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Short Article

EFFECT OF TEMPERATURE ON THE INFECTION OF SUGAR BEET PLANTS BY BEET SEVERE CURLY TOP VIRUS AND ON RECOVERY OF VIRUS-INFECTED PLANTS *

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Abstract

The effect of various temperatures on the infection of sugar beet plants by Iranian isolate of Beet severe curly top virus (BSCTV-IR) was evaluated. Presence of BSCTV-IR in agroinoculated plants was assayed at 7, 14 and 21 days post-inoculation (dpi) using PCR. Based on the infectivity assay, the optimum temperature for BSCTV-IR infection was 25°C at which the mean period between inoculation and the first virus detection was the shortest and the virus concentration was the highest. No virus was detected in plants incubated at 35°C until 21 dpi. To evaluate the effect of temperature on recovery of BSCTV-IR-infected plants, symptomatic plants were incubated at the aforementioned temperatures. The first new leaf showing recovery phenotype emerged 7 dpi at 35 ºC. This period for plants incubated at 30, 25-30, 25 and 20ºC was 12, 18, 25 and 28 days post-incubation, respectively. Thus, recovery occurred faster as the temperature raised. Re-inoculation of recovered plants with the same virus induced no symptoms in newly emerging leaves, indicating the stability of the phenotype of recovered plants against re-infection by the same virus. Quantitative analysis using real-time PCR showed significant decline in viral DNA in either recovered or new leaves emerged after re-inoculation compared to symptomatic leaves.

Keywords: *Beta vulgaris*; Geminivirus, Recovery phenotype, Resistance, Temperature.

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Introduction

Curly top is a devastating sugar beet disease caused by a number of closely related virus species in the genus *Curtovirus,* family *Geminiviridae* (Stanley *et al*., 2005). Typical curly top symptoms are leaf curling, vein swellings and outgrowths (enations) on the lower leaf surface, upright standing of leaves, phloem necrosis and decreased plant growth (Sutic *et al*. 1999).

Symptom remission or recovery of curly top disease in sugar beet and other host plants has been known for many years (Bennett, 1971; Benda and Bennett 1967). The recovered plants showed disappearance or attenuation of symptoms on newly emerged leaves. The rate of recovery is influenced by the variety of the host, the severity of the virus strain and the environmental factors affecting the rate of development of both plant and virus (Benda and Bennett, 1967; Chellapan *et al*., 2004; Chellapan *et al*., 2005). Chellapan *et al*. (2005) demonstrated that symptoms are less severe and virus titer is lower in geminivirus-agroinfected plants incubated at higher temperatures. Meanwhile, geminivirus-induced RNA silencing increased by raising the temperature from 25°C to 30°C. Recovered tissues are resistant to super-infection by the same virus but are susceptible to infection by unrelated viruses (Carrillo-Tripp *et al*., 2007; Hagen *et al*., 2008). Despite these studies, there is little information on the effect of temperature on viral infection and recovery phenotype in BSCTV-infected sugar beets. The goals of the present study were to characterize the recovery phenomenon on BSCTV-infected sugar beet plants through comparative analysis of viral DNA in symptomatic and recovered tissues and to examine the effect of temperature on the virus infection and recovery of the virus-infected plants.

Materials and Methods

Sugar beet (*Beta vulgaris* L.) var. Zarghan was chosen in this study due to its susceptibility to BSCTV and production of typical symptoms. The inoculum was an infectious clone of the Iranian isolate of BSCTV (BSCTV-IR, Acc. No.: X97203) (Ebadzad Sahraei *et al*., 2008). *Agrobacterium tumefaciens* (strain C58) cultures harboring the infectious construct pBin-1.7BSCTV-IR (Ebadzad Sahraei *et al*., 2008) were grown at 28ºC for 24 hr and diluted to a concentration of optical density of 4.5×10^7 cell ml^{-1}. Sugar beet seedlings at 4-6 leaf stage were inoculated by injection of 50 µl of diluted culture into the crown of each plant. The bacterium harboring intact pBin20 vector was used in mock inoculation.

The agroinoculated plants were kept in four growth chambers with constant temperatures of 20, 25, 30 and 35ºC, 18/6 light/dark photoperiod, and 50% relative humidity. Nine plants were used per treatment. Disease symptoms appearance was evaluated at 7, 14, 21 and 30 days post-inoculation (dpi) and the presence of viral DNAs in the inoculated plants was analyzed by PCR at symptom evaluation dates. The number of days between the date of virus inoculation and the first virus detection was recorded.

DNAs were extracted from inoculated plants using cethyltrimethylammonium bromide (CTAB) solution as described previously (Gawel and Jarret, 1991) and were subjected to PCR analysis using two specific BSCTV-IR primers, p358V (5′-GTGGATCAATTTCCAGACAATTATC-3′) and p853C (5′-CCCCATAAGAGCCATATCAAACTTC-3′) (Ebadzad Sahraei *et al*., 2008), designed to amplify a 519 bp fragment of coat protein (CP) of BSCTV-IR.

In recovery experiment, the sugar beet plants showing typical disease symptoms were transferred to growth chambers with aforementioned conditions. A similar set of symptomatic plants were also kept in the greenhouse at 25-30ºC. The intensity of viral symptoms was evaluated on previously symptomatic leaves present before treatment and the newly-emerged leaves and scored as 0 (asymptomatic), 1 (vein clearing), 2 (vein clearing and leaf roll), 3 (enation and leaf roll) and 4 (enations, leaf roll and reduced growth). The presence of viral DNAs in incubated plants were analyzed on symptomatic (S) and recovered (R) leaves at distinct times after incubation by PCR as described above. To determine whether the recovery phenotype could be reversed (i.e., back to the symptomatic phenotype), the recovered plants were re-agroinoculated with the same virus and incubated at the same temperatures. The presence of viral DNAs was analyzed again on already existing S and R leaves and on newly-emerged leaves after re-inoculation (N) 30-35 days post-re-inoculation.

Real-time PCR was used to quantify the viral DNA in S, R and N leaves of re-inoculated sugar

Table 1. Number of BSCTV-infected plants determined by PCR assay of 9 agroinoculated sugar beet plants incubated at indicated temperatures at 7, 14 and 21 days post-inoculation.

Temperature (ºC)	Days post-inoculation		
	7	14	21
	Number of infected plants		
20	0	4	5
25	5	8	9
30	2	6	7
35	0	0	0

beet plants. The S, R and N leaves of five plants from each treatment (20, 25, 25-30, 30 and 35ºC) were dissected and subjected to total DNA extraction as described above and incubated with 50 µg μL^{-1} RNase A (Roche, Germany) at room temperature for 1 h. Real-time PCR was performed in BIOER Line-Gene.K thermocycler using Bioeasy® Real-Time PCR Kit (BIOER, China), with a final concentration of 10 mM fluorescin according to supplier's recommendations. Each 25 µL of reaction mixture contained 2 µL (1.6 ng) of DNA, 4 µM of each primer (see below), 10 µL of 10× Supermix (BIOER) and 1 U of *Taq* DNA polymerase. The resulting data were analyzed according to the method described by Livak and Schmittgen (2001). The primers used for real time PCR consisted of pBSCTV-IR358V (5′-GTGGATCAATTTCCAGACAATTATC-3′) and pBSCTV-IR565C (5′-CATACAACGAACACTTCCTTT-3′) designed to amplify a 206 bp DNA fragment of the viral CP gene. For internal normalization two primers, pEF2-F (5′-GCCTGCAGGTCGACACTAGT -3′) and pEF2-R (5′- CTTCACCATTTTCGCAGCTG-3′), were designed from eukaryotic elongation factor 2 (eEF2) of *B. vulgaris* to amplify a 114 bp DNA fragment from the beet eEF2.

Results and Discussion

The typical disease symptoms appeared within approximately 30 dpi in plants under greenhouse conditions (25-30ºC). However, the appearance of symptoms on inoculated plants maintained at different temperatures was variable (Table 1). At 25ºC the highest ratio of infected plants (5 out of 9 inoculated plants) was found at 7 dpi. It was also the only temperature at which nine plants were found infected at 21 dpi (Table 1). The mean number of days between inoculation and detection of the virus in sugar beet plants at 20, 25, 30 and 35ºC was 17.87, 10.88, 14.75, and 28 days, respectively. Similarly, treatment of 25ºC resulted in the shortest latent period. It was previously shown that temperature as an environmental factor can significantly affect the kinetics of virus replication (Ghosh and Bhattacharyya, 2007). Our results also showed that as the temperatures increased, symptom development in plants slowed down, and no viral infection was detected in plants incubated at 35ºC even 21 dpi (Table 1).

The recovery phenotype was observed in BSCTV-infected plants. The first new leaf showing recovery phenotype (R1, Fig. 1) emerged 7 days after incubation on the plants maintained at 35ºC. The recovery phenotype of this leaf was distinguishable 21 days post-incubation. Subsequent leaves (R2 and R3, Fig.1) were also symptomless. The first new leaf showing recovery phenotype emerged 12, 18, 25 and 28 days post-incubation on plants maintained at 30, 25-30, 25 and 20ºC, respectively. These results showed that the recovery phenotype appeared faster as the temperature was raised (Fig. 2) indicating the effective role of temperature in this phenomenon (Chellapan *et al*., 2005). All recovered plants were re-inoculated with the same virus to determine whether the recovery phenotype could be reversed. Following re-inoculation, no symptoms developed on newly-emerged leaves (Fig. 1. leaf N) 30-35 dpi, or over the duration of the experiments (45 dpi) regardless of the temperature they were incubated at, thus confirming the efficacy of virus resistance mediated by the recovery phenomenon. On the other hand, the control plants which were first mock-inoculated and, in the second stage, BSCTV-IR-inoculated did develop typical symptoms. This indicated that the control plants which were not

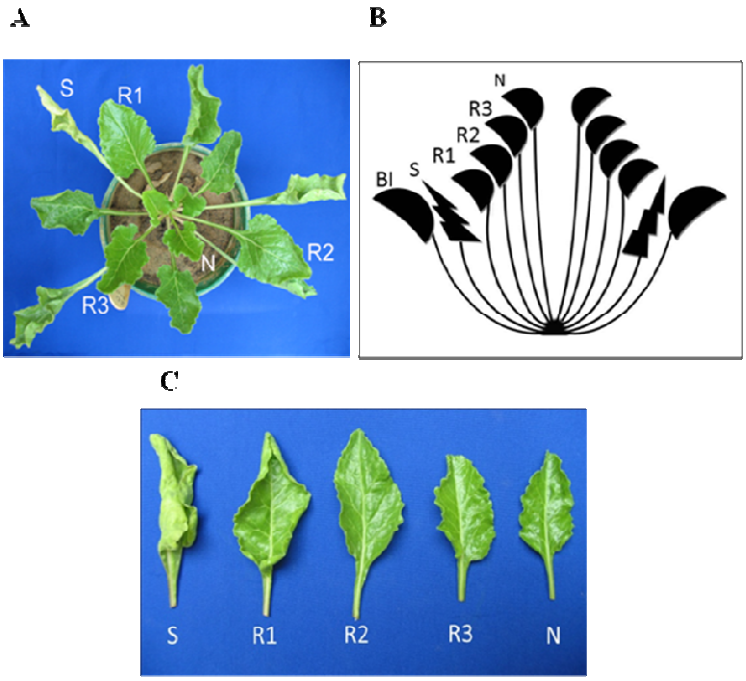

Fig. 1. (A) a BSCTV-infected sugar beet plant incubated at 35ºC, recovered from viral infection and re-inoculated with the same virus. (B) schematic view of leaf positions on the plant. (C) Phenotype of different leaves. B1, symptomless primary leaf before inoculation; S, symptomatic leaf; R1-R3, recovered leaves; N, newly-emerged leaf after re-inoculation. The first new leaf showing recovery (R1) phenotype was emerged 7 days post-incubation.

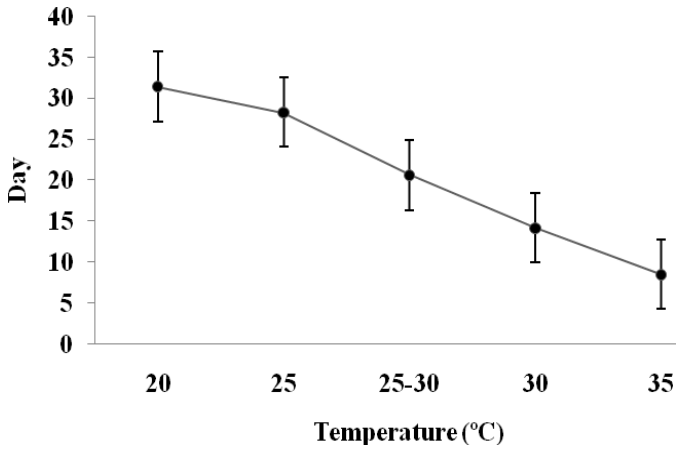

Fig. 2. Mean length of time before the onset of recovery in BSCTV-infected sugar beet plants incubated at various temperatures. Bars indicate standard error values of 9 plants.

recovered from the virus infection and contained no viral DNA were susceptible when inoculated with BSCTV-IR at a relatively old age confirming that the stability of recovery observed in BSCTV-IR-infected plants was not due to the age of plant or its developmental stage. Also, re-inoculation of recovered plants resulted no symptoms in the newly-emerged leaves (N leaf, Fig. 1) suggesting the resistance of recovered plants against re-infection by the same virus (Hagen *et al*., 2008). Regular PCR showed that the viral DNA was present in S, R and N leaves (data not shown).

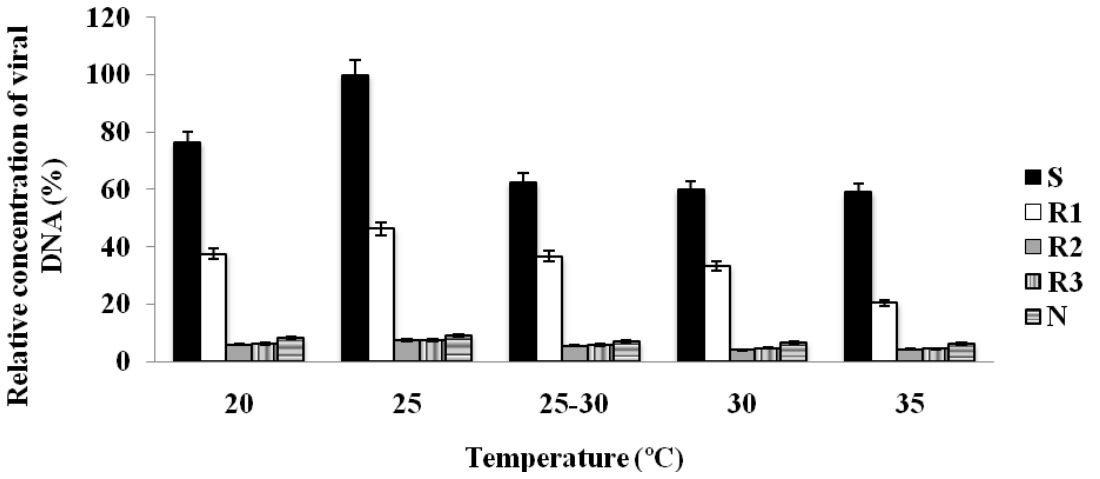

Fig. 3. Relative viral DNA concentration (determined by real-time PCR) in different leaves of BSCTV-infected plants incubated at 20, 25, 25-30, 30 and 35ºC, recovered from viral infection and re-inoculated with the same virus. S, symptomatic leaf; R1-R3, recovered leaves; N, newly-emerged leaf after re-inoculation. Each bar corresponds to the mean value from five plants; maximum value was taken as 100. Error bars refer to standard error of the mean.

However, the intensity of the amplified viral DNA bands visualized in agarose gel electrophoresis and the concentration of viral DNA estimated by real-time PCR in R or N leaves was significantly lower than that of symptomatic S tissues (Fig. 3). Furthermore, the relative concentration of viral DNA of plants incubated at higher temperatures (30 and 35ºC) was lower than that of plants incubated at lower temperatures (Fig. 3). The deteriorative effect of high temperature on the concentration of BSCTV-IR DNA was consistent with the results of Chellapan *et al.* (2005) who observed that concentration of geminivirus DNAs in infected cassava plants at 30ºC was low. Low content of viral DNA in recovered tissues may be the result of RNA silencing machinery activation in plants challenged by viral infection (Carrillo-Tripp *et al*., 2007). Nevertheless, it should be taken into consideration that RNA silencing is not always associated with reduced virus concentration in recovered tissues (Jovel *et al*., 2007). The results of present study showed that temperature does not only affect the development of recovery phenotype in BSCTV-IR-infected sugar beet plants, but high temperature also causes considerable decline in amount of viral DNA in both symptomatic and recovered leaves. Presence of siRNAs as hallmark of RNA silencing should be tested in recovered plants in further experiments.

The ability of BSCTV-infected plants to recover could be used to control the devastating curly top disease. Similarly, the cultivation of plants with genotypes that can rapidly develop the recovery phenotype should be taken into consideration.

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