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Effect of temperature on the Cacao swollen shoot virus (CSSV, Badnavirus) vection by the mealybug *Planococcus citri* to cocoa seedlings in the laboratory

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ABSTRACT

Since the early 2000s, the cocoa industry in Côte d'Ivoire is experiencing the resurgence of the Cacao swollen shoot virus disease (CSSVD). Full-sun cocoa monocultures and low shade plantations are considered as a cause of the rapid spread of CSSVD in the country. The warmer and dryer microclimates prevailing here, aggravated by climate change, would be conducive to vector mealybug outbreaks and would exacerbate CSSVD damage. This has led cocoa sector to encourage shading practices. However, current practices are not supported by sufficient knowledge of CSSVD relationships to microclimate. The virus is transmitted by mealybugs (Hemiptera: Pseudococcidae) through a non-circulative semi-persistent transmission, that means that the virus remains located to the vector mouth parts and that a mealybug remains infectious no more than two days. The present study aims at characterizing the impact of temperature on CSSV vection by mealybugs. The study was conducted in the laboratory of the PRISM department of the Plant Health Institute of Montpellier, in France. The study included three steps: 1) an acquisition period conducted in a growth chamber at 6 different constant temperatures (20, 22, 24, 26, 28 and 30°C) for 24 hours, during which first instars of the mealybug *Planococcus citri* were enclosed in clip-cages on young symptomatic leaves of cocoa seedlings previously artificially infected with a recombinant *Agrobacterium tumefaciens* bacteria containing the cloned sequence of Agou 1 isolate of CSSTBV species ; 2) an inoculation period conducted at the same temperatures, where the young infective mealybug instars were transferred to sprouting cocoa beans and allowed to feed for 48 h ; 3) an incubation period at 25°C, where the cocoa beans were cleared of mealybugs and planted in a tray with potting soil, where they grew until CSSVD symptom onset, which was recorded. Molecular analyses by PCR, with specific primers of Agou 1 isolate, were performed 50 days and 180 days after inoculation period to detect the presence of the virus in cocoa seedlings. Results show that temperature has an effect on CSSV vection by mealybugs. Transmission rate gradually increased from 28 to 36% at 20°C to reach 82 to 95% at 26°C. The trend was not so clear for upper temperatures of 28 and 30°C. Observations on mealybug behavior suggested that the relationships between transmission and temperature could be explained by mealybug activity, which was stronger at higher temperatures. These results are discussed and perspectives are proposed.

Keywords: Thermobiology, Microclimate, Pseudococcidae

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1. Introduction

The cocoa swollen shoot virus disease (CSSVD) is a lethal cocoa disease caused by a complex of virus species from the genus Badnavirus and responsible for varying degrees of severe symptoms on cocoa (Muller, 2016). These virus species have been reported from West Africa only and are absent in Latin America. It is therefore likely that they were transmitted to the crop from African wild host plants related to cocoa (Posnette et al., 1950). CSSVD is a vector-borne disease transmitted to cocoa by some sixteen species of mealybugs (Hemiptera : Pseudococcidae) (Wetten et al., 2016). The virus vection by mealybugs is a non-circulative semi-persistent transmission, that means that the virus remains located to the vector stylets (mouth parts) and that mealybugs remain infectious for a short time, estimated at a maximum of two days (Roivainen, 1976). Cocoa industry in Côte d'Ivoire is experiencing a rapid spread of the disease since the early 2000s. Today, CSSVD leads to the quick destruction of large areas of plantations in most of production zones of the country, often causing farmers to abandon cocoa for other crops (Aka et al., 2020). Lowly diversified cropping systems exacerbated by climate change are being singled out as potentially responsible for the disaster. Based on this, cocoa farmers are currently encouraged by government to diversify their crop by planting shade trees (Anon., 2021). Yet, in our view, current recommendations for shading practices would be more efficient if they rely on a deeper knowledge of the disease epidemiology. Specifically, the impact of micro-climate on the disease vection has not been studied. The present paper describes a preliminary study of the impact of temperature on CSSVD vection by the mealybug *Planococcus citri* (Risso). The method for vection study at different constant temperatures is presented in details and preliminary results on mealybug activity and transmission success as influenced by temperature are presented. These results will help to improve our understanding of the CSSVD epidemiology.

2. Materials and methods

The following experimentation was conducted in the laboratory of the Plant Health Institute of Montpellier (PHIM), located at CIRAD Montpellier, France.

2.1 Plant source

CSSV-infected source plants

Twelve 5-months-old CSSV-infected young cocoa seedlings of variety Amelonado were used as source plants for transmission experimentation. Amelonado variety was chosen because it is susceptible to CSSVD and symptoms are early and clearly visible on seedlings. The young plants grown from seeds, coming from the CIRAD cocoa collection in Kourou (French Guyana), were maintained in a climatic greenhouse at temperature = 25 ± 2°C and RH = 70 ± 10%. After 2 months, CSSV was transmitted to seedlings through injections using a N25 microliter syringe (Hamilton, Switzerland) of 25-50µl of an inoculum of bacteria *Agrobacterium tumefaciens,* previously disarmed, and containing the plasmid pBCPX-2 in which the genome of the CSSV isolate Agou1 has been cloned (Jacquot et al., 1999). CSSV isolate Agou1 originates from Togo and was chosen because it is highly virulent with typical symptoms. CSSVD symptoms (mostly vein reddening) appeared after ≈ 2.5 months on source plants. In addition, source plant infection was confirmed after 2 months through PCR amplification with primers specific to CSSV isolate Agou1 (see PCR-amplification details in section 2.4).

Germinated cocoa seeds

Germinated cocoa seeds were used as plant support to test CSSV transmission by mealybugs, as they have proven appropriate in past studies with good transmission rates reaching 80-100% in optimal conditions (Roivainen, 1976; Dufour et al., 1988). Fresh seeds were extracted from cocoa pods collected from a smallholder CSSVD-free plantation close to Azaguié (N 5.63056°, W 4.08222), and shipped to France. Pods were collected from the common hybrid variety "Mercedes" and, when possible, from one or a few adjacent cocoa trees, in order to get genetically homogenous plant material. Express shipments were done with all required permissions.

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2.2 Insect source

A breeding of *Planococcus citri* (Hemiptera : Pseudococcidae) was initiated in the laboratory of PHIM from two populations of ≈ 400 individuals (100 adult females and 300 larvae), coming from AREFLEC (http://www.areflec.fr/), in Corsica. *P. citri* was chosen because it is one of the major CSSVD vector mealybugs in West Africa and, as a cosmopolitan and polyphagous pest species, it is widely available for experimentation in France. *P. citri* breeding was maintained on pregerminated and organically grown potatoes from variety "Monalisa", in two 26.5 x 13.5 x 15 cm aerated plastic boxes, themselves kept in two 60 x 40 x 80 cm Plexiglas cages 3,5 cm in diameter, in order to avoid any escape of insects. Five to 6 pregerminated potatoes were used in each box and changed every week. The breeding was maintained in a climate chamber at temperature 25 ± 2°C, relative humidity 30 ± 10% and photoperiod 12:12 L:D.

2.3 Experimentation of virus transmission

CSSV transmission by *P. citri* was tested at 6 different constant temperatures, 20, 22, 24, 26, 28 and 30°C, over a total period of 7 weeks, followed by a period of 6 months at 25°C for incubation. For each temperature, transmission was tested in three steps, namely acquisition, inoculation and incubation periods (Figure 1). For acquisition, 100 *P. citri* first larval instars were collected from the laboratory colony and distributed on 4 to 5 young leaves on 3 CSSV-infected source plants, where they were enclosed in clip cages fixed on leaves. Infested source plants were then stored for 24 h in a climate chamber (Binder, Germany) set to one of the 6 constant temperatures, with 80% RH and photoperiod 12:12 L:D. Mealybugs were then transferred to germinated cocoa seeds using a fine camel-hair brush for a 48h inoculation period. For each temperature, the germs of 22 seeds (132 seeds in total for the 6 tested temperatures) were infested, each with 3 potentially infectious mealybugs. Seeds were then kept individually in plastic sterile tubes, 3cm in diameter and 7cm deep, internally lined with absorbent paper to avoid any water condensation. Tubes containing cocoa seeds with mealybugs were stored horizontally for a 48h inoculation period in the climate chamber set to the same constant temperature, HR and photoperiod as for acquisition. After this period, young mealybugs present on cocoa seed germs were immediately counted using a stereomicroscope in order to assess their feeding activity as influenced by temperature. Finally, mealybugs were carefully removed from seeds under a stereomicroscope using a camel-hair brush and by spraying seeds with an organic insecticide. Then seeds were individually sown in growers filled with potting soil and stored in a growth chamber set at 25 ± 2°C, 70 ± 10% HR and photoperiod 12:12 L:D.

2.4 Virus detection in cocoa seedlings

After 6 months, CSSV was detected in seedlings by PCR amplification of DNA extracted from samples of young leaves. Samples of 50 mg were cut from one young terminal leave of each of the seedlings grown from potentially infected seeds. Samples were ground using a grinder (SPEX SamplePrep 2010 Geno/Grinder®, Germany) in 2 ml tubes containing two ceramic balls (2 grindings of 1 min at 1300 rpm). DNA was then extracted using the PlantDNeasy kit (Qiagen, Germany). PCR was carried out using the Taq Phire hot start II (Thermofisher, USA) and the following primer pair: ORF3AF Agou 1 TCGTTATACCAGACACCATGATGAC (534 pb fragment) and ORF3AR Agou 1 ATTTCCATTACTAGATTCTTCCCATAC.

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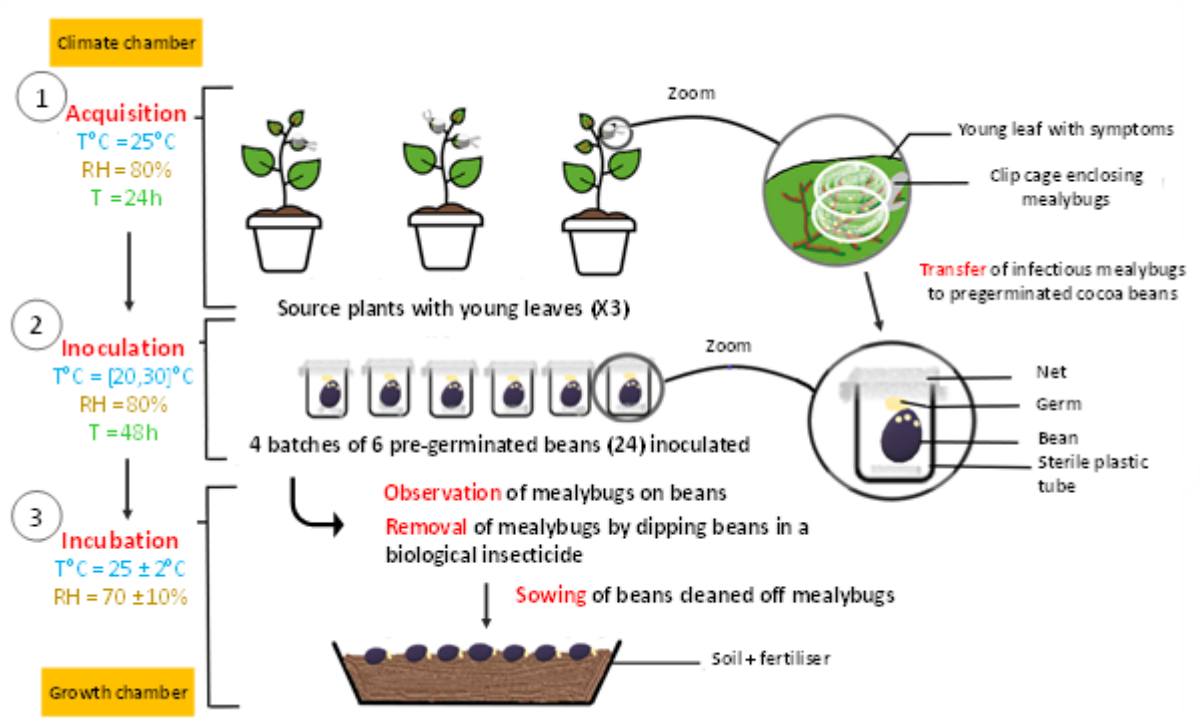

Figure 1: Diagram of the experimental design for testing the impact of temperature on CSSV transmission to germinated cocoa seeds by the mealybug *Planococcus citri*

For each DNA sample, PCR was conducted in 20 µl reaction containing 2 µl of the DNA sample, 4 µl of 5X buffer (provided by the manufacturer), 1.6 µl of dNTPs (at 2.5 mM each), 1 µl of each of the primers (10 µM), 0.4 µl of Phire Hot Start II DNA polymerase (Thermoscientific, USA) and 10 µl of water. Amplification program included a first denaturation step of 30 s at 98°C, followed by 40 cycles of 98°C for 5 s, 52°C (Tm) for 5 s, 72°C for 5 s. A final elongation step was done at 72°C for 10 min. The PCR products were visualized after electrophoresis at 100V for 25 min, by depositing 6 µl of PCR product on a 1% agarose gel in TAE buffer and staining with ethidium bromide.

2.5 Statistical analyses

Impact of temperature on mealybug feeding activity was assessed by comparing the mean number of mealybugs observed on cocoa seed germs at the end of inoculation period (initial number of mealybugs put on germs was 3), for each temperature, using a Kruskal-Wallis test followed by a Wilcoxon test with the Bonferroni correction for pairwise comparisons. Impact of temperature on virus transmission by *P. citri* was assessed by comparing the proportions of CSSV-infected seedlings for each temperature using a Pearson χ^2 test. Statistical analyses were done on R software (version 4.2.1).

3. Results and discussion

3.1 Impact of temperature on mealybug feeding activity

Temperature significantly affected mealybug feeding activity on cocoa seed germs (Kruskal-Wallis $\chi^2 = 17.331$, $df = 5$, $p < 0.01$). Mean number of mealybugs feeding on seed germs significantly increased between 20 and 22°C, and slightly increased again at 24°C, before slightly decreasing to reach a plateau for temperature 26, 28 and 30°C (Figure 2). Observation of the presence of mealybugs on cocoa seed germs at the end of the inoculation period suggests that 20°C is the less favorable temperature for mealybug feeding on cocoa seed germs, while temperatures around 24°C are more favorable.

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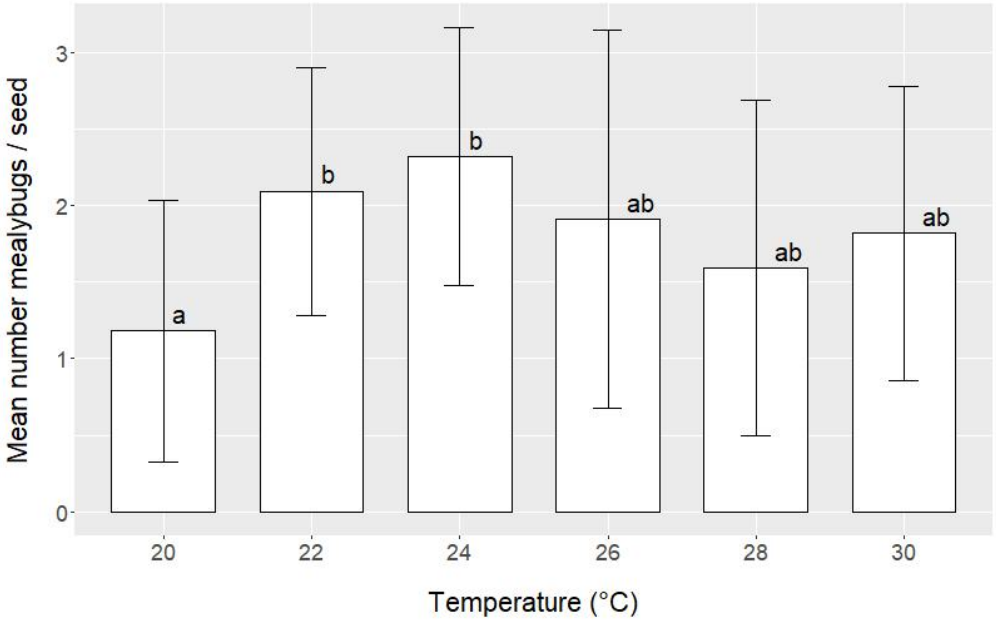

Figure 2: Impact of temperature on the mean number (± SD) of mealybugs P. citri feeding on seed germs at the end of inoculation period (initial number of mealybugs per seed was 3). A same letter topping two different bars indicates no significant difference between values.

Mealybugs like all insects are cold-blooded animals and their development and activity (feeding, reproduction) are strongly affected by the temperature of their environment (Régnière et al., 2012). Positive correlation between temperature and feeding activity has been demonstrated for different herbivore insect species (e.g. Lemoine et al., 2014). In our study, the correlation is not so clear, especially for temperature > 24°C. Our method was primarily aimed at facilitating the virus transmission and we avoided disturbing mealybugs during inoculation period. Feeding activity could have been measured more efficiently using more frequent and accurate observations of mealybug behavior.

3.2 Impact of temperature on CSSV transmission by mealybugs

Temperature significantly affected CSSV transmission to cocoa seeds by mealybugs ($\chi^2 = 35.21$, df = 5, $p < 0.001$). Rate of CSSV transmission significantly increased from 20 to 24°C, with 36.4 and 90.0% respectively. At 24°C, transmission rate reached a plateau, with a maximum value of 95.4% at 26°C, and did not vary significantly for higher temperatures. These results suggest that, in our experimental conditions, a temperature below 22-23°C may reduce the capacity of mealybugs to transmit CSSV virus, while at higher temperatures from 24 to 30°C, their transmission capacity is maximal. For temperatures ≤ 24°C, similarities can be observed between the impact of temperature on mealybug feeding activity and transmission of CSSV. A strong hypothesis may therefore be that temperature may affect virus transmission through mealybug activity. Data on the role that temperature may play in CSSV vection by mealybugs are scarce. Some of past studies used CSSV transmission by mealybugs as a technique to assess the resistance or tolerance of cocoa material to the disease (Legg and Lockwood, 1977; Cilas et al., 1988; Dufour et al., 1994). In these studies, climatic conditions were poorly considered. More generally, rare are the authors that characterized the mechanisms and factors involved in CSSV transmission by mealybugs. Roivainen (1976) is one of them and demonstrated that temperature, among other factors, significantly impacted CSSV transmission by *Formicococcus njalensis*. The author

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reported a maximum transmission rate of ≈ 45% for temperature between 29 and 31°C and a rate of 14% for temperature in the range 24-27°C.

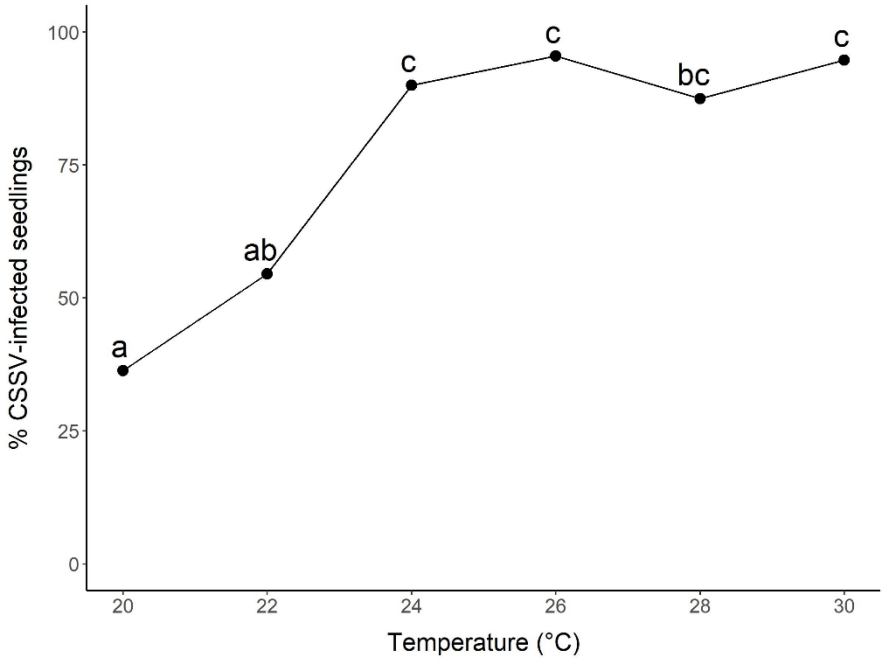

Figure 3: Impact of temperature on the rate (%) of CSSV transmission to cocoa seeds by mealybug P. citri. A same letter topping two different points indicates non significantly different values.

4. Conclusion

The method we developed in the laboratory allows the study of the biology of CSSV vection by mealybugs. Transmission rate as high as 95% was obtained suggesting that the method is adequate for CSSV vection studies by mealybugs. Temperature has an effect on CSSV transmission to cocoa seed by *Planococcus citri* that may be due to more active mealybugs as temperature increases. More globally, mealybug feeding activity may be one of the most important factors affecting CSSV transmission to cocoa. We therefore suggest that more studies be conducted on this subject, with specific methodologies for more accurate feeding behavior characterization, like electropenetrography for example. The preliminary results of the present study will help develop models to better understand and predict the impact of microclimate on CSSV epidemics in cocoa plantations.

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