0981	0.347	0.541	2.457
Grand mean	1.935	4.110	2.435	1.435	1.421	1.317	1.160	1.060	3.035	30.40	56.20
Standard Error	0.0600	0.320	0.084	0.430	0.056	0.086	0.0270	0.0346	0.0870	1.134	2.543
CV%	4.39	2.06	3.48	5.91	5.34	1.025	7.31	8.00	2.80	4.125	6.324

*=Significant at 5% probability level, WRKY, NAC gene, NADPH dehydrogenase, TP = Trehalose phosphatase, O = Oxidoreductase, ADP-Ribose pyrophosphatase, P = Pyrophosphatase, TA = Threonine aldolase, HG = Hydrolase gene, T=Temperature, RH=Relative humidity

The relative expression of NADPH dehydrogenase gene was enhanced a bit to 1.1 fold at 15 days after germination and almost remained same till 45dpi at 34-26°C and RH 60-52%. The gene was expressed to 2.1 folds in severe stage of infection symptoms under the temperature of 24°C and 41% RH.

The relative gene expression of *Trehalose phosphatase* was increased to 1.6 fold as infection initiated at 15dpi under the temperature of 34°C and 60% RH and enhanced to 2 fold at 30dpi under temperature of 33°C and 58% RH. The fold expression was suppressed at later stages of infestation at 45dpi and 60dpi when temperature was reduced as 26-24°C with RH range of 52-41%, respectively.

The relative gene expression of *Oxidoreductase* was enhanced to 1.7 folds later on onset of infection at 34°C and 60% RH. The expression was then gradually reduced during 30dpi-60dpi when temperature and RH were dropped down as 33-24°C and 58-41%, respectively.

The relative expression of *ADP-Ribose pyrophosphohydrolase* gene was increased 2.4 folds at 15dpi when the temperature was 34°C and 60% RH. The expression was reduced to 1.6 folds at 30dpi in moderate CLCuV symptoms when the temperature was 33°C and 58% RH. The expression remained same at 45dpi, then slightly enhanced to 2 folds at 60dpi when the temperature was 24°C and 41% RH.

The relative gene expression of *pyrophosphatase* was enhanced to 2.4 fold at infection initiation when the temperature was 34°C and 60% RH. Then at 30dpi the level of expression suppressed to 0.9 folds at temperature of 33°C and 58% RH and then it slightly reduced in temperature of 26°C with 52% RH and then minor increased at 60dpi when the temperature was 24°C and 41% RH.

The relative gene expression of *Threonine aldolase* was reduced to 0.1 fold at 15dpi under temperature of 34°C and RH 60% after infection as compared to control plants. Then expression was gradually increased to 0.8 fold at 30dpi when the temperature was 33°C with RH 58%. Expression further increased to 1.6 fold and 1.8 fold in later stages of infection at 45dpi and 60dpi when temperature reduced to 26°C and 24°C with RH 52% and 41% respectively.

The relative gene expression of hydrolase was 1 fold in non-infected stage when the temperature was 35°C and relative humidity was 70%. That was remained same in minor symptoms for infection at 15dpi under the temperature of 34°C and 60% RH. Gene expression was enhanced to 4.1 fold at 30dpi in the temperature prevalence of 33°C and 58% RH, and remained the same at 45dpi under the temperature of 26°C with 52% RH. Expression was enhanced to 7 folds when infection was severe at 24°C with 41% RH.

DISCUSSION

We reported the differentially expressed transcripts in *G. hirsutum* var. FH-142 in response to CLCuV infection under various levels of temperature and humidity. Regulation of WRKY gene WAS upregulated at 15dpi when the plants were 95 day old. Gene expression was found to maximum and according to disease ratings scale, the plants were 'resistant' at that time period (as we correlated to the Table 1). *WRKY* DNA-binding transcription factors are involved in regulating the defense mechanisms in host at various levels: instant modulation of target genes, feed-forward or feedback regulatory loops and association with MAP kinases in nucleus, which comprises key regulators of plant defense responses (POKHOLOK *et al.* 2006; MOHAMED *et al.* 2015). Dubey *et al*. (2013) has reported the expression and regulation of *WRKY* during 2 and 24 hours of aphid and whitefly infestation (DUBEY *et al.* 2013). Our samples showed upregulation of this gene at initial level of CLCuV infection at 34°C and 60% R.H. Later on, its expression was reduced. That predicts WRKY as defense related response which was being suppressed by virulent pathogens for its own development.

Upregulation of *NAC* transcription factor was observed in early stages of CLCuV disease development such as 15 and 30dpi, while its level was reduced later at 45 and 60dpi and the temperature and humidity was reduced. However the expression was 2-3 folds more as compared to the control. The *NAC* genes constitute one of the largest families of plant specific transcription factors (TFs) and are present in a wide range of land plants such as *ANAC102* in *Arabidopsis* (CHRISTIANSON *et al.* 2009), *BnNAC* in *Brassica napus* (HEGEDUS *et al.* 2003) and *OsNAC10* from rice (HU *et al.* 2006). This is similar as the *NAC* TFs have an affirmative role in the regulation of plant defense response against different pathogens as well as wounding and

insect feeding. Increase in the expression level of *NAC* genes has been monitored in response to attacks by several pathogens (LIU *et al.* 2014).

NADPH dehydrogenase is activated in respiration cycle during defense response to pathogen in plants. We observed that *NADPH Dehydrogenase* gene was continued to induce after the onset of infection but was greatly enhanced as the infection progress such as 2 fold at 60dpi. It has been also found the higher expression of *NADPH dehydrogenase* in cotton during different level of infestation by sap sucking insects (DUBEY *et al.* 2013). The expression was gradually enhanced by severe CLCuV development under different level of temperature 24-34°C and RH 40-71%. This may be due to the adaptation of plants to adjust the signaling pathways to use them as defense response (O'BRIEN *et al.* 2012). Hence, *NADPH oxidases* centralized at plasma membrane are significant to control biological developments in response to environmental challenges (TORRES *et al.* 2013).

Expression of *Trehalose phosphatase* gene was expressed up to 2 fold higher after 15 and 30dpi of infection when temperature and humidity was decreasing. Trehalose is a non-reducing disaccharide composed of two glucose residues in plants. Our results correlate to the findings (GÓMEZ-ARIZA *et al.* 2007) that the accumulation of TPP has been increased with a certain level of pathogen infection suggesting that plants are capable to restrain sugars as source of carbon and energy which will act as signals or recognized to increase the resistance against infections (MOGHADDAM and VAN DEN ENDE 2012; KHAN *et al.* 2015).

Oxidoreductase are enzymes that catalyze the transfer of electrons from one molecule, the oxidant, to another, the reductant (THAKUR and SOHAL 2013). Our results indicate more than 1.5 fold expression of *oxidoreductase* gene after 15 and 30dpi and that was started to suppress after 45dpi when the temperature and humidity was reduced. That supported the evidence that *oxidoreductase* was positive regulator of plant susceptibility to CLCuV development. While downregulation in later stages predicts that it might be due to expression of defense related genes in response to CLCuV. This leads to the conclusion that disengagement, or inactivation of the photosystem machinery linked to the functions of chloroplast and peroxisome may accumulate the higher levels of ROS affecting the cellular homeostasis (LIAO *et al.* 2014).

Findings of our results demonstrated the differential expression of *ADP-Ribose pyrophosphatase* after the development of CLCuV infection under variable range of temperature and humidity. *ADP-Ribose Pyrophosphotase* is reported as physiological substrate for Nudix Hydrolases (GE and XIA, 2008) that showed several function: redox homeostasis, energy production and responsive during environmental stresses (ISHIKAWA *et al.* 2010). The expression of *Pyrophosphatase* gene was highly expressed over 2 fold as compared to the control at 15dpi and suppressed after that suggests the upregulation leads to accumulation of higher level of soluble sugar that causes induction of PR proteins against pathogens. Evidences suggest that the higher levels of soluble sugars will reduce the sucrose synthesis, increase the starch accumulation and turgor pressure and finally enhance the pathogen related protein expression in certain plants (CHEN *et al.* 2003; AMIN *et al.* 2014).

We observed that *Threonine aldolase* level was enhanced during intense symptoms of CLCuV development at 45 and 60dpi as the temperature and RH was reduced to 26-24°C and 52-41% respectively. This is similar to the observations (DUBEY *et al.* 2013) that the level was enhanced after 24 hours of whitefly infestation. This is correlated with the pathogen infestation that causes the changes in *threonine aldolase* at transcriptional level thus alters amino acids metabolism and reroute its transportation (GIORDANENGO *et al.* 2010). *Hydrolase* related gene

showed maximum upregulation 7 fold than the control at 60dpi at 24°C and RH 41%. Similarly gene was expressed 4 fold than the control at 33-26°C and 58-52% RH respectively. *Hydrolases* are reported as one of major group consists of upregulated genes expressed in response to stress signals after the attack of sap sucking insect. They hydrolyse glucosinolate, which produce toxic compounds and their transportation to phloem lead to the lethality of the sap sucking insects (RADOJČIĆ REDOVNIKOVIĆ *et al.* 2008). The *hydrolases* is also reported in *Brassica oleracea* and *P. rapae* after infestation (BROEKGAARDEN *et al.* 2007).

CONCLUSIONS

The transcripts analysis in local variety of *G. hirsutum* under CLCuV infection showed the regulation of differential expression of defense responsive genes under variable range of temperature and humidity. These findings will be useful to understand the mechanism involved in CLCuV infection and will pave the way for new pathogen management strategies.

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EKSPRESIJA GENA U ODGOVORU NA INFEKCIJU VIRUSOM UVIJENOSTI LISTA PAMUKA *Gossypium hirsutum* U RAZLIČITIM USLOVIMA SPOLJAŠNJE SREDINE

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Izvod

Lisna bolest pamuka (CLCuD) je jedna od pretećih ograničenja proizvodnje pamuka u Pakistanu za koju do sada nije dostupan odgovarajuće sredstvo. Lokalna sorta *Gossipium hirsutum* (FH-142) je gajena u polju i inficirana prirodnim putem CLCuV pod varijabilnim opsegom temperature i vlažnosti. Biljke su pokazale zgušnjavanje vena na površini donjeg lista na 34 ° C i 60% relativne vlažnosti, 15 dana nakon infekcije (dpi) i uvijanje margina lista na 33 ° C sa 58% relativne vlažnosti pri 30dpi. Značajno zatamnjenje listova primećeno je sa smanjenim formiranjem bola na 45dpi pri 26 ° C i 41% relativne vlažnosti. Razvijena je enacija, intezivirano zgušnjavanje i uvijanje lišća i biljke pokazuju patuljast rast na 60dpi na 24 ° C sa 52% relativne vlažnosti. PCR amplifikacija Rep-povezanog gena potvrdila je prisustvo begomovirusa pridruženog CLCuD-u u zaraženim uzorcima. Kvantitativni RT-PCR potvrdio je amplifikaciju i diferencijalnu ekspresiju gena koji odgovaraju gustom stresa na različitim nivoima temperature i vlažnosti. Ova opservacija predviđa da virus uvijenosti lista pamuka (CLCuV) intereaguje sa nekoliko domaćih gena koje su regulisane kako bi biljke postale osetljive ili suzbile druge gene kako bi prevazišle odgovore odbrane domaćina.

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