**Breeding of cocoa (Theobroma cacao L.) genotypes tolerant/resistant to cocoa swollen shoot virus (CSSVs) in cocoa orchards infected by the disease in Côte d'Ivoire**

**Boguinard Sahin Honorine Brigitte GUIRAUD1, Gnion Mathias TAHI1, Inago Caudou TREBISSOU1, Klotioloma COULIBALY1, Xavier ARGOUT2, Olivier FOUET2, Claire LANAUD2**

**1Centre National de Recherche Agronomique (CNRA), Station de recherche de Divo, Programme Cacao, Divo, Côte d’Ivoire, brigo2008@yahoo.fr**

**2Centre International pour la recherche Agronomique et le Développement (CIRAD), UMR Amélioration génétique et adaptation des plantes pérennes (AGAP), Montpellier, France.**

ABSTRACT

The cocoa swollen shoot virus disease (CSSVD) has caused enormous production losses on Ivorian cocoa farms since 2003. In order to identify tolerant or resistant cocoa trees to CSSV, 320 farmers accessions of cocoa trees with good agronomic performances were collected in areas infected by the swollen shoot disease in Côte d'Ivoire. These genotypes were vegetatively propagated on Amelonado rootstocks, a cocoa variety known for its susceptibility to diseases. The agronomic data collected during one year on the plants propagated in the greenhouse mainly concern the presence or absence of CSSV symptoms on the scion and rootstock and the type of symptom.

Three phenotypic groups were identified in the greenhouse. The first group is composed of Potentially Tolerant Accessions to CSSVD (APT) and is composed of 255 cocoa trees that were asymptomatic both in the field and in the greenhouse. The second group called "Potentially Susceptible Accessions" (APS) is composed of 49 cocoa trees that were symptomatic both in the field and in the greenhouse and the third group (PSA/PSA) is composed of 16 cocoa trees that were asymptomatic in the field but symptomatic in the greenhouse The accessions of the three phenotypic groups were then subjected to a molecular diagnostic PCR and qPCR to search and quantify CSSV within them.

Results showed that 53.33% of APTs; 71.42% of APSs and 50% of APT/APSs were positive by PCR molecular diagnosis. By qPCR, 33.33% of the trees in the APT group showed almost zero viral concentration. On the other hand, 27.45% of the trees in the APT group were negative for CSSV by both PCR and qPCR techniques. These results would confirm the tolerance of trees in the APT group to swollen shoot and could also indicate resistance of the disease by these trees. These CSSVD tolerant or resistant cocoa trees could be introduced into multi-location trials in infected areas to confirm their performance against the disease. The use of genotypes having confirmed their resistance to CSSVD as progenitors in the recurrent selection scheme underway in Côte d'Ivoire is considered and discussed.

**Key words:** Breeding, cocoa trees, tolerant, swollen shoot, Côte d'Ivoire

# Introduction

The cocoa tree [Theobroma cacao Linnea (Malvaceae)] is a perennial, tropical plant endemic to South America (Cheesman, 1944). It is a highly prized crop in the world, mainly for its beans used in the manufacture of chocolate, cosmetics, pharmaceutical products and other cocoa derivatives.

For several years, Côte d'Ivoire has maintained its rank as the world's largest producer of merchantable cocoa with a record 2.010 million tons in 2016-2017 (ICCO, 2017). The cocoa sector in Côte d'Ivoire contributes, thus, 15% of the Gross Domestic Product (GDP) and 44% to export revenues (ICCO, 2014).

Despite this significant performance, the current context of Ivorian cocoa farming is marked by numerous production constraints. These include the low level of use of improved plant material (Koua et al., 2018), the aging of the orchard (Assiri et al., 2009; 2016; Koua et al., 2018) and high pest pressure. The latter is due to certain pests such as mirids [Sahlbergella singularis Hagl. (Mirideae)] (Kouamé et al., 2014) and certain diseases such as brown pod rot whose main agent in Côte d'Ivoire is Phytophthora sp (Pythiaceae). The health status of the cocoa orchard in Côte d'Ivoire has deteriorated with the resurgence of cocoa swollen shoot virus (CSSVD) in several production areas of the country. Indeed, after the discovery of the disease in Kongodia and Sankadiokro, new and growing infection foci were observed in several production areas. This led to significant crop losses and abandonment of infected cocoa farms (Ollennu et al., 1989). This disease, which constitutes a serious threat to the sustainability of Ivorian cocoa production, is of particular concern to the cocoa industry and Ivorian agronomic research.

Several control strategies against this disease have been proposed by research. These include agronomic methods based on the systematic uprooting of all trees infected by the disease in order to prevent the proliferation of the infection in the orchard (Dzahini-Obiatey et al., 2006) and chemical control of insect vectors. All of these techniques have proven insufficient, as evidenced by the distress of producers who are increasingly turning to other crops, thus jeopardizing the global cocoa market. As a result, the solution increasingly being considered in Ghana, Togo and Côte d'Ivoire is the search for sources of resistance to the disease.

In this perspective, Ivorian agricultural research through the CNRA has decided to propose sustainable solutions that can counter the expansion of CSSVD. These solutions necessarily involve the selection and supply to producers of varieties that are resistant or tolerant to the disease. Thus, to achieve this objective, an evaluation of potentially CSSVD tolerant genotypes will have to be carried out in order to introduce the best ones in the selection program developed in Côte d'Ivoire (Lachenaud et al., 2001). Indeed, the selection of high-yielding genotypes of cocoa trees resistant to swollen shoot is necessary in order to guide the choice of breeders for the improvement of the species (Ollennu et al., 1989). Thus, according to several authors, genetic control, based on the use of CSSVD resistant or tolerant varieties, combined with appropriate agronomic practices, appears to be an alternative to swollen shoot control (Adu-Ampomah et al., 2003; Ollennu et al., 1989).

In order to search for potential sources of resistance to the swollen shoot virus, surveys and collection of accessions were undertaken in swollen shoot infected farmer cocoa farms. Within the framework of this study, 320 accessions from CSSVD-infected cocoa orchards were evaluated in the greenhouse in order to study their behaviour towards CSSV in a controlled environment. Indeed, it was a question of carrying out a phenotyping on all the accessions reproduced in greenhouse and to follow in the course of time, the possible appearance of characteristic symptoms of the disease. The plants thus evaluated were subjected to a molecular diagnosis to search for the presence of the swollen shoot virus within them. In addition, this work also aimed at quantifying the viral load within the accessions potentially tolerant to the disease.

# II. Materials and Methods

2.1. Plant material

The plant material used for the molecular diagnosis of farmers' accessions against CSSV was composed of 320 genotypes from nine departments (**Table 1**). These were 320 accessions out of 698 collected that had at least eight budded plants per accession after grafting in the greenhouse. These samples belong to the three phenotypic groups highlighted in the greenhouse APT, APS, APT/APS.

2.2 Methods

2.2.1. Vegetative propagation of accessions

Accessions potentially tolerant (APT) and susceptible (APS) to CSSV were vegetatively propagated in the greenhouse. Indeed, five to ten graft wood were taken from each accession (tree) and multiplied by grafting onto Amelonado seedlings. The grafting on susceptible rootstocks aims at evaluating the behavior of this plant material towards the disease once in contact with a susceptible variety (Amelonado).

2.2.2. Data collection in the greenhouse

Two weeks after the grafting of the accessions in the greenhouse, data were collected on a weekly basis. The data collection included 320 accessions that obtained at least eight live plants. The parameters measured were the following :

- the number of budded grafts per accession ;

- the number of plants per accession with CSSV symptoms ;

- the latency time for the appearance of the first symptom per accession.

2.2.3. Collection of leaf samples

Two young leaves (flush) were collected in the greenhouse from each of the grafted accessions. These leaves were kept in envelopes and stored at room temperature before being transported to the virology laboratory of the UMR BGPI of CIRAD in Montpellier (France).

2.2.4. Extraction and purification of viral DNA

Viral DNA extraction was performed on 20 mg of dried leaves in 2 mL grinding tubes (Starstedt). The plant material was ground using a FastPrep-24 robot grinder (Londono, 2011; Mathiesen, 2009). DNA extraction was performed in Eppendorfs tubes (1.5 mL) by using the Qiagen brand "Plant DNeasy kit" DNA extraction kit (Yoshikawa et al., 2011). DNA extraction was performed based on the manufacturer's instructions. Indeed, 20 mg of dry leaves per sample were ground to powder using the ceramic ball mill following 2 runs of 20 s. The grind was dispersed in 400 µL of extraction buffer (AP 1). Four (4) µL of RNAse (100 mg/mL) was added to each tube to remove all traces of RNA. The tubes were then vortexed and incubated for 10 min at 65°C, while shaking occasionally. DNA was extracted with the addition of 130 µL of lysis buffer (P 3) to each tube. The mixture was incubated on ice for 5 min. This step precipitates detergent, proteins, and polysaccharides. The tubes were then centrifuged at 8000 rpm for 5 min to remove abundant precipitate. The supernatant was then transferred to a Qiashredder column (lilac) placed on 2 mL tubes and centrifuged at 8000 rpm for 2 min. The DNA is precipitated with the addition of 1 mL of buffer AW 1 to each tube. 650 µL of the mixture is then transferred to DNeasy columns placed on 2 mL tubes. This was followed by centrifugation at 8000 rpm to dissociate the DNA from impurities. The DNA was washed in 2 steps with 900 µL of AW 2 buffer followed by centrifugation. Finally, the columns were placed on new eppendorfs and the DNA was eluted in 100 µL of buffer AE pre-warmed to 65°C. PCR inhibitors were then removed from the DNA using the PCR Inhibitor Removal Kit (ZymoResearch). This kit was used in addition to the extraction protocol to further purify the samples after extraction. It was composed of arrays containing recovery columns (membranes) that were peeled off their caps and centrifuged at 8000 rpm for 3 min in order to activate them. The columns were placed on 1.5 mL Eppendorfs tubes. For each sample, 100 µL of DNA was deposited on the corresponding columns and centrifuged at 8000 rpm for one minute.

2.2.5. Performance of PCR with microsatellite-specific primers (SSR)

Polymerase chain reaction (PCR) was performed in Mastercycler thermal cyclers (Eppendorfs) with cocoa microsatellite primers (mTcCIR 351, mTcCIR 331) (Lanaud *et al.,* 1999; Fouet et al., 2011) and the CSSV-specific primers (Badna ¼ CSSV Deg 2; UNI 1 F/R) (**Table 2).** The microsatellite primers were used to ensure good quality and quantity of cocoa genomic DNA within the total extracted DNA. The CSSV primers were used for molecular diagnosis of viral DNA within the extracted total DNA.

PCR was performed in 0.2 mL Eppendorfs tubes with two cocoa-specific microsatellite primers (mTcCIR 331; mTcCIR 351). The sequences of the two primers used were presented in Table 2. PCR was performed following the protocol of Allegre et al. (2012). Five (5) µL of 1:10 diluted DNA is mixed with 20 µL of a reaction mixture consisting of 8.3 µL of pure water, 5 µL of Promega buffer (5 X), 1 µL of dNTP (5000 µM); 0.25 µL of Forward primer (10 µM); 0.25 µL of Reverse primer (10 µM); 0.2 µL of Taq polymerase (5 U/µL) (**Table 3**). This mixture was used to perform the PCR (Touch Down program). It started with an initial denaturation step at 95°C for 5 min, followed by 10 cycles at 95°C for 30 s, 58°C to 48°C (-1°C/cycle) for 45 s and 72°C for 45 s. This was followed by 25 cycles of 95°C for 30 s, 48°C for 45 s and 72°C for 45 s. The PCR was completed with a terminal elongation step at 72°C for 8 min (Allegre et al., 2012). Positive and negative controls were included in the molecular analyses to ensure the efficiency of the PCR diagnosis.

2.2.6. Performance of PCR with CSSV-specific primers

CSSV primers were used for molecular diagnosis of swollen shoot disease samples. PCR was performed in 96-well plates and 0.2 mL tubes. It was performed with the CSSV-specific primer pair (Badna ¼ CSSV deg 2), drawn in the reverse transcriptase region of the virus genome. The sequences of the primers used for the molecular diagnosis of CSSV were presented in Table 4. Two kits were used for PCR to improve the molecular diagnosis. These were the Promega kit which was used first on all samples and the Phire kit which was used second on only the negative samples (**Table 4**).

In the first approach ; 5 µL of DNA was added to 20 µL of a reaction mixture consisting of 11.8 µL of pure nuclease-free water, 5 µL of Promega buffer (5 X), 1 µL of dNTP (10 mM), 1 µL of Forward primer (10 µM); 1 µL of Reverse primer (10 µM); 0.2 µL of Taq polymerase (5 U/µL) Table 5. This mixture was used to perform the PCR (Touch Down program). It started with a denaturation step at 95°C for 5 min, followed by 94°C for 30 s, 57°C for 30 s, 72°C for 30 s (35 cycles) and 72°C for 10 min (**Table 5**).

In a second approach, the Thermo Scientific Phire Green Hot Start II DNA Polymerase kit was used to improve the diagnosis of samples negative to the first PCR. The composition of the PCR mix is presented in Table 6. Two (2 µL) of 1:10 diluted DNA were added to 18 µL of a reaction mix consisting of 11.2 µL of pure water, 4 µL of Phire Green Reaction Buffer (5 X); 0.4 µL of dNTP (10 mM); 1 µL of forward primer (10 µM); 1 µL of reverse primer (10 µM); 0.4 µL of Phire Hot Start II DNA Polymerase (5 U/µL). The PCR program used for this kit began with an initial denaturation step at 98°C for 30 s, followed by 98°C for 30 s, 60°C for 30 s, 72°C for 15 s (35 cycles) and 72°C for 1 min. Positive and negative controls were included in the molecular analyses to ensure the efficiency of PCR diagnosis (**Table 6**).

2.2.7. Electrophoresis

Electrophoresis was performed on a 1% agarose gel in 0.5 X TAE (Tri-Ethyl Acetate) buffer. Eight (8) µL of the PCR mix per sample was placed in each well on the gel. Five (5) µL of ladder (Invitrogen) was also placed in the first well of each line to serve as a control for band size determination. Electrophoresis was performed in a "Mupid" type electrophoresis tank following a migration at 100 V for 30 min. After migration, the gel was deposited for 10 min in a solution containing ethidium bromide (BET) and rinsed with pure water for 2 min. The gel was then placed on the UV camera transilluminator connected to a computer and the images were viewed using vision-capt software.

2.2.8. Performance of qPCR

The qPCR was performed using the TaqMan method. It was conducted in 96-well plates with the LightCycler 480 instrument. The TaqMan second contained a fluorescent dye at its 5' end and a Quencher at its 3' end. Two types of seconds and two primer pairs were used. The first second and the first primer pair (named SSP) were specific to cocoa and were used as controls to ensure the presence of genomic DNA in quantity and quality. Thus, the use of the cocoa-specific second and SSP primers allowed the detection of "false negatives" due to poor quality of the extracted DNA. The second probe and pair of primers (named Bd 2) were specific to the CSSV genome and were therefore used for the molecular diagnosis qPCR of the disease. This primer pair was drawn on the "movement protein" area of the virus genome. Positive and negative controls were included in the molecular analyses to ensure the efficiency of the qPCR diagnosis. The experimental protocol for qPCR using LightCycler v 1.5.0 is presented in Appendix 5. To perform qPCR, 3 µL of DNA was added to 11.5 µL of a reaction mixture composed of 1.5 µL of pure nuclease-free water; 7.5 µL of Sybergreen buffer (2 X); 1.5 µL of forward primer (5 µM); 1.5 µL (5 µM) of reverse primer. The plate containing the 394 samples was placed in the Light cycler after centrifugation at 1500 rpm for 30 s. The qPCR started with one cycle of denaturation, 45 cycles of amplification, 1 cycle for melting curve development and one cycle of cooling (**Table 7**).

2.2.9. Molecular data collection and analysis

The results of the molecular PCR diagnosis (marked by the presence or absence of viral DNA in a given sample), revealed by agarose gel electrophoresis according to the appearance or not of bands at the size of 626 bp, allowed the diagnosis of CSSV virus within each sample analyzed. The sample was said to be virus positive if the band appeared at the expected size after electrophoresis. Thus, PCR data were collected in terms of presence (positive diagnosis) or absence (negative diagnosis) of band for each DNA sample (corresponding to each accession).

The diagnostic qPCR data were generated by LightCycler v1.5.0 software. They were materialized by the assessment of the Threshold Cycles (Ct) marking the appearance of the amplified products according to the number of amplification cycles. In addition, the assessment of the curve shape, the color of the qPCR signal (red or green) and the value of the threshold cycles (Ct) allowed the diagnosis of DNA samples by qPCR. Thus, a sample was diagnosed as qPCR positive when three conditions were met. The first condition is that the curve for that sample, output by the light cycler v1.5.0 software must be a well-marked sigmoid shape. The second condition is that the Ct value must be lower than 40 and the last condition is that the signal emitted by the software is of red color. Failure to meet any of these conditions implies a negative diagnosis of the sample to CSSV. On this basis, qPCR data were collected in terms of the presence of virus (positive diagnosis) or absence of virus (negative diagnosis). In addition, the viral load was estimated based on the Ct value. The closer the Ct value is to 40, the lower the viral load in the sample.

# III. Results and Discussion

3.1 Results

3.1.1. Assessment of accessions resistance in the greenhouse

Three phenotypic groups were identified in the greenhouse out of a total of 320 accessions observed. The first group is composed of 253 APT (i.e. 93.36% of the 271 accessions analyzed) free of disease symptoms on all their plants. However, 18 APTs (6.64%) had at least one plant with disease symptoms. This new phenotypic group (group 3), highlighted in the greenhouse, and which includes individuals free of symptoms in the field but with disease symptoms in the greenhouse, was called "APT/APS". Furthermore, in the second group, 38 accessions (i.e. 77.55% of the 49 accessions analyzed) had symptoms of the disease on at least one foot per accession. Thus, more than one year after observation of the accessions propagated in the greenhouse, three phenotypic groups were observed.

**Group 1** **(APT)** is composed of genotypes that did not show symptoms of CSSV in the field and in the greenhouse. Indeed, all the accessions of this group were free of symptoms of the disease at the time of their collection in the field and after one year of observation in the greenhouse following their multiplication by grafting on the variety Amelonado.

**Group 2** **(APS)** is composed of genotypes with CSSV symptoms in the field and in the greenhouse. All the accessions of this group were symptomatic during their sampling in the field and, after one year of observation in the greenhouse.

**Group 3 (APT/APS)** is composed of genotypes free of symptoms of the disease in the field but which once in the greenhouse, after grafting on the susceptible variety Amelonado, have foliar symptoms of the disease. Figure 1 shows the manifestations of CSSVD symptoms observed on plants in the field and in the greenhouse. No significant difference was found between the symptoms in the field (**Figure 1**)

3.1.2. Detection of CSSV in farmers' accessions through molecular PCR diagnosis

The results of the molecular PCR diagnosis of each of the 320 samples were presented. Samples with red colored bands were diagnosed as PCR positive because the bands that appeared on the gel, after impregnation with BET and visualization under ultraviolet (UV) light, were observed at the expected size (626 bp). The results of the molecular analyses were presented in the form of virus detection rates within the samples analyzed. Thus, Table 8 presents the rate of CSSV detection by PCR of samples according to localities and phenotypic groups. Analysis of this table indicates that 55.93% of all samples analyzed were PCR positive with the Badna ¼ CSSV deg 2 primer. The PSA group (group 2) had the highest rate of infected samples with 71.42%. While the APT group presented 53.33% of infected samples compared to 50% for the APT/APS group (**Table 8**).

3.1.3. Detection of CSSV in farmers' accessions through molecular diagnosis by qPCR

The results show the molecular qPCR diagnosis performed with the second control SSP and the CSSV Bd 2 probe on each of the 349 samples. The number of qPCR positive samples per locality and per phenotypic group is presented in **Table 9**. Analysis of the table shows that compared to the PCR technique, an increase in the detection rate of CSSV by qPCR was observed in the APS and APT/APS groups with 79.59% and 93.75% respectively. In addition; 53.13% of all samples were positive for CSSV qPCR diagnosis. Samples from the APT/APS group had the highest rate of qPCR positive samples with 93.75%. They were followed by the PSA group (79.59%) and finally the PTA group (45.49%).

3.1.4. Detection of CSSV in farmers' accessions through molecular diagnosis by PCR and qPCR

**Table 10** presents the number and detection rate of samples positive for both PCR and qPCR molecular diagnosis according to localities and phenotypic groups. Analysis of the table shows that 26.25% of samples were positive for both PCR and qPCR diagnosis. Individuals in the PSA group had the highest percentage of PCR and qPCR positive samples with 55.1%. They were followed by samples from the APT/APS group (50%) and finally those from the APT group (19.22%).

**Table 11** shows the number and percentage of samples negative in both PCR and qPCR based on accessions and phenotypic groups. Analysis of the table indicates that 23.75% of all accessions were negative in both PCR and qPCR. Individuals from the APT group had the highest percentage of PCR and qPCR negative samples (27.45%). They were followed by accessions from the APS group (10.2%) and finally those from the APT/APS group (6.25%).

3.1.5. Quantification of viral load of accessions

Table 12 groups the samples according to their viral load (viral concentration). This viral load was materialized by the value of the threshold cycles (Ct). The lower the Ct value, the higher the viral load in the sample. Analysis of the table indicates that only 5.1% of the samples in the APT group had a high viral concentration (Ct values between 20 and 25 Ct) and 33.33% had a very low viral load (Ct values above 40 Ct). In contrast, the APT/APS group had 0% of samples with high viral concentration.

3.2. Discussion

The main objective of this study was to identify CSSVD tolerant and resistant cocoa trees. The results of this study showed that 6.64% of the potentially tolerant accessions (PTA) showed symptoms of the disease. Thus, three phenotypic groups were highlighted in the greenhouse, within the evaluated population. First, accessions that were symptom-free in the field and in the greenhouse (APT), second, accessions that were symptomatic in the field and in the greenhouse (APS), and third, accessions that were asymptomatic in the field but that, once in the greenhouse, showed symptoms of CSSVD (APT/APS). This new phenotypic group (APT/APS) revealed in the greenhouse is an indicator of the presence of the virus within the APTs and of the possibility of the existence of a form of tolerance to the disease essentially materialized by the latency time.

Furthermore, the absence of CSSVD symptoms on plants in the field and in the greenhouse could be explained by a phenomenon of evasion at the level of cocoa trees in the field, materialized by the non-contact of the plant with the pathogen or by a form of tolerance/resistance of these cocoa trees to the vector (mealybug) or to the virus (Dzahini-Obiatey & Fox, 2010). In the latter case, the accession could perform well despite the presence of the virus within it. In other words, the plant would have the virus without showing the symptoms of the disease. Some authors explain this by the change in climatic conditions that could lead to a decrease in the appearance of symptoms (Allegre et al., 2012; Dahal et al., 1997). On the other hand, the observed variation in symptom onset could be explained by the characteristics of different CSSV isolates and species (Kouakou et al., 2012; Abrokwah et al., 2016). Indeed, these authors showed that swollen shoot disease is caused by different virus species whose symptom expression could vary from one strain to another.

The presence or absence of CSSV within symptom-free plant material can be verified by molecular analyses of plant material (Muller *et al*., 2001; Kouakou *et al.,* 2012; Abrokwah *et al.,* 2016). Molecular analyses of all plant material evaluated in the greenhouse could provide the real diagnosis of the accessions and conclude on their behavior towards CSSV.

The results obtained in this work indicated that 53.33% of APT; 71.42% of APS and 50% of APT/APS are positive to molecular diagnosis by PCR. This would mean that 53.33% of the PTAs observed in the greenhouse actually had the virus within them but did not show symptoms of the disease. Thus, the absence of CSSVD symptoms on the plants could suggest a tolerance of these accessions to the disease. The 46.67% of APTs diagnosed as PCR negative could suggest a dodging phenomenon at the plant level, a tolerance to mealybugs or a very low viral load difficult to detect by simple PCR diagnosis (Muller et al., 2001). According to these authors, the detection of CSSV in symptomless plants is very difficult because of the low sensitivity of some primers. In addition, the uneven distribution of the virus in plants (and leaves) and the low viral concentration in symptomless leaves (Muller et al., 2001).

In addition, the results indicated a better sensitivity of the primers with samples from the PSA group than from the PTA. This was materialized by the superiority of detection rate with samples from the PSA group (71.42%) than those from the PTA group (53.33%). These results could indicate a not very high detection rate in these groups. There are several possible reasons for these results. First, at the time of sampling, many of the plants in these groups had relatively old leaves that were free of disease symptoms. This could be the reason for a decrease in virus load or concentration in the samples (Muller et al., 2001). Secondly, the low detection rate could be explained by the uneven distribution of CSSV viral concentration within the leaves (Dzahini-Obiatey & Fox, 2010). The low detection rate of the virus within the samples could also be due to an error made in the characterization of symptoms on the field samples (Abrokwah et al., 2016). Indeed, CSSV symptoms present in different ways, making it difficult to accurately tell the presence or absence of the disease through simple field phenotyping. Finally, the low detection rate could be attributed to a problem with the sensitivity of the primers used (Kouakou et al., 2012; Chingandu et al., 2017). Indeed, the primers used in PCR, are sequences of about 15 to 25 base pairs drawn on very precise regions of the genome and specific to certain groups of isolates (Chingandu et al., 2017). Thus, given the high molecular variability of the virus observed in some countries such as Côte d'Ivoire (Kouakou et al., 2012) and Ghana (Abrokwah et al., 2016), the primers used might not be versatile or polymorphic enough to detect and amplify viral DNA from all isolates.

On the other hand, the results indicated that compared to the PCR technique, an increase in the detection rate of CSSV in qPCR was observed in the APS and APT/APS groups with 79.59% and 93.75%, respectively. This could show the good sensitivity of the qPCR technique in symptomatic samples. These results are similar to those obtained by Poitras & Houde (2002) who showed that the sensitivity of qPCR diagnosis is higher than that of PCR diagnosis. According to these authors, qPCR technology, based on the detection and quantification of a fluorescent indicator whose emission is directly proportional to the quantity of amplicons generated during the reaction, does not require any post-amplification manipulation. As a result, the problems of post-PCR contamination by amplicons are significantly reduced. In addition, qPCR relies on the ability to monitor the amount of DNA present in the reaction at any time and not at the end as in PCR (Kubista et al., 2006). Thus, this specific feature of qPCR could improve detection within a sample. In the TPA group, the results showed a decrease in detection rate from 53.33% (with PCR diagnosis) to 45.49% (with qPCR diagnosis). These results could be explained by the fact that the PCR primers used (Badna 1/4 CSSV Deg 2) are broad spectrum (degenerate). They thus have the possibility to hybridize to another region of the genome that may vary slightly. Furthermore, the conserved motifs corresponding to the replication enzymes used to design these primers are common to some retro-transposons identified largely in the cocoa genome (Argout et al., 2017). Indeed, according to these authors primers used in PCR could often amplify retro-transposons that have similar motifs or sequences to CSSV isolates (Harper et al., 1999). Thus, these degenerate primers used in PCR are more versatile, but may otherwise show false positives. In contrast, the primers used in qPCR, although also degenerate, have been designed in the movement protein region making them more specific for detecting only viral sequences corresponding to CSSV.

Taking into account%). These results are similar to those of Kouakou et al. (2012) who showed that symptomatic plant material may have a better detection rate for CSSV than asymptomatic cocoa trees. Furthermore ; 19.22% of individuals in the APT group were positive for CSSV in both PCR and qPCR. This could mean that regardless of the detection method used; 19.22% of the samples in group 1 (asymptomatic field and greenhouse samples) have the virus in them. These results confirm the tolerance of these TPAs to CSSV. Indeed, this tolerance would be described by the fact that some APTs could have the virus in them without having symptoms of the disease in the field and in the greenhouse. Also ; 27.45% of the samples in the APT group were negative in PCR and qPCR. This would mean that these asymptomatic field and greenhouse samples do not have the virus in them. This could suggest, on the one hand, a form of resistance of the plant to the virus or to the mealy bug and, on the other hand, a phenomenon of evasion, i.e. the absence of contact between the plant and the pathogen.

The molecular diagnosis qPCR also allowed the quantification of the viral load within the samples. This quantification was carried out by the appreciation and comparison of the threshold values of the Threshold Cycle (Ct) and also by the appreciation of the curves materializing the appearance of the amplification products. The results of this study showed that 34.94% of the TPAs had very low viral loads with Ct values between 35 and 40. Also; 33.33% of APTs have near zero viral concentration with Ct values much higher than 40. The very low and near-zero viral loads identified in 34.94% and 33.33% of APTs, respectively, could clearly explain the absence of symptoms in these accessions (Muller et al., 2001). Thus, all accessions in the APT group that have low or no virus load within them could be described as resistant to CSSVD. This could be a potential resistance of the plant to the vector or virus. All of these accessions could be subject to a dodging phenomenon that would suggest non-contact between the plant and the virus. Simultaneously the PCR and qPCR molecular diagnosis according to the origins and phenotypic groups of the accessions, the analysis of the results shows that the individuals of the APS group have the highest percentage of positive samples in PCR and qPCR with 55.1%. They are followed by samples from the APT/APS group (50%). These results are similar to those of Kouakou et al. (2012) who showed that symptomatic plant material may have a better detection rate for CSSV than asymptomatic cocoa trees. Furthermore ; 19.22% of individuals in the APT group were positive for CSSV in both PCR and qPCR. This could mean that regardless of the detection method used; 19.22% of the samples in group 1 (asymptomatic field and greenhouse samples) have the virus in them. These results confirm the tolerance of these TPAs to CSSV. Indeed, this tolerance would be described by the fact that some APTs could have the virus in them without having symptoms of the disease in the field and in the greenhouse. Also ; 27.45% of the samples in the APT group were negative in PCR and qPCR. This would mean that these asymptomatic field and greenhouse samples do not have the virus in them. This could suggest, on the one hand, a form of resistance of the plant to the virus or to the mealy bug and, on the other hand, a phenomenon of evasion, i.e. the absence of contact between the plant and the pathogen. The molecular diagnosis qPCR also allowed the quantification of the viral load within the samples. This quantification was carried out by the appreciation and comparison of the threshold values of the Threshold Cycle (Ct) and also by the appreciation of the curves materializing the appearance of the amplification products. The results of this study showed that 34.94% of the APTs had very low viral loads with Ct values between 35 and 40. Also ; 33.33% of APTs have near zero viral concentration with Ct values much higher than 40. The very low and near-zero viral loads identified in 34.94% and 33.33% of APTs, respectively, could clearly explain the absence of symptoms in these accessions (Muller et al., 2001). Thus, all accessions in the APT group that have low or no virus load within them could be described as resistant to CSSVD. This could be a potential resistance of the plant to the vector or virus. All of these accessions could be subject to a dodging phenomenon that would suggest non-contact between the plant and the virus.

# Conclusion

This work allowed a better appreciation of the behaviour of potentially tolerant (APT) and susceptible (APS) cocoa trees in the greenhouse with respect to swollen shoot. It appears from this study that three phenotypic groups have been identified in the greenhouse. Group 1 is composed of accessions that were symptom free in the field and in the greenhouse (APT). Group 2 consists of accessions that were symptomatic in the field and in the greenhouse (APS) and finally, group 3 consists of accessions that were asymptomatic in the field but that, once in the greenhouse, had CSSV symptoms (APT/APS). This new phenotypic group (APT/APS) revealed in the greenhouse constitutes an indicator of the presence of the virus inside the APTs and of the possibility of the existence of a form of tolerance to the disease essentially materialized by the latency time. These results could confirm the possibility of finding within the analyzed population, a potential tolerance or resistance to CSSVD.

The second objective of this study was to investigate and quantify CSSV in farmers' accessions evaluated in the greenhouse and found to be potentially tolerant to swollen shoot. It constitutes an important approach in the understanding of the defense mechanism deployed by cocoa genotypes against the disease.

From this study, it was found that 19.22% (PCR and qPCR positive) and 27.45% (PCR and qPCR negative) of the APT accessions were qualified as tolerant and resistant to swollen shoot disease respectively. Five levels of infection were revealed based on Ct values. In addition, 34.94 and 33.33% of APTs had very low and almost no virus load, respectively, which could explain the absence of symptoms on the plants.

This study allowed us to draw a conclusion on the susceptibility to CSSV of potentially disease-tolerant farmer cocoa accessions in Côte d'Ivoire. It allowed the identification of sources of resistance to the disease that could be introduced in a multilocal evaluation trial in CSSVD-infected areas in order to confirm the characteristics of these genotypes.

However, in view of the significant genetic variability existing within this virus species, it is appropriate to identify the isolates present within the population and to highlight the phylogenetic relationships existing between them.

# References

**Abrokwah F, Dzahini-Obiatey H, Galyuon I, Osae-Awuku F & Muller E (2016).** Distribution géographique de la variabilité moléculaire du cacao swollen shoot virus au Ghana. Plant Disease 100 : 2011-2017.

**Allegre M, Argout X, Boccara M, Fouet O, Roguet Y, Berard A, Thevenin JM, Chauveau A, Rivallan R & Clement D (2012).** Découverte et cartographie d'un nouveau panel d'étiquettes de séquences exprimées, de polymorphismes de nucléotides simples et de répétitions de séquences simples pour les études génétiques à grande échelle et la sélection de Theobroma cacao L. DNA Resources 19 : 23-35.

**Argout X, Martin G, Droc G, Fouet O, Labadie K, Rivals E, Aury JM & Lanaud C (2017).** Le génome Criollo du cacao v2.0 : une version améliorée du génome pour les études génétiques et de génomique fonctionnelle. BMC Genomics 18 (1) : 730.doi: 10.1186/s12864-017-4120-9.

**Assiri AA, Yoro GR, Deheuvels O, Kebe BI, Keli ZJ, Adiko A & Assa A (2009).** Les caractéristiques agronomiques des vergers de cacaoyers (Theobroma cacao L.) en Côte d'Ivoire. Journal of Animal & Plant Sciences 2 (1) : 55-56.

**Assiri AA, Deheuvels O, Keli ZJ, Kebe BI, Konan, A & Koffi N (2016).** Identification des caractéristiques agronomiques pour le diagnostic et la prise de décision de régénération des vergers de cacaoyers en Côte d'Ivoire. African Crop Science Journal 24 (3) : 233-234.

**Cheesman E (1944).** Notes sur la nomenclature, la classification et les relations possibles des populations de cacaoyers. Tropical Agriculture 21 : 144-159.

**Chingandu N, Kouakou K, Aka R, Ameyaw G, Gutierrez OA, Herrmann HW & Brown JK (2017).** La nouvelle espèce proposée, le cacao red vein virus, et trois espèces de badnavirus précédemment reconnues sont associées à la maladie du swollen shoot du cacao. Virology Journal 14 (1) : 199. doi : 10.1186/s12985-017-0866-6.

**Dahal G, Hugues JD & Thottappilly G (1997).** Effect of temperature on symptom expression and reliability of banana streak badnavirus detection in naturally infected plantain and banana (Musa spp,). Plant Disease 82 : 16-21.

**Dzahini-Obiatey H, Ameyaw GA, & Ollennu LA (2006).** Contrôle de la maladie des pousses gonflées du cacao par l'éradication des arbres infectés au Ghana : Une enquête sur les zones traitées et replantées. Crop Protection 25 : 647-652.

**Dzahini-Obiatey H & Fox RTV (2010).** Signes précoces d'infection dans les graines de cacao inoculées par le Cocoa swollen shoot virus (CSSV) et découverte des cotylédons des plantes résultantes comme sources riches de CSSV. African Journal of Biotechnology 9 (5) : 593-603.

**Fouet O, Allegre M, Argout X, Jeanneau M, Lemainque A, Pavek S, Boland A, Risterucci AM & Tahi GM (2011).** Caractérisation structurelle et cartographie de marqueurs EST-SSR fonctionnels chez Theobroma cacao. Tree Genetics & Genomes 7 : 799-817.

**Harper G, Dahal G, Thottappily G & Hull R (1999).** Detection of episomal banana streak badnavirus by IC-PCR. Journal of Virological Methods 79 : 1-8.

**ICCO (Organisation internationale du cacao) (2014).** Bulletin trimestriel des statistiques du cacao, vol, n° 4, année cacaoyère 2012/2013.

**ICCO (Organisation internationale du cacao) (2017).** Bulletin trimestriel de statistiques du cacao, n°3, année cacaoyère 2016/2017.

**Koua SH, Coulibaly NAMD & Alloué-Boraud WAM (2018).** Caractérisation des vergers et des maladies de cacao de Côte d'Ivoire : cas des départements d'Abengourou, Divo et Soubré. Journal of Animal & Plant Sciences 35 (3) : 5706-5714.

**Kouakou K, Kébé BI, Kouassi N, Aké S, Cilas C & Muller E (2012).** Distribution géographique de la variabilité moléculaire du Cocoa swollen shoot virus en Côte d'Ivoire. Plant Disease 96 : 1445-1450

**Kouamé N, N'Guessan F, N'Guessan H, N'Guessan P & Tano Y. (2014).** Variations saisonnières des populations de mirides du cacaoyer dans la région de l'Indénié-Djuablin en Côte d'Ivoire. Journal des Biosciences Appliquées 83 : 7595-7605

**Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, Sindelka R, Sjogreen B, Strombom L, Stahlberg A & Zoric N (2006).** La réaction en chaîne par polymérase en temps réel. Molecular Aspects of Medecine 27 (2-3) : 95-125.

**Lachenaud P, Eskes AB, N'goran JAK, Clement D, Kebe I, Tahi GM & Cilas C (2001).** Premier cycle de sélection récurrente en Côte d'Ivoire et choix des géniteurs du second cycle, pp 11-22. In : Actes de la 13e conférence internationale sur la recherche sur le cacao, Kota Kinabalu, Sabah (Malaisie).

**Lanaud C, Risterucci A, Pieretti I, Falque M, Bouet A & Lagoda PJL (1999).** Isolation et caractérisation des microsatellites chez Theobroma cacao L. Molecular Ecology 8 : 2141-2143.

**Londono D (2011).** Les lipoprotéines bactériennes peuvent se disséminer depuis la périphérie pour enflammer le cerveau. American Journal of Pathology 3 : 1891-1908.

**Mathiesen G (2009).** Analyse à l'échelle du génome de la fonctionnalité du peptide signal chez Lactobacillus. BMC Genomics 10 : 425, doi : 10.1186/1471-2164-10-425.

**Muller E, Jacquot E & Yot P (2001).** Détection précoce du cacao swollen shoot virus à l'aide de la réaction en chaîne par polymérase. Journal of Virological Methods 93 : 15-22.

**Ollennu LAA, Owusu GK & Thresh JM (1989).** Spread of cocoa swollen shoot virus to recent plantings in Ghana. Crop Protection 8 : 251-264.

**Poitras E & Houde A. (2002).** La PCR en temps réel : principes et applications. Reviews in Biology and Biotechnology 2 : 2-11.

**LIST OF TABLES AND FIGURES**

Table 1. Locations and phenotypic groups of cocoa samples used for molecular diagnosis against CSSV

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Départements** | Groupe 1 (APT) | Groupe 2 (APS) | Groupe 3 (APT/APS) | Total |
| **Agnibilékro** | 2 | 0 | 0 | 2 |
| **Bangolo** | 9 | 0 | 0 | 9 |
| **Bouaflé** | 99 | 38 | 8 | 145 |
| **Duekoue** | 25 | 0 | 0 | 25 |
| **Issia** | 6 | 0 | 1 | 7 |
| **Meagui** | 56 | 10 | 4 | 70 |
| **Sinfra** | 55 | 1 | 3 | 59 |
| **Soubre** | 3 | 0 | 0 | 3 |
| **Total** | **255** | **49** | **16** | **320** |

Table 2. Sequences of two cocoa SSR primers used for total DNA quantification.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Amorces** | **Brin Forward (F)** | **Brin Reverse (R)** | **Tm (°C)** | **Taille (pb)** |
| mTcCIR 331 | CCC CTC CAT TTG GTT TC | CAT GAC CTC CGC CTG T | 56,2 | 222 |
| MTcCIR 351 | CCA AAG AAA CAG GAA GCA A | GAA GTT AGG GGC ATG GG | 56,4 | 270 |

Tm : hybridization temperature

Table 3 Volume of products and reagents used for PCR with SSR primers

|  |  |
| --- | --- |
| **Réactifs** | **Volume (µL) 1 X** |
| Eau pure | 8,3 |
| Tampon Promega (5 X)  dNTP (5000 µM) | 5  1 |
| Cacao Forward primer (10 µM) | 0,25 |
| Cacao Reverse primer (10 µM) | 0,25 |
| Taq polymerase (5 U/µL) | 0,2 |
| ADN (1 ng/µL) | 5 |
| **Total** | **25** |

Table 4. Primer pair sequences used for molecular diagnosis of CSSV

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Amorces** | **Brin Forward (F)** | **Brin Reverse (R)** | **Tm (°C)** | **Taille (pb)** |
| Badna ¼ CSSV Deg 2 | CCATCCCTTGGACHGCNTTYTGGGT | TTACATACGGCNCCCCAHCCYTCCAT | 57 | 626 |

Table 5. Volume of products and reagents used for PCR with Taq Promega

|  |  |
| --- | --- |
| **Réactifs** | **Volume (µL) 1 X** |
| Eau pure sans nucléase | 11,8 |
| Tampon 5 X Promega  dNTP 10 mM | 5  1 |
| CSSV Forward primer, 10 µM | 1 |
| CSSV Reverse primer, 10 µM | 1 |
| Taq polymerase Promega | 0,2 |
| DNA Template | 5 |
| **Total** | **25** |

Table 6. Volume of products and reagents used for PCR with Taq Phire

|  |  |
| --- | --- |
| **Réactifs** | **Volume (µL) 1X** |
| Eau pure sans nucléase | 11,2 |
| 5X Phire Green Reaction Buffer,  Mix dNTP (10 mM) | 4  0,4 |
| CSSV Forward primer, 10 µM | 1 |
| CSSV Reverse primer, 10 µM | 1 |
| Phire Hot Start II DNA Polymerase | 0,4 |
| DNA Template | 2 |
| Total | 20 |

Table 7. Volume of products and reagents used for qPCR.

|  |  |
| --- | --- |
| **Solution stock** | **1 R° en µL** |
| Matrice CDN | 3 |
| Amorces F+R à 5µM | 1,5 |
| H2O | 3 |
| 2 X Sybergreen | 7,5 |
| **Total** | **15** |

Table 8. Number of PCR positive cocoa samples (detection rate) with Badna ¼ CSSV Deg 2 primer by locality and phenotypic group

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Origines des accessions** | **Nombre d’échantillons positifs (%)** | | | |
| **APT** | **APS** | **APT/APS** | **Total** |
| Agnibilékro | 0 (0 %) | 0 | 0 | 0 (0 %) |
| Bangolo | 6 (66,67 %) | 0 | 0 | 6 (66,67 %) |
| Bouaflé | 55 (55,56 %) | 27 (71,05 %) | 5 (62,5 %) | 87 (60 %) |
| Duekoué | 9 (36 %) | 0 | 0 | 9 (36 %) |
| Issia | 0 (0 %) | 0 (0 %) | 0 (0 %) | 0 (0 %) |
| Meagui | 21 (37,5 %) | 7 (70 %) | 2 (50 %) | 30 (42,85 %) |
| Sinfra | 43 (78,18 %) | 1 (100 %) | 1 (33,33 %) | 45 (76,27 %) |
| Soubré | 2 (66,67 %) | 0 | 0 | 2 (66,67 %) |
| **Diagnostic positif PCR** | **136/255**  **(53,33 %)** | **35/49**  **(71,42 %)** | **8/16**  **(50 %)** | **179/320**  **(55,93 %)** |

*Table 9. Number of qPCR positive cocoa samples (detection rate) with Bd 2 probe by locality and phenotypic group*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Origines des accessions** | **Nombre d’échantillons positifs (%)** | | | |
| **APT** | **APS** | **APT/APS** | **Total** |
| Agnibilékro | 1 (50 %) | 0 | 0 | 1 (50 %) |
| Bangolo | 4 (44,44 %) | 0 | 0 | 4 (44,44 %) |
| Bouaflé | 45 (45,45 %) | 31 (81,58 %) | 7 (87,5 %) | 83 (57,24 %) |
| Duekoué | 6 (24 %) | 0 | 0 | 6 (24 %) |
| Issia | 2 (33,33 %) | 0 | 1 (100 %) | 3 (42,86 %) |
| Meagui | 11 (19,64 %) | 7 (70 %) | 4 (100 %) | 22 (31,43 %) |
| Sinfra | 46 (83,64 %) | 1(100 %) | 3 (100 %) | 50 (84,75 %) |
| Soubré | 1 (33,33 %) | 0 | 0 | 1 (33,33 %) |
| **Diagnostic positif** | **116/255**  **(45,49 %)** | **39/49**  **(79,59 %)** | **15/16**  **(93,75 %)** | **170/320**  **(53,13 %)** |

*Table 10. Number of cocoa samples positive in both PCR and qPCR (Detection rate) by locality and phenotypic group)*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Origines des accessions** | **Nombre d’échantillons positifs (%)** | | | |
| **APT** | **APS** | **APT/APS** | **Total** |
| Agnibilékro | 0 (0 %) | 0 | 0 | 0 |
| Bangolo | 3 (33,33 %) | 0 | 0 | 3 (33,33 %) |
| Bouaflé | 21 (21,21 %) | 21 (55,26 %) | 5 (62,5 %) | 47 (32,41 %) |
| Duekoué | 1 (4 %) | 0 | 0 | 1 (4 %) |
| Issia | 0 (0 %) | 0 | 0 (0 %) | 0 (0 %) |
| Meagui | 2 (3,57 %) | 5 (50 %) | 2 (50 %) | 9 (12,86 %) |
| Sinfra | 21 (38,18 %) | 1 (100 %) | 1 (33,33 %) | 23 (38,98 %) |
| Soubré | 1 (33,33 %) | 0 | 0 | 1 (33,33 %) |
| **Diagnostic positif** | **49/255 (19,22 %)** | **27/49 (55,1 %)** | **8/16**  **(50 %)** | **84/320 (26,25 %)** |

*Table 11. Number of samples of cocoa trees negative in both PCR and qPCR (Detection Rate) by origin and Detection rate) by origin and phenotypic group*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Origines des accessions** | **Nombre d’échantillons négatifs (%)** | | | |
| **APT** | **APS** | **APT/APS** | **Total** |
| Agnibilékro | 1 (50 %) | 0 | 0 | 1 (50 %) |
| Bangolo | 2 (22,22 %) | 0 | 0 | 2 (22,22 %) |
| Bouaflé | 21 (21,21 %) | 4 (10,53 %) | 1 (12,5 %) | 26 (17,9 %) |
| Duekoué | 10 (40 %) | 0 | 0 | 10 (4 %) |
| Issia | 4 (66,67 %) | 0 | 0 (0 %) | 4 (57,14 %) |
| Meagui | 26 (46,43 %) | 1 (10 %) | 0 (0 %) | 27 (38,57 %) |
| Sinfra | 5 (9,09 %) | 0(0 %) | 0 (0 %) | 5 (8,47 %)) |
| Soubre | 1 (33,33 %) | 0 | 0 | 1 (33,33 %) |
| **Diagnostic négatifs** | **70/255 (27,45 %)** | **5/49 (10,20 %)** | **1/16 (6,25 %)** | **76/320 (23,75 %)** |

*Table 12. Number of samples (percentage) according to different viral loads and phenotypic groups*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Groupes phénotypiques** | **Effectifs analysés** | **Nombre d’échantillons (%)** | | | | | |
| **Charge virale** | | | | | |
| **20 et 25 Ct** | **25 et 30 Ct** | | **30 et 35 Ct** | **35 et 40 Ct** | **Plus de 40 Ct** |
| **APT** | 255 | 13 (5,1 %) | | 5 (1,96 %) | 9 (3,53 %) | 89 (34,9 %) | 85 (33,33 %) |
| **APS** | 49 | 5 (10,20 %) | | 6 (12,24 %) | 4 (8,16 %) | 24 (48,98 %) | 9 (18,37 %) |
| **APT/APS** | 19 | 0 (0 %) | | 2 (12,5 %) | 3 (18,75 %) | 10 (62,5 %) | 1 (6,25 %) |
| **TOTAL** | 320 | 18 (5,63 %) | | 13 (4,06 %) | 16 (5 %) | 123 (38,44 %) | 95 (29,69 %) |

APT: Potentially Tolerant Accessions; PTA: Potentially Susceptible Accessions; APT/APS: Potentially Tolerant Accessions that have shown symptoms of the disease in the greenhouse; Ct: Treshold Cycle

**LIST OF FIGURES**

|  |  |
| --- | --- |
| E:\PHOTOS SYMPTÖMES\IMG-20190109-WA0005 (1).jpg  A | E:\PHOTOS SYMPTÖMES\IMG-20190109-WA0001 (1).jpg  B |

Fig 1. Symptomatic manifestation of swollen shoot disease on a field (A) and greenhouse (B) accession (A) and in the greenhouse (B)