

## Susceptibility of *Vasconcellea cauliflora* to Papaya ringspot virus.

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### Abstract

This research proved the susceptibility of *Vasconcellea cauliflora*, a specie considered as a virus resistant to *Papaya ringspot virus* (PRSV-P). The transmission of the disease was done by mechanical inoculation of a severe strain PRSV-P on 60 days old plants. Under greenhouse conditions with average values of 25.15°C and 78.68% RH, 8.3% of plants inoculated with the severe strain showed apical necrosis. At la Facultad de Agronomía (UCV), Maracay (440 msnm) under field conditions, 8.6% of inoculated plants showed typical symptoms of the disease seven days after being transplanted. Subsequently, an inoculation test was done in three growth chamber conditions: T1=29°C and 6300 lux; T2= 27°C and 5500 lux and T3= 25°C and 4200 lux; the daily time was fixed at 16 h, and the relative humidity was about 75.5%. Systemic symptoms were observed in 82, 86 and 47% of plants in treatments T1, T2 y T3 respectively. These results were consistent with the ELISA test. The susceptibility of *V. cauliflora* to the local severe strain of PRSV-P was influenced by temperature and light intensity conditions. This question the role of this species in the search for PRSV-P resistance genes in papaya breeding programs.

**Key words:** *Vasconcellea cauliflora*, virus resistance, PRSV-P, ELISA.

## Introduction

Worldwide the illness produced by the virus of the ringspot of papaya (PRSV-P) constitutes the most important obstacle by the destructive effects caused in the production of papaya (*Carica papaya* L.). PRSV-P is a *Potyvirus* transmitted by aphids in a non persistent way, which produces mosaic, chlorosis and distortion in leaves, damage of greasy pots in petioles, stems and very typical pots in ring shape in fruits. The illness reduces yields and can also cause the death of plants (11, 26, 27).

The main attention toward problems of genetic improvements of papaya is focused on the resistance search against PRSV-P (11, 20, 21, 28). In the germplasm of papaya, moderate resistance levels have been found (6), but these cultivars resistant to the virus have horticulture inferiors characteristics to the ones of the commercial cultivars (9). Later cited by Jensen in 1937 (15), found in *Carica cauliflora* Jacq., recently reclassified as *Vasconcellea cauliflora* by Badillo (2), was immune to the illness. However, Conover in 1962 (4), informed for the first time about the susceptibility of *V. cauliflora* at

PRSV-P. In investigations about the resistance of PRSV-P in caricaceae, Horovitz and Jiménez in 1967 (14) consider the behaviour of *V. cauliflora* as resistant to the virus. To the moments, most research done in different countries support the immunity or resistance of the wild specie *V. cauliflora* to PRSV-P (14, 18, 21, 25), which is mostly used in the genetic improvements of papaya (20, 21), for the obtaining of inter-specific or inter-generic hybrids (14, 16, 21, 23) through the rescue of fecundated ovules and hybrids embryos in-vitro (18, 21, 24)

In a research previously done about hosts of the severe and normal strain of the virus of the papaya ringspot (PRSV-P), *V. cauliflora* was included, in which were observed similar symptoms to those of a hypersensitivity reaction in a minority of inoculated plants with a severe PRSV-P (13). This investigation was planned to know more the relation of susceptibility of *V. cauliflora* to the inoculation of the severe strain of PRSV-P, and also to identify the origin of the possible relation.

## Materials and methods

**Sowing and maintenance of plants.** Ripe fruits coming from a wild population of *V. cauliflora*, were collected in the National Park Henry Pittier (Aragua state). Seeds were directly sowed in polyethylene bags of 1 L of capacity, having a sterile mix

of sand, land and organic manure in 1:1:1 v/v proportion. Plants (three x bag) were kept in a shed against insects until these were two months old. These were irrigated, fertilized and protected with pesticides every 15 days as a preventive measure. Essays

were done in the Phytopathology area of la Universidad Central de Venezuela in Maracay (10°16'13"N and 67°36'18"O), from March-November, 1999).

**Viral isolations.** A severe strain of the local PRSV-P was used, collected at la Facultad de Agronomía (UCV – Maracay) and characterized through bio-essays (13), which was multiplied on papaya as a natural host of the virus and kept at -20°C in the virology laboratory.

**Mechanical inoculation.** 24 plants of 60 days old (dde) of *V. cauliflora* plus an inoculated witness with distilled water were inoculated. In the mechanical inoculation process, a frozen portion of tissue of *C. papaya* infected with the viral strain was taken, which was macerated in a sterile and cool mortar with phosphate of potassium 0.01 M with pH 7, in a 1:10 (p/v) proportion. The obtained juice was softly rubbed with the fingers in four leaves of the apical area, previously sparkled with Carborundum 600. Subsequently, the inoculated leaves were washed with water to eliminate the excess. All the material was identified and kept in a shed against insect, with an average temperature of 25.15°C and 78.68% HR for a month.

**Sow in the field.** After a month, plants of *V. cauliflora* inoculated with the severe PRSV-P and the witness inoculated with water, were acclimatized in a greenhouse with an average temperature of 29°C and 75% HR for a week and later, were transplanted in a smallholding of 64 m<sup>2</sup>. The sow

distance was of 2m between plants and 3 m between rows on a ridge, in reason of three plants per point. Conventional agronomical practices were done as for example fertilization, irrigation and weed control, fungus and insects.

**Bio-essay with temperature treatments and light intensity.** With a completely randomised design, 144 plants of *V. cauliflora* that were mechanically inoculated with the severe strain of PRSV-P were put on a growth chamber and exposed to three temperature and light treatments in reason of 48 plants per treatments: T1 at 29°C and a light intensity of 6300 lux (103 uEm<sup>2</sup> 1<sup>1</sup>), T2 at 27 °C and a light intensity of 5500 lux (85 uEm<sup>2</sup> 1<sup>1</sup>) and T3 at 25 °C and a light intensity of 4200 lux (62 uEm<sup>2</sup> 1<sup>1</sup>). T1 was obtained combining two fluorescent bulbs (F72T12 / High D of 56 W, Philips), eight incandescent bulbs of 100 W, T2 with two fluorescent bulbs and six incandescent, and T3 with two fluorescent bulbs and four incandescent, all located at 60 cm height from the roof. In each temperature treatment a witness was included (12 inoculated plants with water). The relative humidity was of an average 75% HR, the photoperiod of 16 h of light and 8 h of darkness. After a months with the different treatments, the inoculated plants were relocated in a greenhouse for another month.

**Bio-essay of re-inoculation in *C. papaya*.** After 30 days the inoculation of the bio-essay with treatments, leaves of *V. cauliflora*

were sowed produced by PRSV-P, 5 g of infected tissue was used to inoculate 10 healthy plants of *C. papaya* cv. Cartagena Amarilla of 60 dde, these plants were evaluated for 60 days in a shed against insects in an average of 25.15°C and 78.68% for a month, through a mechanical inoculation process similar to the one described.

**Serological test.** In order to detect the virus in *V. Cauliflora* with the severe PRSV-P, an ELISA case made in France (Sanofi-Pasteur) was used, with a multi-faceted polyclonal developed for PRSV-W. The concentration of the substrate 4-nitrofenilphosphate was modified, due to the pale colouring observed with the chromogen quantity suggested in the protocol; the rest of the procedure was the one suggested by the commercial entity. 16 composed samples were taken (3 pl. / bag) from each treatment, recollecting those leaves that showed the best symptoms. The value was considered as white, samples were put at random and twice to avoid false positives, as well as healthy and sick controls (PRSV-W) that were part of the commercial ELISA case. After one hour of inoculation the reaction was measured in nm (nanometer of optical density), using the ELISA reader (Multiskan EX).

**Measured variables.** In field conditions, percentage of plants with symptoms were counted once, seven days after the transplant of the

inoculated plants. With the aim of determining the effect of the temperature and light intensity treatments in the inoculated plants with the viral strain, 48 plants/treatment were evaluated (16 composed samples for ELISA) plus witnesses at the time when symptoms appeared. The information was qualitatively and quantitatively taken, considering the following variables: 1) Absence of symptoms: asymptomatic plants, negative ELISA and 2) with symptoms: smooth and strong mosaic, pale and strong dappled, chlorosis, deformation and positive ELISA. A positive sample was considered when it had a twice superior optical density than the mean obtained with witnesses. Values of optical density were analysed using the Statistics program 7.0 (Analytical Software, FL. USA).

**Environmental conditions.**

A) During the test with *V. cauliflora* in a shed and growth chamber, the temperature and relative humidity were daily measured with thermal-hydrogen (Göttingen. Typ. 252) and the light intensity was measured with quantometer (Li-cor mod. Li-185B). B) For the field test, the information provided by Azkúe (1) of CENIAP, doing a comparison in the parameters of temperature, precipitation, evapotranspiration and relative humidity from May-July of 1999, in relation to the same period in 1998 and from 1987-97, registered at CENIAP Maracay (10°LN 67°LO).

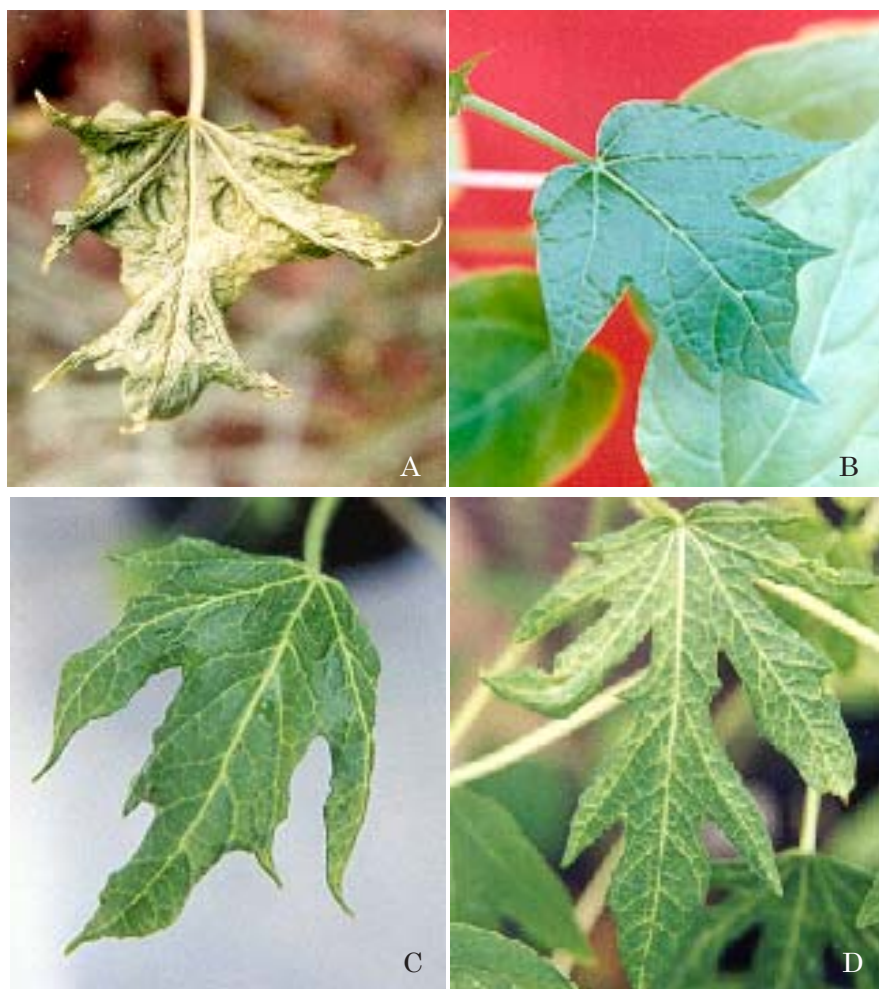
## Results and discussion

**Mechanical inoculation and sow in the field.** 14 days after the inoculation (ddi), 8.3% of *V. cauliflora* plants inoculated with the severe PRSV-P showed signs of infection with symptoms of apical necrosis very similar to a reaction of hypersensitivity, these symptoms persisted for a month during the time kept in the shed (an average of 25.15°C and 78.68% HR). The total of the 24 plants inoculated with the severe strain of PRSV-P were transplanted to the field, seven days after the transplant (ddt) 8.6% showed symptoms, increasing at 16.6% within 15 ddt. Within 30 ddt, a 50% was registered, keeping until 45 ddt; 100% of this infection was reached at 55 ddt. Until that time, the observed symptoms were leaves with mosaic of a very intense green to olive green, wrinkled, venal chlorosis with swelling, an irregular deformation of the leaf blade with a thread-like look (figure 1A). The *V. Cauliflora* witness showed signs of infection at 58 ddt, observing plants with stunted, mosaic, chlorosis and deformation of leaves.

**Bio-essay with treatments of temperature and light intensity.** The witness inoculated with water did not show any symptoms in plants exposed to treatments in conditions of growth chamber (figure 1B), while there was a visible reaction of *V. cauliflora* in the inoculation of the severe strain PRSV-P in the different tested treatments. After 12 ddi the inoculated plants receiving these treatments produced foliar symptoms, as for example pale to market dapples,

mosaic, distortion or deformation of leaves, flaccidity or bulkiness of veins (figure 1C). Within 15 ddi, were 82% of infected plants, showing symptoms of the illness. In T2, 86% of the infected plants was evidenced showing symptoms, while T3 had 47% of infected plants with symptoms of PRSV-P. The observed symptoms were generalized, keeping in the same proportion and incrementing its intensity during the corresponding 30 days of the evaluation. Contrary to the typical symptoms produced by PRSV-P in *C. papaya*, in this specie greasy dapples were not observed in stalks or petioles, which normally appear in this stage of the illness (19). All temperature and light treatments favoured the manifestation of the symptoms of the illness, but differentiated between them by the proportion of affected plants. Passed 30 ddi, plants were kept in a greenhouse (an average of 29 °C and 75 HR) where symptoms were market.

**Re-inoculation essay in *C. papaya*.** Sap was extracted from the different treatments, from samples of tissues of *V. cauliflora* leaves infected with PRSV-P, which was mechanically re-inoculated on papaya cv Cartagena Amarilla, reproducing symptoms from smooth to severe mosaic with deformation of the lamina and bulkiness in the veins have been observed. The transmission of the virus was efficient in 80% of the inoculated plants (8/10), observing the symptoms 12 ddi after in shed conditions (figure 1D).

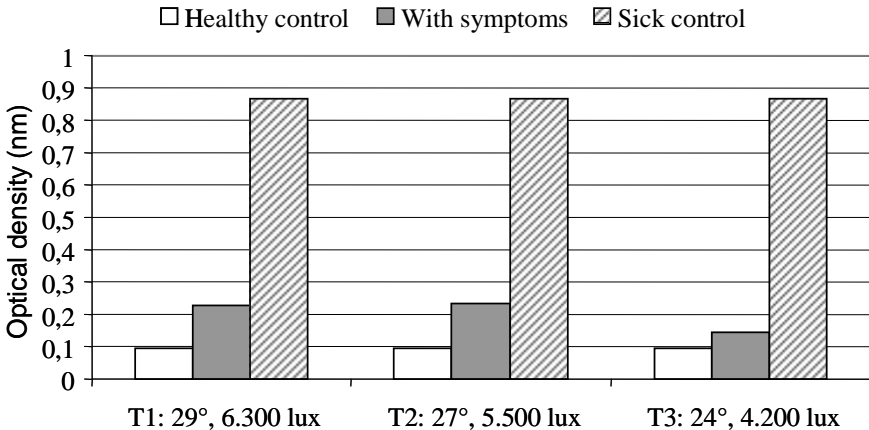


**Figure 1. Induced symptoms by mechanical inoculation of the local severe PRSV-P. A) Leaves with wrinkling, reduction of the lamina and filiform appearance in *V. cauliflora*, 30 ddt in field. B) *V. cauliflora* leaf without symptoms of the witness inoculated with water in growth chamber. C) *V. cauliflora* leaf with flaccidity, bulking and venal lighten at 21 ddi, belonging T2 in growth chamber. D) Leaf of *C. papaya* with wrinkling, bulking of veins and deformation at 21 ddi, in the re-inoculation of the severe PRSV-P through the infected tissue of *V. cauliflora*.**

The research done by Lange in 1961 (17) was considered in growth conditions. Its results indicated that the optimum of temperature measured in increment of dry matter was between 17°C and 30°C, while the maximum elongation of plants occurred in almost 30°C of temperature. This information was extrapolated and compared with the register of the climatologic station, designing thus the three temperature treatments used in this research. Another important information used in this research is that temperature has a market effect on the concentration of the virus, the optimum for the accumulation depends on both, of the quantity of pathogen and the host (10). An increment of temperature usually increases the replication rate and movement of the virus through the

plant (10, 22). Results showed that the transmission of the virus through the mechanical inoculation in growth chamber conditions was efficient.

**Serological test.** Plants of *V. cauliflora* inoculated with the severe strain of PRSV-P that were exposed to treatments 1 and 2, showed symptoms and were positive in the serological reaction, while plants that are part of T3, had weak symptoms and values but with a tendency to be positive. The witness inoculated water did not show any symptoms, and as well as the healthy control were negative, meanwhile the sick control was positive (figure 2). Values of optical density obtained in the ELISA test were analysed using the non parametric method of Kruskal and Wallis, detecting differences ( $p=0.05$ ), indicating that there were significant differences between treatments. In



**Figure 2.** Reaction of *V. cauliflora* towards PRSV-P (ELISA) within 22 days of being inoculated, in relation to the temperature and light intensity treatments, in fixed conditions of 16 hours of light and 75.5% of average HR in growth chamber.

the mean comparison three homogeneous groups were differentiated, the sick control had an average value of 0.865 nm forming a group. T1 and T2 formed an intermediate group with average values from 0.227 nm and 0.235 nm respectively. T3 with approximately 0.145 nm is part of the healthy control, which had an average control of 0.096 nm, therefore the fixed parameters to consider the reaction of the virus in this treatment as positive are not fulfilled.

It is important to mention the role of the ELISA immunoenzymatic diagnose technique developed by Clark and Adams (3) and some variations done to this technique in the detection of PRSV-P (12, 13). Using this technique in essays with *V. cauliflora*, the security and confidentiality incremented in the obtained results, because the first necrosis symptoms of the top in shed conditions with an average of 25.15 °C and 78.68 % HR, a hypersensitivity reaction was considered, and the weak reactions of ELISA in these plants caused the observation and follow-up of the development of the illness in the shed and in the field.

#### **Environmental conditions.**

In shed conditions during February-March, 1999, the temperature was kind of stable. In March, the highest peak was of 25.7°C, while the lowest peak was registered in the same month with 24.52°C indicating few variations in the daily thermal potential, or stable conditions. In relation to the relative humidity, it has a similar behaviour registering the highest peak in February which

was of 80.18% and the lowest peak of 77.49% in the same month. In the comparative analysis of the predominant climatic conditions in the field essay during the period of May 1st to July 30, 1999, the maximum temperature was of 32.1°C in June, 1999, while in May, 1998 was of 31.9°C and 32°C for the same month but in the period of 87-97. The minimum temperature was of 15.6°C in May, 1999, and of 19.6°C in July, 1998, while in the period of 87-97 was of 18.1°C in July. The distribution of the total precipitation during the period under study was of 489.2 mm in 1999, in 1998 was of 397 mm and in the time of 87-97 of 361.6 mm. In the same period the total evapotranspiration was of 691.8 mm in 1999, in 1998 was of 393.6 mm and in the period of 87-97 was of 411 mm. The relative humidity was of 64.4% for 1999 and of 80% for both 1998 and the period of 87-97. According to these analysis, it can be deduced that in 1999, when experiments were done, this was atypical in relation to the average values of the register of 10 years for the area, and these conditions allowed to observe the symptoms induced by PRSV-P in *V. cauliflora* in the field, fact which was not previously cited.

In growth chamber conditions during October, 1999, the highest temperature peaks were registered at 2 pm in 38°C; 37.5°C and 33.5°C in treatments T1, T2 and T3 respectively; the lowest peaks were registered at 7 am in 24.5°C; 23°C and 22°C in treatments T1, T2 and T3 respectively. The average temperature was of 29°C, 27°C and

25°C in treatments T1, T2 and T3 respectively. During the night there was a fall, but it kept near 25°C most of the night. The relative humidity was between 71.4% and 78.5%. The photoperiod of 16 h light supplemented with bulbs, contributed to keep these conditions. It must be also considered that the virus concentration varies with the type of tissue, age of the plant, of the tissue, and also of the development stage of the illness. Besides, temperature has an important role in the growth of the plant; and this at the same time influences the movement of the virus, the concentration of each infected cell and sometimes the percentage of infected cells, affirming that the appearance of symptoms not always agrees to the higher concentration of the virus (10, 22). This explains why it was observed an increment in the proportion of plants with symptom, once these were put in shed conditions (29°C and 75% HR) from November to December, 1999.

The fact that *V. cauliflora* is sensitive to the inoculation with the used severe strain of PRSV-P, depending on temperature and light, redefines the role of this specie as resistant to the illness (14, 18, 21, 25). Due to the behaviour of *V. cauliflora* in growth chamber conditions, greenhouse and field, it is thought that an increment in the temperature over a point of the normal growth conditions of the plant, increases the multiplication rate of the virus, reducing the appearance time of the first symptoms. Therefore, changes in the temperature in which plants develop, might conduct to the selective

multiplication of some strains adapted to specific conditions (10, 22, 27). However, it must be explained which was the critical factor between the temperature and light in growth chamber conditions that influenced in the best transmission and multiplication of the virus expressed in the typical symptoms of the infection caused by PRSV-P, due to in the different treatments positive responses were obtained, it would had been interesting to compare in fixed conditions, the critical temperatures that were found (29, 27 and 25°C) and vary the light conditions and relative humidity.

In order to explain the existent controversy about *V. cauliflora* as a resistance specie to PRSV-P, it is obligatory to mention Conover (4, 5), who reports for the first time the susceptibility of *V. cauliflora* towards PRSV-P in Florida (USA). The most cited research about the behaviour of *V. cauliflora* as resistant to PRSV-P was published by Horovitz and Jiménez in Venezuela, in 1967 (14). In this research, the viral strain as well as the vegetal material used in the different essays coming from Aragua state, Venezuela, agree to the origin of strain and the vegetal material used in the study with Horovitz and Jiménez (14) and Malaguti *et al.* (19), the difference in the results obtained in this research to those cited is basically due to the used severe strain PRSV-P, which behaved with higher virulence maybe due to the environmental conditions present during the experiments, this last aspect is considered as a weakness of these studies since experimental

conditions are not detailed or described. Besides, in the adaptation tests of some caricaceae species, the crossing results of *C. (V.) goudotiana* x *C. (V.) cauliflora*; *C.(V.) monoica* x *C. (V) goudotiana* and *C. (V) monoica* x *C.(V) cauliflora* produced viable seeds, but plants were susceptible to the virus though the resistance was expected, inferred at least by *V. cauliflora* due to its dominant character, because this was

considered as the only one resistant, in that moment these investigators did not find a satisfactory response for this phenomenon (28). However, other investigators observed that only *V. cauliflora* and its hybrids did not exhibit the symptoms of the viral illness (23). Genetically speaking, the cited research has different results, which might be discussed considering the influence of the two environmental factors and/or strains.

## Conclusions

It is concluded that the results of this investigation agree to those obtained by Conover (4, 5), Cook (7) and Cook and Milbrath (8) who showed that *V. cauliflora* is susceptible to the severe strain of the papaya ringspot virus (PRSV-P) and authors differed from the rest of the investigators that consider this specie as resistant to the virus (14, 18, 21, 25). Finally, *V. cauliflora* might show

the typical symptoms of the illness produced by PRSV-P in experimental conditions and field, or might behave as an asymptomatic host, so plant breeders and biotechnologists should be alert of this multiplication condition and persistence of the virus in this specie, hence they do not spend time or effort in its use as a resistance source for the virus.

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