

Article

High-Throughput Virus Screening in Crosses of South American and African Cassava Germplasm Reveals Broad-Spectrum Resistance against Viruses Causing Cassava Brown Streak Disease and Cassava Mosaic Virus Disease

Samar Sheat ^{1,*}, Xiaofei Zhang ²  and Stephan Winter ^{1,*} 

¹ Plant Virus Department, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, 38124 Braunschweig, Germany

² Alliance of Bioversity International and CIAT, The Americas Hub, Cali 763537, Colombia; xiaofei.zhang@cgiar.org

* Correspondence: samar.sheat@dsmz.de (S.S.); stephan.winter@dsmz.de (S.W.); Tel.: +49-5312993780 (S.W.)

Abstract: Screening cassava lines for resistance against viruses causing cassava brown streak disease (CBSD) is cumbersome because of the unpredictable and erratic virus infections in the slow plant infection processes that are frequently not associated with distinct leaf symptoms and because of the reliance on the assessment of root necrosis as an indicator of plant resistance/tolerance. The selection of resistant candidates thus extends over several growing cycles and is still associated with uncertainties about the stage of virus infection. To reduce the time for selection of resistant crosses and the uncertainties associated with field screening, we have developed a fast-forward virus screening workflow to assess cassava seedlings from crosses of cassava brown streak- and cassava mosaic virus-resistant parents. After passing through an intensive and precise virus infection routine, cassava seedlings that carried resistance against cassava brown streak and mosaic viruses were identified. Taking the results of 195 seedlings from 18 crossing families together, it became evident that resistance against the viruses causing CBSD is a dominant trait. The protocol developed for virus resistance screening in cassava can be readily adopted. It shifts resistance evaluation from the field to the nursery and replaces the erroneous and lengthy virus infection and screening process with a method of precision and speed.

Keywords: grafting; virus resistance; CBSD; CMD dominant trait; virus indexing



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1. Introduction

Among many traits considered for improving cassava crops in Africa, virus resistance is an invariable prerequisite. All cassava (*Manihot esculenta* subsp. *esculenta*) varieties grown on the continent require resistance against cassava mosaic begomoviruses (CMB) causing cassava mosaic disease (CMD) and against cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV), collectively termed U/CBSV, causing cassava brown streak disease (CBSD).

Breeding for resistance against cassava mosaic viruses and brown streak viruses has been pursued since the Amani program in 1930 [1]. Then, CMD resistance was obtained by crossing *Manihot esculenta* with its relative, *Manihot glaziovii* Muell.-Arg, to achieve virus resistance and good root yields in subsequent backcrosses [1,2]. CMD1 resistance is of a recovery type, recessive and polygenic. The CMD2 resistance later identified in the West African cassava landrace TME 3 [3] provides extreme resistance against all known viruses causing CMD [4–6] and is now the predominant cassava mosaic virus resistance source. Cassava varieties such as TME 204 (syn. TME 419/Obama) express a broad-spectrum resistance against all eleven African begomoviruses species causing CMD, including the Indian and Sri Lankan cassava mosaic viruses (ICMV and SLCMV, respectively). CMD2

is monogenic, has a dominant inheritance, and is very stable and consistent in conferring virus resistance [7–9]. The CMD3, described in the elite cultivar TMS 97/2205 selected from crosses of TMS 30572 (CMD1-resistant type) and TME 6 (CMD2-resistant type) and is a quantitative trait locus [3,7,9–11] and similarly confers very high resistance against the viruses causing CMD.

In contrast to mosaic virus resistance, there is no resistance against viruses causing CBSD in African cassava varieties, and all of the common landraces and improved cassava varieties eventually succumb to this devastating tuber disease. We recently identified virus resistance in South American cassava germplasm [12], screening 238 germplasm accessions from the core cassava diversity held at the International Center for Tropical Agriculture (CIAT), Cali, Colombia, for resistance against U/CBSV. Most lines were highly susceptible to virus infections, others had a differential resistance against CBSV and susceptibility against UCBSV, while some cassava germplasm accessions were virus-free in upper plant parts but restricted CBSV to the roots. Finally, after a stringent screening process, three cassava lines did not become infected at all. However, virus tests under laboratory and field conditions also revealed that CMB resistance was essentially absent in South American cassava lines. Thus, cassava mosaic virus resistance needed to be introgressed to complement cassava brown streak virus resistance with cassava mosaic virus resistance.

The success of breeding cassava for virus resistance requires sources of resistance in readily flowering genotypes to produce viable crosses and seeds and a straightforward screening process to identify resistant progenies. Within the framework of our NextGen Cassava Partnership (<https://www.nextgencassava.org>, accessed on 23 February 2022), crossing efforts were initiated in 2019 at the International Center for Tropical Agriculture, CIAT, Colombia, to combine the resistances from African and South American sources into hybrids with dual virus resistance. When seeds from the first crosses became available in 2020, the most appropriate virus screening workflow was to be developed that would lead to the identification of broad-spectrum virus resistance, also considering that the inheritance of the cassava brown streak virus resistance was unknown.

Screening for resistance against CMB is straightforward because planting seedlings into infection hotspots where ample numbers of whitefly (*Bemisia tabaci* Gen.) vector insects are present can guarantee virus infections from viruliferous whiteflies [13]. Virus symptoms become readily visible and scoring for disease incidence and severity allows the elimination of symptomatic plants and a selection of resistant candidates during the first or second planting season [14]. In contrast, screening for resistance against cassava brown streak viruses under field conditions is cumbersome because of the unpredictable virus transmission from whitefly populations and the slow virus infection processes. Since virus infections are often not associated with distinct leaf symptoms [15], the extent of root necrosis is commonly used as an index of resistance/tolerance [16,17], which hampers early assessment. In addition, the complex infection biology of the cassava brown streak viruses presents an inherent risk of mistaking plants with healthy-appearing roots or low disease severity scores as resistant or as being free from infection. Consequently, to improve accuracy, the selection of promising U/CBSV resistance candidate lines extends over several growing cycles [16].

Based on the success of our CBSD resistance screening workflow [12], we have tested a fast-forward virus screening protocol to assess crosses between cassava brown streak virus- and cassava mosaic virus-resistant parents for dual virus resistance. Passing cassava seedlings through an intensive and precise virus infection routine allowed an accurate assessment of resistance against mosaic and brown streak viruses in approximately nine months—from seedling infection to a final verdict on resistance status. The components tactics were: controlled and effective plant infections to eliminate CBSV-susceptible lines early followed by mosaic virus resistance testing with pre-selected lines only. By shifting the resistance evaluation from the field to the nursery, the erroneous and lengthy infection and screening process was replaced with a method of precision and speed.

2. Materials and Methods

2.1. Cassava Viruses

All cassava plants for this study were maintained under glasshouse conditions at temperatures between 26 °C to 32 °C and >75% relative humidity, with additional light provided during German winter conditions. The virus isolate, CBSV-Mo83 (DSMZ PV-0949, GenBank accession FN434436), was maintained in the cassava variety TMS 96/0304. A cassava TME 117 infected with East African cassava mosaic virus Uganda (DSMZ, PV-1302, GenBank accession OL444942, OL4449943) was used. To accelerate the screening process, the TME 117 was mixed-infected with UCBSV (GenBank accession MW961202). All infected plants were propagated with stem cuttings to produce sufficient virus inoculum for the graft infections.

2.2. Cassava Seeds and Seedlings

Crosses among parents with complementary traits to combine both resistances were made. The CMD-resistant materials were either clones that were recently evaluated for resistance against SLCMV [17], derived from crosses between TMS30555 (NGA5 in CIAT germplasm bank) and TME3 [3] having CMD2 resistance or progenies of C-clones (GM) that were selected based on CMD2 linked markers. The cassava clones chosen to provide resistance against CBSV were either CoL40, with immunity against all cassava brown streak viruses, or PER221 and COL144, with resistance only to CBSV. In addition, PER353 was used, which restricted virus infections to the roots. The virus resistance status of all parental cassava lines (Table 1) was confirmed by subjecting the respective lines to the stringent infection procedures developed for CBSV, UCBSV, and EACMV. The best parental lines—COL40 and C33—combine immunity against all cassava brown streak viruses (COL40) and CMD2-type resistance acting against all known cassava mosaic viruses (C33).

Table 1. Resistance status of cassava lines used as crossing parents at CIAT. The virus resistance status was determined experimentally at DSMZ following earlier studies [12,18].

Cassava Line	CBSV	UCBSV	EACMV
COL 40/DSC 118	resistant	resistant	recovery
COL 2182/DSC 167	resistant	resistant	susceptible
PER 221/DSC 250	resistant	susceptible	susceptible
COL 144/DSC 120	resistant	susceptible	susceptible
PER 353/DSC 260	resistant/root restr.	resistant/root restr.	susceptible
C19	susceptible	susceptible	susceptible
C33	susceptible	susceptible	resistant
C39	susceptible	susceptible	?
C243	susceptible	susceptible	susceptible
C413	susceptible	susceptible	resistant
GM7673-3	susceptible	susceptible	?
GM1055B-1	susceptible	susceptible	?
GM10055B-2	susceptible	susceptible	?

? resistance status not known; root restr, root restricted.

Cassava seeds from crosses made at CIAT (Table 2) were subjected to hot water treatment at 65 °C for 3 min to synchronize and increase the germination rate, transplanted into soil, and kept at 38 °C and 70% humidity in a growth chamber. Seedlings emerging after approximately 14 days were grown until 3 to 5 leaves had developed, then potted into 2 L rose containers and transferred to a foil tunnel in the glasshouse for adjustment. The hot water treatment was repeated for seeds that had not germinated to increase the number of seedlings. After 3 to 4 weeks, two-node cuttings were taken from seedlings and rooted for further propagation.

Table 2. Seeds from families (Entry) obtained from crossing cassava lines with known U/CBSV resistance status.

Entry	Mother	Father	Seeds
1	PER 353	GM 7673-3	14
2	GM10054B-1	PER 221	15
3	GM10054B-1	PER 353	15
4	GM10054B-2	PER 353	15
5	GM10055B-2	PER 353	15
6	GM10062-1	PER 353	15
7	C 33	PER 221	14
8	C 33	PER 353	15
9	C 39	PER 353	12
10	C 243	PER 353	15
11	C 413	PER 353	15
12	COL 40	C 33	8
13	COL 144	GM 7673-3	15
14	COL 144	GM10055B-1	15
15	COL 144	GM10055B-2	15
16	COL 144	C 19	15
17	COL 144	C 33	15
18	COL 144	C 39	15
Total			258

2.3. Virus Infections

Virus infections were established in cassava seedlings, as described in [18], using a side-grafting approach. Scions having 2–4 buds were cut from CBSVs-infected TMS 96/0304 plants and side-grafted onto the stems of the plants to be tested (1.1) for virus response (Figure 1(2)). The inserted scions at the joint interface were protected from desiccation by a layer of parafilm for approximately 10 days, with the parafilm removed thereafter. Grafted plants were kept for 3 days in a closed tunnel at high humidity, after which the foil was gradually opened. After approximately 14 days, plants were transferred from the tunnel and maintained under glasshouse conditions for symptom inspection. Four weeks after, grafting symptoms developing on rootstocks indicated CBSV-susceptible seedlings (1.3). Seedlings that did not show symptoms at this time point (1.4) were considered as resistant candidates.

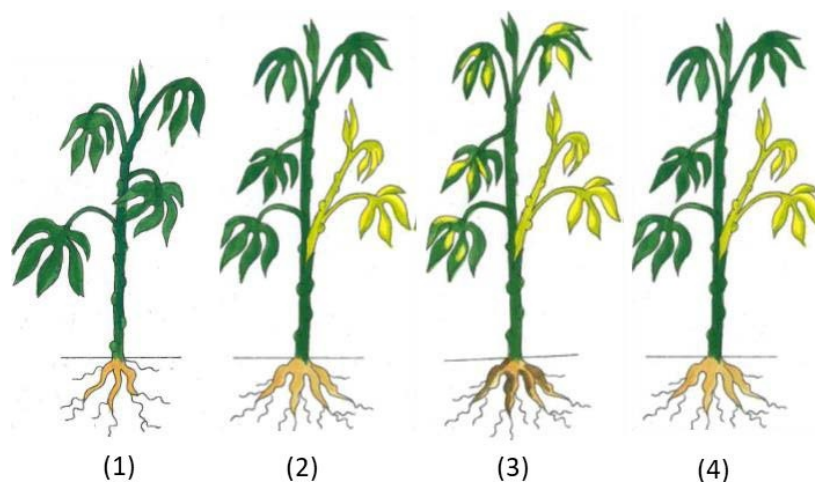


Figure 1. Introducing CBSV infections in 8-week-old cassava seedlings using scion grafting. Virus infections were introduced into cassava seedlings (1) by graft insertion of virus infected scions (2). A few weeks after grafting, typical symptoms for CBSV developing on leaves of rootstocks confirmed virus-susceptible seedlings (3), while the absence of symptoms in rootstocks indicated putative virus resistance (4).

EACMV-UG/UCBSV mixed infections were introduced only to those cassava plants previously graft-infected with CBSV that were free of virus symptoms and tested negative in qRT-PCR. Similar to CBSV grafting, scions from EACMV-UG/UCBSV mixed-infected plants were side-grafted onto the plants (Figure 2) and subsequently monitored for virus symptoms.



Figure 2. Introducing EACMV infections by side-grafting of scions (open circle) from EACMV-UG/UCBSV mixed-infected cassava.

2.4. Symptom Assessment and Virus Detection

For U/CBSV resistance screening, symptoms on leaves and roots were scored using a simplified scoring frame for the purpose. This was defined as: S for virus symptoms with S0, no symptoms on leaves; S− /S+, moderate symptoms on roots/no virus on the leaf; S+, mild symptoms on leaves only; S++, severe symptoms on leaves; and S+++, very severe symptoms on leaves. For EACMV scoring, a similar scale was used; however, because of the absence of symptoms on tubers, only leaf symptoms were recorded: S0, no symptoms on leaves; S?, inconspicuous symptoms on leaves; S+, moderate mosaic symptoms on leaves; S++, severe mosaic symptoms on leaves; S+++, severe mosaic symptoms with leaf distortion.

Total RNA from cassava leaves, stems, and tubers was extracted and used to detect U/CBSV by qRT-PCR, as described in our previous study [12]. For EACMV detection, total plant DNA was extracted from cassava leaves using a DNAeasy Plant Mini Kit (Qiagen, Germany), essentially following the manufacturer's protocol, and subjected to qPCR for EACMV detection, following an established protocol [19].

2.5. Resistance Screening Protocol

Each of the seedlings was subjected to a propagation step that included rapid bud multiplication. The virus screening protocol (Figure 3) adopted simplified our previous protocol developed for resistance discovery [12], with emphasis on the precision and

speed of the process. Considering the highly effective infection method and the known virus response of the crossing parents, infection with the severe isolate CBSV-Mo83 would result in early symptom development in phase 1, allowing us to eliminate susceptible cassava seedlings rapidly. Approximately 14 days after grafting, CBSV-infected plants were inspected for virus symptoms on stems and leaves. Symptom monitoring was carried out every three days and continued until symptoms became visible, the evaluation phase ending with virus testing by qRT-PCR and the removal of infected plants. Cassava seedlings that remained symptomless and virus-free for four weeks were subjected to screening phase 2, with grafting of a scion of an EACMV-UG/UCBSV mixed-infected plant onto the symptomless rootstock. Symptoms indicating virus infections were monitored regularly for five months, complemented by qRT-PCR/qPCR tests for virus detection, and plants with symptoms and viruses were eliminated. Detection of any of the viruses was taken as proof of the susceptibility of a cassava seedling and even the discovery of a virus in a single plant prompted the exclusion of the line from further testing. Six months after grafting, virus indexing was repeated with leaf, stem, and tuber materials to finalize the identification cycle (Figure 3). In phase 2 of the resistance screening, seedlings that tested virus-free during the first infection cycle were subjected to the second confirmation round of virus screening with more plants (biological replicas). In parallel with the greenhouse testing, phase 1 candidate seedlings were brought to the field in an epidemic zone (Plaine de la Ruzizi, DR Congo) to evaluate virus resistance and agronomic traits in natural environments (Figure 3).

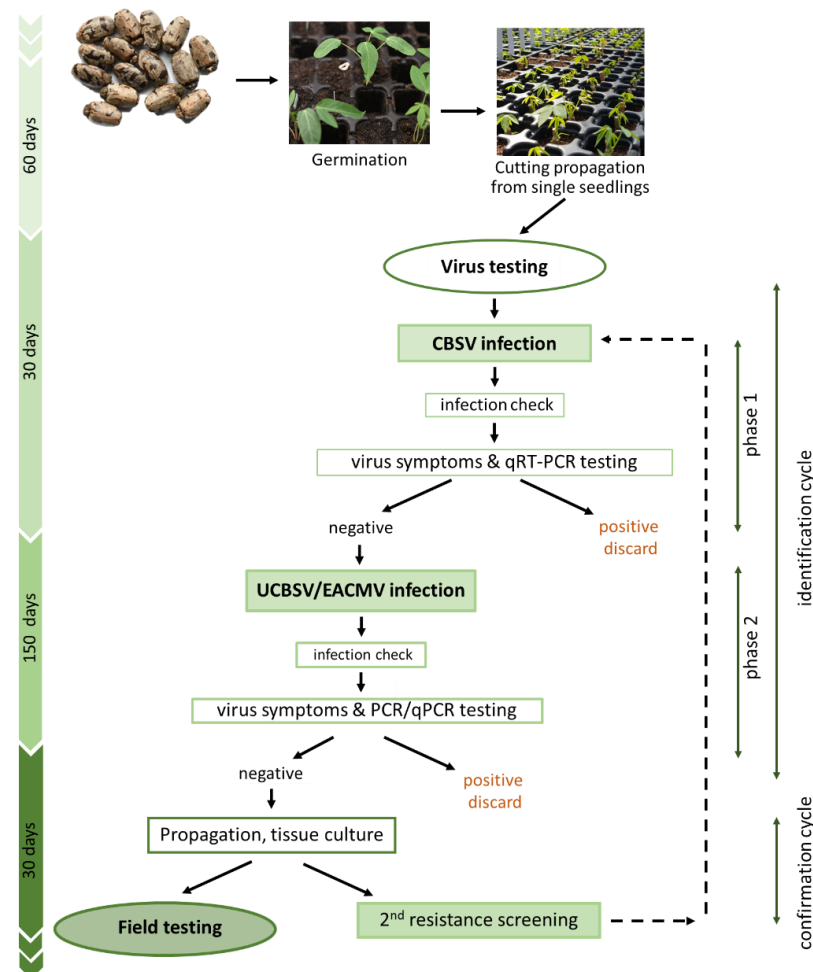


Figure 3. Testing for U/CBSV and EACMV virus resistance in cassava seedling families. The confirmation cycle consists of a further laboratory screening phase and parallel field testing to reduce evaluation time.

3. Results

The cassava crosses made by CIAT in the framework of our NextGen Cassava partnership resulted in 18 families comprising various parental combinations (Table 2). From 258 seeds received for virus testing, 195 germinated and, finally, 180 seedlings were subjected to virus infections.

3.1. Infection of Cassava Seedlings with CBSV, UCBSV, and EACMV-UG

Most of the cassava seedlings were susceptible to CBSV infections and developed systemic CBSV symptoms ranging from vein clearing and chlorosis to wilting of branches and leaves during the first month after grafting (MAG) (Table S1). Virus infections were confirmed by qRT-PCR on 124 seedlings, while 56 seedlings remained symptomless and viruses were not detected. However, further CBSV infections became evident during the following month, and, from the 56 plants testing negative 1 MAG, after six months, 41 of 180 cassava seedlings tested (23%) remained free of CBSV (Table 3).

Table 3. Virus resistance screening of 18 cassava seedling populations generated from crosses between U/CBSV- and EACMV-resistant parents. Evaluation of resistance against UCBSV and EACMV was only performed with the 41 cassava lines that had CBSV resistance.

Entry	Family	Seedling	CBSV Resistance		CBSV/UCBSV/EACMV Resistance
			1 MAG	6 MAG	6 MAG
1	GM13472	9	1	1	1
2	GM13473	12	7	6	
3	GM13474	14	5	2	
4	GM13475	12	5	3	
5	GM13477	9	3	2	2
6	GM13478	13	2	2	
7	GM13481	9	5	2	
8	GM13482	11	6	5	
9	GM13483	9	1	0	1
10	GM13484	12	5	2	
11	GM13485	13	2	2	
12	GM13487	5	2	1	
13	GM13489	13	4	3	1
14	GM13490	3	1	1	
15	GM13491	6	0	0	
16	GM13493	10	2	3	
17	GM13494	12	4	4	1
18	GM13495	8	2	2	
Total		180	57	41	5

Cassava seedlings that remained virus-free 1 MAG (Figure 4a) were subjected to phase 2 for EACMV-UG and UCBSV testing. Following graft infection, seedlings became virus-infected, showing cassava brown streak virus and/or EACMV symptoms, or remained free of symptoms.

Finally, after six months, five seedlings (Table 3) were free of any virus and the leaves, stems, and tubers of those lines were symptomless and virus-free. None of the viruses became established despite heavy infections in the grafted scions (Figure 4b).

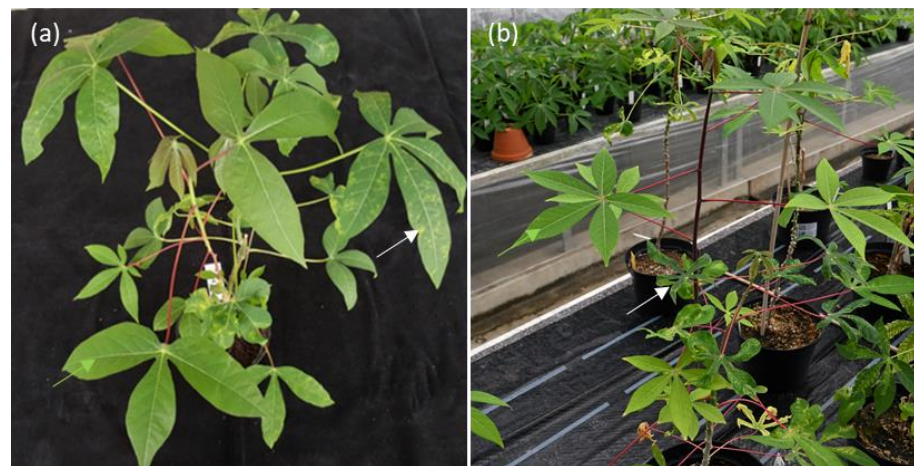


Figure 4. Virus resistance testing of cassava seedlings under controlled glasshouse conditions. (a) Typical CBSV symptoms (white arrow) of CBSV-infected scion (green petiole) used for graft infection. Virus-infected rootstock (red petiole) remains free of symptoms (white arrow). (b) Absence of symptoms (green arrow) in CBSV-resistant rootstock (from (a)) after graft infection with UCBSV/ EACMV indicates broad-spectrum resistance against EACMV and U/CBSV. Typical EACMV symptoms on EACMV-infected scion (white arrow).

The parental lines chosen for the crossing experiments had either CBSV resistance and were susceptible against EACMV or were resistant against EACMV and susceptible against CBSV. A CBSV-resistance phenotype—absence of symptoms and virus in F1 seedlings—in 16 families (except families 9 and 15, Table 3) thus indicated a dominant inheritance pattern and heterozygous alleles. Subsequent virus screening against UCBSV and EACMV-UG resulted in a few seedlings from four families that showed a combined resistance against all viruses tested, confirming that both cassava brown streak virus and cassava mosaic virus resistance have dominant inheritance.

3.2. Mounting a Precise and Efficient Screening Strategy to Identify Virus Resistance in Cassava

With the confirmation screening for virus resistance not final, the stringent virus screening protocol that followed (Figure 3) identified cassava candidates with resistance against all viruses. In 5 out of 180 (3%) seedlings from crosses between South American U/CBSV-resistant cassava and African CMB-resistant cassava lines, a broad-spectrum resistance was found already within an approximately six-month phase 1 of virus infection, monitoring, and indexing (Table 3). The second round of virus screenings for resistance confirmation has not yet been finalized; however, the most promising candidates are part of a preliminary field trial in a virus epidemic zone on the Plaine de la Ruzizi, DR Congo. The infection controls readily became infected with EACMV, showing prominent symptoms of the disease (Figure 5), but there were no signs of virus infection in the crosses selected for broad-spectrum resistance against cassava brown streak and cassava mosaic viruses.



Figure 5. Virus resistance testing of cassava seedlings under field conditions. Typical mosaic and leaf curling symptoms (white arrows) on leaves of cassava seedling 17-5, family 17 confirm cassava mosaic virus infections introduced by viruliferous whiteflies and sensitivity of the South American cassava.

4. Discussion

Progress in breeding for CBSD resistance is hampered by a lack of resistance sources and a complicated virus infection biology that prolongs field screening and resistance identification in promising candidate lines over several years. We searched, with limited success, most of the African cassava lines for cassava brown streak virus resistance and finally discovered natural resistance in South American cassava germplasm [12]. The identification and characterization of extreme virus resistance in some genotypes [20] of this germplasm can be considered a breakthrough in cassava brown streak virus resistance research.

The breeding cycle for cassava is long and can take six years to develop a new variety with high heritability traits and additional years to evaluate promising clones before release [21,22]. Since crosses from heterozygous progenitors are very diverse and every F1 seedling is genetically distinct, field evaluation in multilocation trials extends over many years following conventional breeding strategies [23]. Resistance against cassava mosaic virus was achieved in a six-year workflow by eliminating sensitive (symptomatic) genotypes in multiple cycles of plant growth and selection to identify virus-resistant genotypes [23,24]. Starting from botanical seeds of crosses in year one and F1 seedlings developing in a nursery in year two, seedlings with virus resistance were selected and then passed through further multiplication and single row trials (SRTs) in year three. In year four, between 100 and 300 genotypes were evaluated in preliminary yield trials (PYTs), with 40 to 50 genotypes being selected for advanced yield trials (AYTs) in year five. The best 10 to 30 genotypes then reached the uniform yield trials (UYTs) in year six [23,24]. Critical factors in screening for cassava mosaic virus resistance are efficient virus infection and an unequivocal identification of diseased plants. For CMB, the natural vector of the viruses, *B. tabaci*, is highly effective in transmitting and spreading the disease [25,26]. As shown in Figure 5, virus symptoms develop readily, and as soon as six weeks after planting pronounced symptoms of virus infection identified susceptible lines. Resistance selection is straightforward because only a few viruliferous whiteflies are needed for disease spread and pronounced symptoms develop readily in susceptible cassava lines. Moreover, the widely used CMD2-resistance trait provides extreme resistance and plants eventually recover from a symptomatic phase, after which infections stall and no further virus establishment takes place [3,5,7,8,27].

Similar to CMD, wild *Manihot* species were used in the Amani program to introgress resistance against the viruses causing CBSD into cassava [1]. The Tanzanian cassava variety Namikonga is a result of interspecific hybridization and backcrossing [2]. It has enhanced resistance levels and good agronomic performance and is used as a parent in African resistance breeding programs. Nevertheless, progress in CBSD-resistance breeding [16,28,29] is limited. Reports on current breeding and selection efforts reveal a lack of defined resistant sources and targets and, consequently, guide an ambiguous resistance phenotyping. Since time of infection relative to symptom incidence and severity is not taken into consideration [29,30], scoring the severities of leaf and tuber symptoms rather shows the variability of symptom expression across environments. Genome-wide association studies (GWASs) and genomic selection (GS) have been introduced to advance cassava resistance breeding [14,29]. However, the powers of these methods can only unfold when precise phenotyping can be based on defined virus infections.

We think that current approaches to evaluate CBSD resistance need to be critically improved by addressing major uncertainties concerning virus transmission, plant infections, and especially the starting time of the disease. *B. tabaci* transmits ipomoviruses in a semi-persistent mode, limiting a rapid field spread because viruses are not circulating in the insect and are lost with sucking and probing [31]. In contrast to cucumber vein yellowing virus, a related ipomovirus, the transmission of U/CBSV is very inefficient and a minimum of 25 whiteflies and long acquisition and inoculation periods are required to achieve plant infections [31]. This has tremendous implications for virus screening under field conditions because disease progress is erratic and virus spread only occurs when high numbers of whiteflies are present seasonally, after long or short rainy seasons. Furthermore, the slow infection processes associated with unclear symptomatology contribute to significant difficulties in evaluating incidence and the severity of virus infections.

Our study reduced the uncertainties of disease evaluation associated with natural virus infections by grafting scions of defined virus sources at defined time points to infect cassava precisely and eliminate susceptible lines early. While for particular cassava genotypes it can take many months until virus infections establish, in crosses between known CBSV-susceptible and -resistant parents, infections became apparent after two to four weeks. In our infection experiments, seedlings of several families did not become infected (Table 3), which provided proof that CBSV resistance in the selected South American cassava lines is a dominant trait in a heterozygous background. Unfortunately, this study employed only low numbers of seedlings from each family, which does not allow the accurate determination of Mendelian segregation. However, with the exception of two families, plants were found in all groups that did not become infected with CBSV, and this confirmed our findings from our earlier study. In our fast-forward screening approach, seedlings that did not become infected with CBSV were subsequently subjected to UCBSV/EACMV infections to assess whether the resistance would also hold also against further viruses. This procedure was possible because virus interactions between CBSV and UCBSV that would lead to cross protection cannot be assumed, hence a subliminal CBSV infection carried over from phase 1 (Figure 3) would not interfere with the evaluation of genetic resistance against UCBSV.

At the outset of our experiments, it was utterly unclear whether a resistance phenotype—absence of symptoms and absence of virus—would become evident in F1 hybrids. This would indicate that the CBSV resistance provided by the South American parental germplasm, COL 40, COL 144, COL 2182, PER 221, and PER 353, would be a dominant trait and, indeed, CBSV resistance was evident in some F1 crosses. Assuming that the parental lines providing EACMV resistance did not have CBSV-resistance alleles, the susceptible/resistant ratio observed (23%) was, however, far from the expected segregation pattern (50%). We provisionally explain this deviance with the low number of seedlings tested from each family, pending a revision of the inheritance pattern when numbers of seedlings can be tested that are sufficient for a statistical evaluation. Nevertheless, the fact that a resistance phenotype can be seen at such an early stage in F1 hybrids facilitates the resistance screening process enormously.

On the basis of our earlier work [12], we can predict that UCBSV infections will establish in seedlings from crosses with COL144, PER221, and PER353 and that only seedlings of family 12, COL40 × C33 (Table 2), would carry broad-spectrum resistance against all cassava brown streak virus species. Indeed, seedlings derived from COL40 (Table 3, family12) were protected against all U/CBSV isolates. Since the CMD2-type resistance provided by C33 is also a dominant trait, we found that one out of five seedlings in this family (Table 3) carried broad-spectrum resistance against EACMV and U/CBSV. Two further resistant germplasm lines, COL2182 and PER556 [12], that were not part of this study exhibit broad-spectrum immunity against U/CBSV similar to COL40 and would equally qualify as parents for resistance crosses.

The fast-forward approach in our virus screening workflow was possible because the resistance characters of the parents were well known. Notwithstanding the inheritance of the resistance trait and detrimental effects that may be inherent in the use of South American germplasm, the encouraging results from our crosses emphasize that virus resistance can be achieved in cassava when virus-resistant sources exist and are used in crosses. A precise infection process then supports an unambiguous resistance evaluation that may be broad-spectrum or limited to species and strains. A clear time point of the initiation of the infection will permit the association of foliar leaf symptoms and root necrosis with disease progress. Lastly, crosses lacking U/CBSV resistance will fall early to show virus symptoms and thus can be immediately exempt from further testing.

Our high-throughput virus screening challenge was to achieve a robust identification of susceptible lines, reduce the number of plants to be further tested, and identify virus resistance in breeding populations precisely. This would prevent a carryover of susceptible clones to the next breeding stage and dramatically reduce numbers and consequently the costs of field evaluations. Thus, our CBSV screening protocol will increase the selection accuracy and intensity and lead to increased genetic gains in breeding programs. This requires a collection of reference viruses ready for graft inoculation and highly efficient infection methods that guarantee virus infections and allow early identification and elimination of susceptible plants. A branch developing from an infected scion then presents a constant source of virus inoculum to the rootstock and these stringent conditions increase heritability. Starting the screening with a virulent virus allowed us to eliminate more than 75% in the first phase of screening and within one month to further accelerate the testing by simultaneously screening for two viruses.

The parents for the 18 seedling families were not only chosen for virus resistance traits and, indeed, as a best bet, only seedlings of family 12 would have been chosen because of defined virus resistances in the parental lines. The final seedling 12-1, with immunity against CMB and U/CBSV, and four other candidates selected are currently being subjected to a confirmation round of testing. This comprises virus assays with more plants and field trials in unreplicated plots to assess their agronomic performance.

In summary, by subjecting crosses from South American cassava lines to a stringent and fast-forward screening protocol, we confirmed the resistance against U/CBSV identified in our earlier study [12]. We selected hybrids with dual resistance against U/CBSV and CMD and provided evidence that, as with CMD2, resistance against U/CBSV is a dominant trait. The protocol that we present here for U/CBSV-resistance screening shortens the selection process and provides the precise phenotyping data required to support GWAS and GS and the genetic characterization of CBSV resistance.

Supplementary Materials: The following is available online at <https://www.mdpi.com/article/10.3390/agronomy12051055/s1>, Table S1: Symptoms of CBSV, UCBSV, and EACMV infections in cassava seedlings.

Author Contributions: Conceptualization, methodology, validation, S.S. and S.W.; writing—review and editing S.S., S.W. and X.Z.; investigation, S.S.; project administration, S.W.; funding acquisition, S.W. All authors have read and agreed to the published version of the manuscript.

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