

## **INFLUENCE OF SOME FACTORS INVOLVED IN GEMINIVIRUS-PEPPER PLANT INTERACTION.**

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### **ABSTRACT**

The effect of some epidemiological factors, such as inoculation with different amounts of viral inoculum of the geminivirus Pepper Huasteco Virus (PHV) and pepper plant phenological stages when is inoculated, was evaluated. For the first factor four viral DNA doses 1, 2, 10 and 15 µg, are corresponding to  $1.565 \times 10^{14}$ ,  $3.13 \times 10^{14}$ ,  $1.565 \times 10^{15}$  y  $2.347 \times 10^{15}$  viral genomes respectively. Second factor included inoculation with 10 µg of viral DNA in the next phenological stages in the plants: 4-8 true leaves, 40 days after transplanting, anthesis or pre-flowering stage and first fruit setting.

These trials were carried out at greenhouse conditions (26-28°C) with four families of peppers; Ancho, Pasilla, Habanero and Sonora Anaheim. Experimental design was a completely randomized. The variables measured were infectivity, syndrome severity and amount of viral DNA in the plant. DNA amplifications by PCR were done to confirm the virus presence and ELISA tests to determine viral concentration. The strongest severity on the syndrome expression occurred on inoculated plantlets with 4-8 true leaves; and the highest viral concentration was observed on anthesis stage plants inoculated with 2 µg viral DNA ( $3.13 \times 10^{14}$  viral genome).

**Keywords:** Geminivirus, amount of viral inoculum, pepper, epidemiology, phenological stage.

### **INTRODUCTION**

It has been known since mid 1980's that in Mexico Solanaceas are infected by geminivirus, which are transmitted by white flies (*Bemisia tabaci* Genn. o *B. argentifolii* Bellows and Perring) (Garzón-Tiznado *et al.*, 1993; Torres-Pacheco *et al.*, 1996b). Geminivirus are characterized for having one or two single circular DNA chain genome molecules with a size of 2.5 and 2.9 kb each.

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A geminivirus looks like two identical regular polyhedrons fused by one of their sides (Lazarowitz *et al.*, 1992). Geminivirus are classified in four genera Mastrevirus, Curtovirus, Begomovirus y Topocuvirus, according to the transmittal vector, the host plant and their genome organization. It is estimated that the losses caused by geminivirus diseases at the mexican altiplane can be up to 40% in pepper (Garzón-Tiznado *et al.*, 2002).

In Mexico, the diseases associated to this type of virus have been called “chino” of tomato in Sinaloa and atigrada pepper plant in Puebla. Garzón-Tiznado *et al.* (1993) and Torres-Pacheco *et al.* (1993) established the etiology at molecular level of the geminiviral disease “rizado amarillo” in pepper crop south Tamaulipas. Geminiviruses such as Chino of tomato (CdTV), Pepper Huasteco Virus (PHV) and Peppper Golden Mosaic Virus (PepGMV, former Texas Pepper Virus TPV) have been involved in this type of diseases (Garzón-Tiznado *et al.*, 1993; Torres-Pacheco *et al.*, 1996).

In order to rationally control these pepper viral diseases, epidemiologic analysis followed etiology studies to establish the bases to obtain resistant varieties (Garzón-Tiznado *et al.*, 1993; Méndez-Lozano *et al.*, 2003 Godínez-Hernández *et al.*, 2001; López-Anaya *et al.*, 2003a; López-Anaya *et al.*, 2003b).

Therefore, efforts have been oriented to determine the geminivirus distribution in productive regions in México (Torres-Pacheco *et al.*, 1996), alternative host plants (Garzón-Tiznado *et al.*, 2002) and even the interaction between geminiviruses (Torres-Pacheco, 1997; Méndez-Lozano *et al.*, 2003).

However, it is necessary to complement the information related to the conditions for the infection process, the virus amount used to inoculate or transmit, and the plant phenological stage when it is more susceptible to infection or when the disease is more severe. Among the most conspicuous geminivirus that attack pepper in México is the PHV (Torres-Pacheco *et al.*, 1996), which was first reported in 1993 by Garzón-Tiznado *et al.* This virus belongs to the Begomovirus genus, which is transmitted by the white fly, infecting dicotyledonous and its genome is formed by two molecules.

However, some critical factors to assess damage severity remain to be defined. Because of this, the work was focused on evaluation of the effects associated with disease severity such as amount of viral inoculum and the plant age at the time of infection. An intuitive supposition that the more developed is the plant, the more resistant is to any stress (Balsubrahmanyam *et al.*, 2000) remains to be proven. It was reported that tomato inoculated with TSWV was differentially affected in both disease incidence and yield, this was also observed in transgenic plants.

The objectives if this work were to evaluate the effects of amount of viral inoculum and plant phenology on the syndrome development in pepper plants inoculated with Pepper Huasteco Virus (PHV).

## MATERIALS AND METHODS

Factors evaluated consisted of amount of viral inoculum (AVI) 1, 2, 10 and 15 µg of viral DNA, ( $1.565 \times 10^{14}$ ,  $3.13 \times 10^{14}$ ,  $1.565 \times 10^{15}$  y  $2.347 \times 10^{15}$  viral genomes) and plant phenological stages (PPS) at the inoculation point (plantlets 4-8 true leaves, 30-40 days after transplant, anthesis and initial fruit set).

Experimental design consisted in a completely randomized design with 5 plantlets per experimental unit and three replicates. Treatments design linked each experiment in Baconian way, keeping one factor constant in each treatment analysis. ANOVA Fisher was made to determinate factor significance and statistics differences among doses and phenological stages were determined by Tukey's standardized range test. Variables studied were Infectivity, according to percentage of infected plants per genotype, Disease Severity, using a pre determined scale (Torres-Pacheco *et al.*, 1997; Godínez-Hernández *et al.*, 2001). Viral Presence, by immunoenzymatic analysis (ELISA using the AGDIA protocol), and PCR (Mullis and Faloona 1987).

With PCR, PHV common region was amplified (aprox. 200 bp) to confirm presence or absence of PHV viral DNA (denaturalization 94 °C 1 min; Annealing, 55 °C 1 min; extension 72 °C 2 min for 35 cycles). Primers used were 240 and 241 (Torres-Pacheco *et al.*, 1996a). Virus inoculation was performed by biolistics (Garzón-Tiznado *et al.*, 1993) and plant DNA extraction using Dellaporta protocol (1983). In this work were used next genotypes ancho 30-15, pasilla Sel 12, Sonora Anaheim and habanero UX-SMH-55. Conditions in greenhouse were at 25-30 °C.

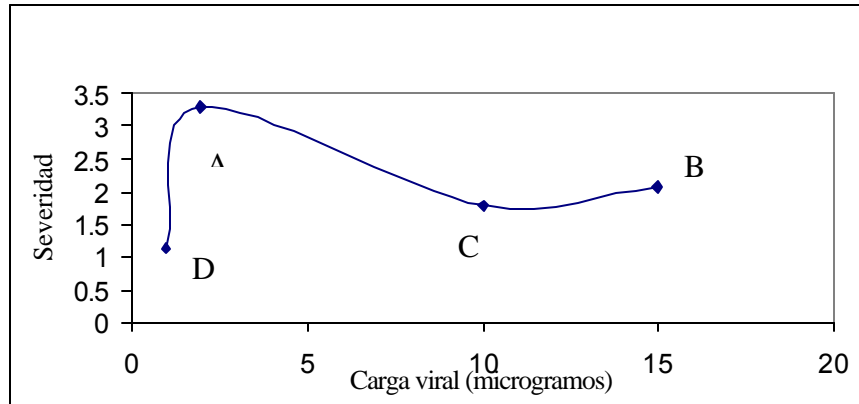
## RESULTS AND DISCUSSION

Amount of Viral Inoculum Effect. Evaluation of infectivity, disease severity and viral concentration were done on inoculated plants. A) Infectivity. Inoculation with 2 µg of viral DNA ( $3.07 \times 10^{14}$  viral genomes) induced the strongest infection (59.3%) as opposed to 10 µg (29%) and 15 µg (20%), in which it was expected a higher infection rate because of the number of viral genomes ( $1.565 \times 10^{15}$  and  $2.312 \times 10^{15}$ , respectively). Inoculation with 1 µg viral DNA caused less infection rate than 2 µg, but more than 10 µg or 15 µg (Table 1)

**Table 1. Effect of amount of viral inoculum on pepper infectivity.**

Amount of viral inoculum (µg)	Number of viral genomes	Infectivity (%)
1	$1.541 \times 10^{14}$	31.2
2	$3.07 \times 10^{14}$	59.3
10	$1.541 \times 10^{15}$	29.0
15	$2.312 \times 10^{15}$	20.0

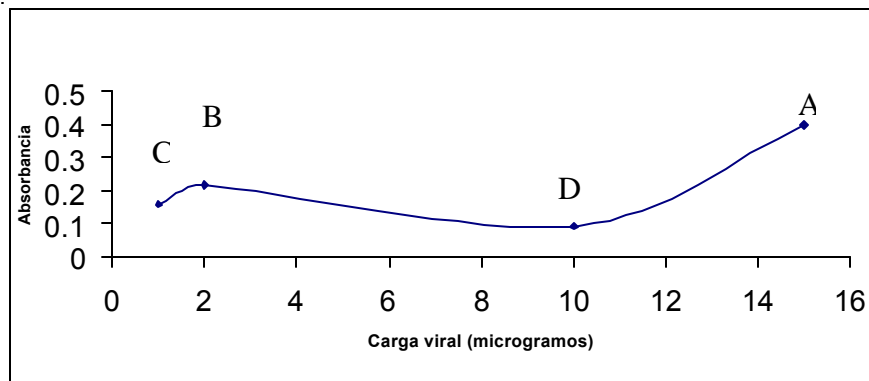
Geminiviruses are multiplied in the host nucleus. Gene function, expression and regulation are dependent on host cell DNA, RNA polymerase, transcriptional factors, etc. This implies a high dependence to the equivalent host processes (Torres-Pacheco, *et al.*, 1996a). Therefore, the results shown above might imply that at high concentrations (10  $\mu\text{g}$  and 15  $\mu\text{g}$ ) a competition for the nuclear membrane and plasmodesmata entrance sites may occur. Different letters indicate significant statistical difference at  $\alpha=0.01$ , C.V.=20.03%



**Figure 1. Disease severity on pepper populations inoculated with amounts of viral inoculum.**

It is possible that in such competition, viral molecules interfere with one another having a low efficiency in the interaction virus-cell as a result. On the other hand, 1  $\mu\text{g}$  inoculum would be extremely low concentration and therefore no infectivity could take place. It could also be a phenomenon more related to the amount of inoculum carried on membrane making it glutinous and less effective at the biolistics inoculation implementation.

B) Plant Disease Severity. The strongest syndrome severity was observed at 2  $\mu\text{g}$  concentration followed by 15  $\mu\text{g}$ , 10  $\mu\text{g}$ , and 1  $\mu\text{g}$  and the lightest at 1  $\mu\text{g}$ , and no difference was observed at 1 and 10  $\mu\text{g}$  (Figure 1). A possible explanation might be similar to infectivity results as viral genomes competition.



**Figure 2. Viral concentration in plants inoculated with different amounts of viral inoculum.**

However, 1 µg might infect adequately unless we are dealing with a very low concentration. There was no correlation between the viral amount and disease severity in the incubation conditions of inoculated plants. On this regard, in natural conditions few vectors may infect an important amount of inoculum or, on the other hand, many vectors may be innocuous (Mann, *et al.*, 1997). Different letters indicate significant statistical difference at  $\alpha = 0.01$  y C.V = 18.17%

C) Viral concentration in inoculated plants. Plants inoculated with 15 µg viral DNA had the highest viral concentration followed by 2 µg viral DNA. These results appear to oppose to traditional experiments where 10 µg was the concentration used to inoculate pepper plants (Garzón-Tiznado, *et al.*, 1993; Godínez Hernández *et al.*, 2001., Anaya López *et al.*, 2003), however, in our experiments 10 µg resulted to be less efficient than 2 µg viral DNA. Interestingly, these data do not correlate to the severity symptoms where 2 µg viral DNA produced higher efficiency in disease severity followed by 15 µg viral DNA. Apparently, 2 µg was more efficient in mechanical terms per event or viral cell, and 15 µg was more efficient in terms of number of inoculated events (Figure 2).

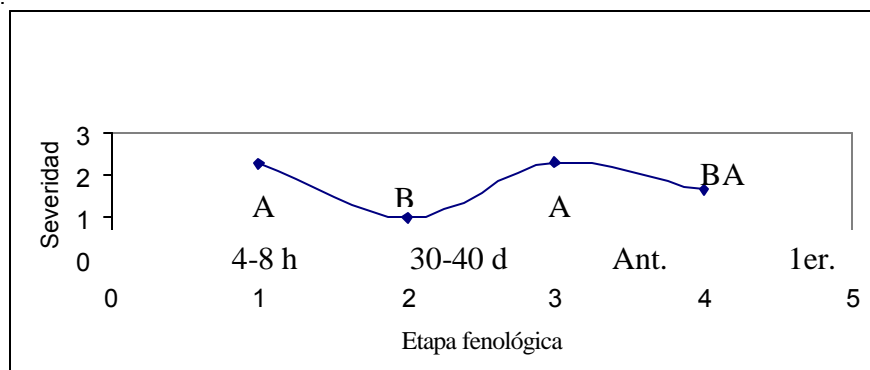
Effect of phenological stage. A) Infectivity. During anthesis stage it was observed more infected plants inoculated with 10 µg viral which was constant in all phenological events (Table 2).

This could obey to the amount of meristems, and the flowering and vegetative buds. In other words, at this stage most of the plant cells are at the S phase and anthesis and fructification stages have more meristematic points than any other stage and therefore, the viral DNA has better chance to get in specific sites during cellular division and so to increase plant infection probability.

**Table 2. Infectivity on inoculated pepper plants with PHV (10 µg viral DNA) at different phenological stages.**

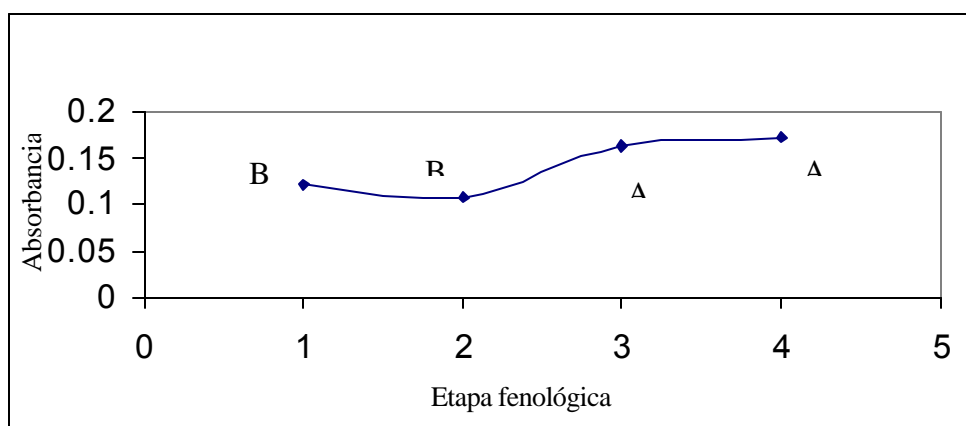
Phenological stage	Infectivity (%)
4-8 true leaves	37.5
30-40 days after transplant	25.0
Anthesis	71.4
Initial fruit set	44.8

B) Plant Symptoms Severity. Plantlets with 4-8 true leaves showed more severity, however there was no statistical difference respect to anthesis (Figure 3). Different letters indicate significant statistical difference at  $\alpha = 0.01$  y C.V.= 20.03%



**Figure 3. Disease severity on pepper plants at different phenological stages.**

According to Torres Pacheco *et al.*, (1996a) plantlets would be induced to phase S in tissues where normally this does not occur, producing form and functional disturbs. In regard to anthesis, where there are numerous growth points (cells in S phase), plants get infected accordingly, and consequently the rate of replication causes disturbance because of photosyntates reduction (Godínez Hernández *et al.*, 2001). Different letters indicate significant statistical difference at  $\alpha=0.01$  y C.V=35%

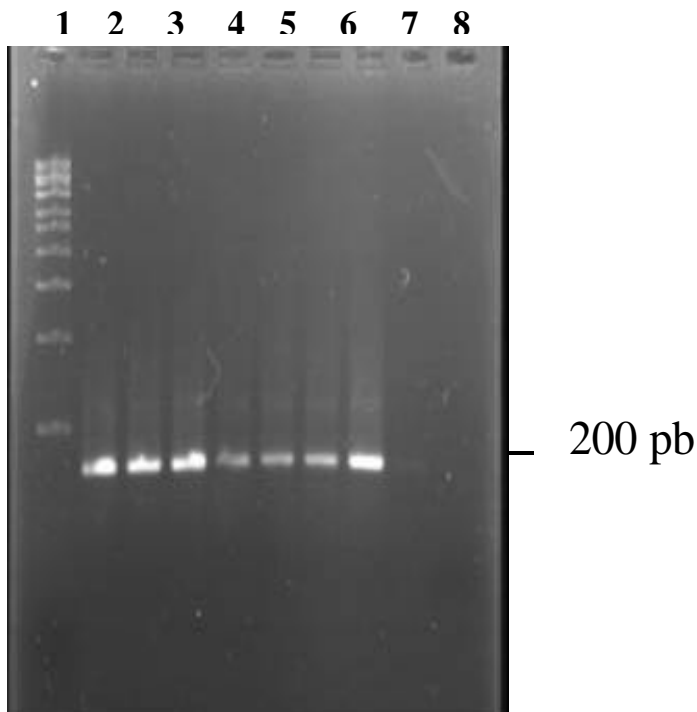


**Figura 4. Viral concentration in pepper plants at different phenological stages.**

C) Viral concentration in inoculated plants. It was observed that the amount of virus was significantly favored at anthesis stage and the beginning of fruit setting in pepper plant (Figure 4).

This is consistent with the idea that there is a considerable amount of meristematic cells in this stage such as flowering and vegetative buds, having the conditions for virus replication during S phase (Accotto *et al.*, 1993).

It is worth to notice that in all three variable responses (infectivity, plant symptoms severity, and viral concentrations) anthesis stage was the best plant stage for the viral syndrome development. The viral viral detection was confirmed with PCR (Figure 5).



**Figure 5. PCR amplification of the Pepper Huasteco Virus in apical leaves from pepper plants inoculated via biolistics and showing symptoms xx days after inoculation. Lane 1: xx bp ladder; Lane 2: Positive control PHV; Lane 3: Pepper plant with rate 5 severity; Lane 4: Pepper plant with rate 4 severity; Lanes 5 and 6: Pepper plant with rate 3 severity; Lanes 7 and 8: Pepper plant with rate 2 severity; Lane 9: Pepper plant with rate 1 severity; Lane 10: Negative control. Lanes 6 and 8: *C. chinense*. Lanes 3, 4, 5, 7, and 9: *C. annuum*. Arrow indicates 200bp PHV fragment**

## CONCLUSIONS

The amount of viral inoculum influence the infectivity, symptoms severity and virus amount in the plant. For practical terms the expression of these variables are induced at higher levels at 2 µg viral DNA ( $3.13 \times 10^{14}$  viral genomes).

Susceptible to infection in terms of severity occurred at plantlet and beginning of flowering stages. The main infection and inoculum propagation risks were observed when the plant started the anthesis stage.

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