BILL & MELINDA GATES foundation

Final Narrative

Use this form to provide your final update to your foundation program officer regarding the results achieved for the entire project. In addition, please provide your perspective on key lessons learned or takeaways and input on the foundation's support of your work to ensure that we can capture and share learnings as appropriate both internally and externally.

The Final Narrative must be submitted in Word, as PDFs will not be accepted.

General Information

Investment Title	NextGen Phytosanitation: Rapid Elimination of Viruses from RTB Plants for Crop Improvement and Seed Systems		
Grantee/Vendor	International Potato Center		
Primary Contact	Jan Kreuze	**Investment Start Date**	September 29, 2016
Feedback Contact[1]	Jan Kreuze	**Investment End Date**	December 31, 2020
Feedback Email[1]	j.kreuze@cgiar.org	**Reporting Period Start Date**	September 29, 2016
Program Officer	Jim Lorenzen	**Reporting Period End Date**	December 31, 2020
Program Coordinator	Randy Shigetani	**Reporting Due Date**	March 31, 2021
Investment Total	$2,385,906.00	**Opportunity/Contract ID**	INV-009425/OPP1130216
Remaining Funds (If applicable)	$		

[1] Feedback Contact/Email: the full name and email of the contact whom foundation staff queries for various surveys.

Submission Information

By submitting this report, I declare that I am authorized to certify, on behalf of the grantee or vendor identified on page 1, that I have examined the following statements and related attachments, and that to the best of my knowledge, they are true, correct and complete. I hereby also confirm that the grantee or vendor identified on page 1 has complied with all of the terms and conditions of the Grant Agreement or Contract for Services, as applicable, including but not limited to the clauses contained therein regarding Use of Funds, Anti-Terrorism, Subgrants and Subcontracts, and Regulated Activities.

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Progress and Results

1. Final Progress Details

Provide information regarding the entire investment's progress towards achieving the investment outputs and outcomes. In addition, submit the Results Tracker with actual results as requested.

1. Summary of progress completed toward the deliverables

SUMMARY

The project ran one year longer than originally anticipated, the loss of one of the project partners midway through the project, and delays in the final phases of the project due to the COVID-19 pandemic. Despite these facts, overall the project was successful in accomplishing its goals.

1. It was immediately apparent that growth and multiplication of *in vitro* cultures of all target crops, sweetpotato, yam, and cassava would be a major bottleneck and limitation to meeting the goals of the project. Therefore, major emphasis was placed on the testing of tissue culture (TC) parameters to improve the growth rates and recovery of meristems, critical for the development of phytosanitary clean plants. The main findings include:

- A reduction of macronutrients by one-fourth gave similar meristem regrowth rates in sweetpotato to simply replacing the NH4NO3 by KNO3 (elimination of NH4 in the medium) and resulted in 4–15X higher meristem regrowth rates in 55% of accessions tested. These results suggest that NH4 levels in the sweetpotato TC medium are too high and can be reduced, although results are accession specific. Experiments with reduction of nutrients for meristem regrowth in yam and cassava were performed slightly differently: by reduction of total medium components (macronutrients, micronutrients, and vitamins). In no case did a total reduction of medium components result in better or faster meristem recovery in either yam or cassava.
- Altering the hormone concentration and balance (auxin/cytokinin) did not result in better meristem growth or recovery for either cassava or yam.
- Although sweetpotato meristems from virus-infected plants had a significantly higher survival rate, there was no significant difference in the final recovery of plants between plants infected by a virus or not infected.
- The addition of charcoal to the medium had no significant effect on the regrowth of cassava meristems; however, it did have a significant enhancement in regrowth in 3/11 of yam accessions.
- The addition of antioxidants (glutathione and ascorbic acid) to the medium had no significant enhancement in regrowth in yam, yet in 2/11 of cassava accessions, ascorbic acid significantly enhanced the regrowth rate from meristems.
- Preliminary data indicated that a dark period immediately after sweetpotato meristem excision did not significantly improve meristem survival, although a later dark period (28–35 days post-excision) may increase survival. There was no benefit of a dark period on regrowth from either cassava or yam meristems.
- The most significant result for enhancing growth of sweetpotato meristems is the inclusion of a liquid culture phase during the regrowth process, which can reduce the time for the generation of plants from meristems from 27 weeks to 18 weeks, a 33% gain in efficiency.
- The most significant results with cassava and yam were that the addition of 25 mg/L and 40 mg/L of AA to the MS media used for cassava, and the addition of 4 g/L of activated charcoal to the MS media used for yam, can improve the regrowth rate.
- Overall, the effect of genotype is significant in all experiments.

2. Although the recovery of plants from meristems is a factor in the speed at which phytosanitary clean plant material can be obtained, the processes for elimination of viruses as well as the detection of viruses in plant material are also critical factors in the generation of virus-free planting materials. To facilitate the cleaning of plant material, the use of resistance inducers or antivirals, both *in vitro* and as a topical spray on greenhouse plants, was tested. In conjunction, the use of thermotherapy on greenhouse plants (as is routine in cassava) was tested for sweetpotato and yam. The main findings include:
   - Thermotherapy (4 weeks at 36°C) of sweetpotato greenhouse plants, followed by introduction of shoot tips into *in vitro*, is a successful method for the generation of virus-free material.
   - Topical spraying of sweetpotato greenhouse plants with salicylic acid (70 mg/L) or lactoferrin (100 mg/L), followed by introduction of stem segments into *in vitro*, is a successful method for the generation of virus-free plants, although at a lower frequency than thermotherapy.
   - Preliminary results suggest that topical spraying lacotoferrin on cassava could be effective in eradication of African cassava mosaic virus (ACMV) but not East African cassava mosaic virus (EACMV).Topical spraying of Actigard on cassava showed a lessening of symptoms, and a reduction in ACMV was detected in polymerase chain reaction (PCR).
   - The supplement of salicylic acid (5 mg/L), ribavirin (30 mg/L), or lactoferrin (500 mg/L) to *in vitro* culture medium is a successful method for the generation of virus-free sweetpotato plants *in vitro*. The same three compounds had no conclusive beneficial effects in yam.
   - Responses in sweetpotato to the antiviral compounds, on both greenhouse plants and *in vitro*, is specific to both genotype and the virus or virus-combinations present in the plants.
3. A main component of the project was the testing and further development of small RNA sequencing and assembly (sRSA) for the detection of viruses. sRSA was compared with standard indexing to confirm efficacy against the current protocols to determine if it could be used to shorten and streamline the diagnostics of phytosanitary

cleaning to confirm plants clean of viruses in a reasonable time period. Key findings from these experiments include:

- In a comparison of six different sRNA extraction and library preparation protocols with sweetpotato, all methods gave good virus detection; however, extraction with the E.Z.N.A. kit performed the best across the six sweetpotato viruses analyzed. The extraction in a CTAB reduces virus genome coverage.
- In a comparison of the large RNA fraction versus the small RNA fraction, small RNA sequencing is much more sensitive for most sweetpotato viruses.
- The average minimum number of unique small RNA reads for sRSA for sweetpotato viruses could be calculated as ~40,000 for sweet potato chlorotic stunt virus (SPCSV), ~20,000 for SPV2, ~5,000 for sweet potato collusive virus (SPCV), and below that for the other viruses tested.
- Both spike-in with synthetic sequences and a Capsicum baccatum sample infected with an endornavirus proved effective as a control for library quality and contamination, respectively, in sweetpotato. The Capsicum baccatum control was subsequently used successfully to standardize samples in the comparison of standard indexing and sRSA results.
- The comparison with sweetpotato of standard indexing and sRSA ,with Capsicum baccatum for contamination control, showed congruence between the two methods 99% of the time. The remaining 1% (1 plant) of the time could be explained by undefined symptoms of indicator plants in the standard indexing. Results were not as clear at the International Institute of Tropical Agriculture (IITA), as almost ~35% of the comparisons of sRSA with the standard PCR tests were not congruent. However, the abovementioned controls had not yet been implemented at IITA and will certainly strongly improve the results (as they enable to distinguish contaminations from true positives effectively).
- Transfer of the sRSA technology to IITA was greatly enhanced by two IITA staff members spending time in the Kreuze lab.

4. The identification of inhibitors of sweet potato feathery mottle virus (SPFMV) as a means to control the synergistic viral disease caused by the co-infection with SPCSV was further investigated in this project by the University of Helsinki. The idea was to identify and develop inhibitors which could be used to lessen the severity of the disease caused by these two viruses. Key findings include:
   - Identifying and building a structural model for the functional analysis and catalytic site(s) of the RNase3 from SPCSV.
   - Developing a screen and then screening potential RNAse3 inhibitor compounds. The screening started with 6,620 compounds, which were reduced first to 109 compounds, then to 30, and finally to five candidate inhibitors for extensive testing. These five compounds all reduced the accumulation of SPCSV by two- to eightfold and SPFMV by two- to fourfold in co-infection.
   - A high throughput phenotyping platform was used to monitor photosystem II (PSII)-related and found a strong correlation with a reduction in PSII activities in infected plants relative to the controls due to downregulation of genes causing a clear disruption of photosynthetic capacity. This led to the conclusion that ΦPSII and qP are sensitive parameters for the quantification of virus effects.
   - The *in silico* similarity of the five identified inhibitors of RNase3 was used to identify new compounds with structural similarity which may be potential novel inhibitors of RNase activity. By the end of the project, 59 compounds with potential were identified for future study. But there was not enough time to test all for phytotoxicity or effects on viral accumulation.
5. A key element in the detection of viruses using the sRSA technology is the development of software which can be used to identify the viral sequences. VirusDetect was an existing program, yet was difficult to use and required larger computing power and bandwidth than is usually available in many of our partner countries. The upgrading of this program was therefore a principal objective of the project. Key progress with this upgrade included:
   - Initial progress with made with the integration of VirusDetect with the Chipster platform. While this successfully made the program more user friendly and enhanced its ability to run on laptops, it still resulted in a relatively heavy program. Many elements were not needed for VirusDetect, and it was still not user-friendly enough for many partners.
   - Progress with hiring a post-doc to upgrade VirusDetect to a operate with a Windows interface proved difficult and delayed the project by a year; ultimately, a programmer was found to work on the project. VirusDetect has now been completely reformatted into a Windows-compatible platform: VirusDetect-Windows (VDW).

- The program was also upgraded to include the ability to use spikes of known viruses or sequences as a quality control measure. This has proved invaluable at standardizing library data.
- VDW v0.94 can be downloaded from: https://research.cip.cgiar.org/virusdetect/.

6. Unfortunately, midway through the project, the principal investigator (PI) from the University of Wisconsin-Madison (UW-M) left the university. UW-M withdrew from the project, and thus a portion of its phase of the research could not be completed. Despite this, the QuickChip portion of the project was successfully transferred to CIP; however, its results reporting interface was insufficient to enable easy field testing, and CIP did not have the technical expertise to make the necessary adjustments. And although we started a student project with the University of Technology (UTEC) in Peru to address this issue, the COVID-19 pandemic prevented its advancement. Nevertheless, we have re-taken the initiative in early 2021 to try to advance the QuickChip development with UTEC using other support.

IN VITRO

The effect of macronutrient levels on the regrowth of meristems

Sweetpotato

The effect of macronutrient level in the growth media of sweetpotato meristems (0.25–0.35 mm) was assessed with a set of nine *in vitro* accessions. Meristems placed on culture medium supplemented with ¼-strength MS macronutrients had a significantly higher average regrowth rate (37.5%), compared with the control full-strength macronutrient treatment (21.0%) and ½-strength macronutrient treatment (30.9%). No statistical difference was observed between ¼-strength and full-strength medium without ammonium (32.7% regrowth rate). The control showed a significantly higher average survival rate of 67.5% compared with the ¼-strength macronutrient medium (57.1%), yet many of the meristems in the control treatment did not develop into complete *in vitro* plants. The control treatment also showed significantly lower rooting rates (26.4%) and number of leaves (2.6) compared with the ¼-strength macronutrient medium (41.5% and 3.6, respectively) (Annex 1, Table 1).

Eight of nine sweetpotato accessions had the highest regrowth rate with ¼-strength (four acc.) or with full-strength macronutrient concentration replacing NH_4NO_3 by KNO_3 (four acc.). One accession (CIP 420865) showed its highest regrowth rate (65.6%) with full-strength (Control) and ½-strength macronutrient concentration; however, this accession also had a high recovery rate with the other two treatments (57.8–61.1%). Seven of nine sweetpotato accessions had 4–15 times higher (18.9–48.9%) regrowth rates on ¼-strength medium relative to the control (Annex 1, Table 2).

A comparison of virus-infected (HS0) and virus-free (HS2) sweetpotato inventories of the same accession showed the virus-infected material had a significantly higher median survival rate of (80%), compared with the clean virus-free inventory (60%). Although an interesting observation, there were no statistical differences in regrowth, rooting rate, and number of leaves between virus-infected and virus-free material (Annex 1, Table 3, and Figure 1).

Cassava and yam

The *in vitro* culture medium for cassava and yam meristems is also MS, yet in contrast to the experiment in sweetpotato whereby only the macronutrients were reduced, in cassava and yam the total mineral-based mixture containing macro-elements (potassium, phosphorus, calcium, magnesium, nitrogen); micro-elements (sodium, molybdate, iodine, boron, zinc, magnesium, cobalt, copper, and iron); and vitamins (thiamine, pyridoxine, etc.) were decreased by ½, ¼, and ⅛.

The establishment rate of the meristem explants of all the cassava and yam accessions was lower on the treatments than the control (full-strength MS medium) after 12 weeks of growth (Annex 2, Figures 1 and 2). The genotype effect is significant, but there was no interaction between genotype and treatment (Annex 2, Table 1). Meristem establishment did not reach 100% in any of the treatments. Although occasionally superior regrowth was seen compared with the control in some yam accessions, the effect was not significant (e.g., TDa 1052).

The effect of growth hormone (auxin/cytokinin) variations on meristem regrowth in cassava and yam

Two growth regulators, NAA and BAP, used in meristem regrowth media of cassava and yam, were adjusted (Annex 2, Tables 2 and 3). Results of this experiment demonstrated nonsignificant effects from altered concentrations of two hormones in MS media despite some improvement in a few accessions of cassava (e.g., Tme 213; Annex 2, Figure 4). As with virtually all experiments, a significant genotype effect was observed (Annex 2, Table 4).

The effect of activated charcoal on i*n vitro* meristem regrowth in cassava and yam

Three levels of activated charcoal (1, 2, and 3 g/L) were added to the meristem regrowth culture media of cassava and yam and their effect compared with the control (no activated charcoal) (Annex 2, Figure 5). After 4 weeks, regrowth of

cassava meristems cultured on activated charcoal varied principally by a genotype x treatment effect (Annex 2, Table 5). No significant differences in regrowth rates were observed between cassava meristems grown in media with or without activated charcoal, except for accessions Tme 3907 exposed to 4 g/L of activated charcoal and Tme 581 exposed to 2 g/L (Annex 2, Figure 6). The regrowth of yam meristems cultured on activated charcoal also was affected by a genotype x treatment interaction (Annex 2, Table 5). The addition of 4 g/L of activated charcoal to the culture media improved yam meristem regrowth rate for three accessions (TDa 1052, TDr 2188, and TDr 4750) compared with the control treatment (Annex 2, Fig. 7). The same favorable effect was also noticed with the treatment of 1 g/L for accession TDa 1052 and 2 g/L for TDr 4750. These results demonstrate that the addition of activated charcoal improves meristem regrowth of several yam accessions.

The effect of antioxidants (glutathione and ascorbic acid) on the regrowth of cassava and yam meristems

Two antioxidants, glutathione - AG (0, 0.49 and 0.74 mg/L) and ascorbic acid - AA (0, 25 and 40 mg/L), were added to the MS media for cassava and yam. Results showed that in cassava, there were interactions between genotype and dose (Annex 2, Table 6), whereas some accessions had nonsignificant enhancement of regrowth after 4 weeks (Annex 2, Figures 8 and 9).For yam meristems cultured on medium supplemented with antioxidants, there was also a genotype and treatment effect (Annex 2, Table 7) with little enhancement of regrowth (Annex 2, Figures 10 and 11).

The effect of dark periods for meristem growth on the regrowth of meristems

Sweetpotato

Sweetpotato meristems (0.25–0.35 mm) from five accessions were exposed to darkness at different phases of the regrowth cycle: (1) without darkness (control), (2) first seven days in darkness, (3) from day eight to 14 in darkness, and (4) from day 29 to 35 in darkness. For each accession, two different health status inventories were tested, virus-free (HS2) and virus-infected (HS0).

A single repetition with a reduced sample size of six meristems per treatment was performed, but COVID-19 limitations did not allow time for additional repetitions. On the basis of preliminary data, no statistical differences were observed for regrowth (31.7–41.7%), rooting rate (35.0–41.7%), and number of leaves per plant (3.2–4.1). However, based on these preliminary data, a significantly higher survival rate (95.0%) was observed with the late exposure to darkness (from day 28–35), compared with the control treatment without a darkness period (80.0%) (Annex 1, Table 4). Clearly, a follow-up of this experiment is needed.

Cassava and yam

The effect of a dark period on cassava and yam meristem regrowth was tested by keeping meristem explants on MS media in the dark conditions for either seven or 14 days. Routine exposure to a 12 h/day photoperiod from the start of the culture was used as a control. Observations of the regrowth rate were made after 12 weeks. The results showed that cassava meristem regrowth was affected by genotype and the light regime treatment, without an interaction of those two factors. In contrast, yam meristem regrowth was influenced by the dark period during the first days of culture with an interaction with the genotype (Annex 2, Table 8); however, the regrowth rates were lower than in the control treatment. Therefore, we conclude that exposure to dark conditions for the first two weeks of meristem culture does not improve meristem regrowth rates in cassava and yam (Annex 2, Figures 12 and 13).

The effect of a liquid treatment on sweetpotato meristem growth

Sweetpotato meristems (0.25–0.35 mm) from 10 accessions were treated with CIP's routine meristem culture protocol during the first five weeks of maintenance. They were then placed on three different culture media: (1) control (routine protocol; solid medium), (2) liquid culture medium without agitation (samples were placed on a filter paper for support), and (3) agitated liquid medium (80 rpm). Only virus-infected (HS0) samples were assessed in this experiment.

After 18 weeks, no significant differences were observed in the survival rates between the three treatments (75.0–77.7%). However, full-plant recovery rates were significantly higher in the liquid medium treatment with agitation (59.3%) compared with either the liquid medium without agitation (30.0%) or the solid medium control (20.3%). The rooting rate on liquid medium with agitation (59.7%) was also significantly higher response than on the other two media (27.3–41.3%) (Annex 1, Figure 2).

Nine of 10 sweetpotato accessions showed good recovery on agitated liquid medium (36.7–90.0%). The one accession with a low recovery rate (6.7%) had no meristems recovery on the control treatment. Additionally, the control treatment showed low recovery for six of 10 accessions; for three accessions no recovery was recorded (0%). On liquid medium without agitation, four accessions had a low recovery rate (<= 13.3%) and two accessions had no recovery (0%) (Annex 1,

Table 5)**.**

On the basis of these results, CIP's standard protocol for meristem culture is in the process of being changed from solid to liquid medium with agitation (after five weeks on solid medium). Using the modified protocol, **the time required to recover complete *in vitro* plants from sweetpotato meristems is reduced from 27 to 18 weeks (gain of efficiency of 33%) on average.**

The application of thermotherapy to sweetpotato greenhouse plants for virus elimination pre-*in vitro*

Thermotherapy of greenhouse sweetpotato plants was tested for virus elimination in a preliminary experiment (single repetition) with six virus-infected sweetpotato accessions (Annex 1, Table 6). Greenhouse plants were exposed to thermotherapy (36°C) for four weeks and controls remained at ambient temperature (28°C), followed by shoot tip excision from apical and axillary buds and introduction into *in vitro*.

Apical and axillary sweetpotato shoot tips showed similar regrowth rates for both the thermotherapy and control treatments. After 27 weeks, shoot tips coming from thermotherapy-treated greenhouse plants had a slightly higher full-plant recovery rate (84.7–93.1%) compared with the control treatment (73.6–80.6%) (Annex 1, Figure 3).

Sweetpotato greenhouse plants treated with thermotherapy (36°C) had a higher percentage of virus-free plants (55.1%), compared with the control treatment (42.6%). These preliminary results showed that SPFMV could be eliminated successfully (100%), as could a synergistic infection with three viruses: begomovirus (BGV), sweet potato vein clearing virus (SPVCV), and SPCV (73.7%).Nevertheless, the cleaning rates for SPCSV (16.7%), begomovirus (27.0%), and the synergistic infection by begomovirus and SPCV (40.0%) were at a lower level (Annex 1, Table 6). These results suggest that there could be a differential cleaning effect based on the virus or viruses present.

Topical application of antiviral compounds as a spray to greenhouse plants for virus elimination

Sweetpotato

The feasibility of spraying antiviral compounds on sweetpotato greenhouse plants for virus elimination was assessed with six virus-infected accessions. The plants were sprayed with (1) salicylic acid (70 mg L^{-1}), (2) lactoferrin (1000 mg L^{-1}), (3) Control 1 (only water), and (4) Control 2 [Water + Tween (0.2 $mL.L^{-1}$)]. Antivirals and controls were sprayed at a plant age of ~11, 12, 13, and 14 weeks (post-transplanting to pots). Ten days after the final spraying, apical and subapical (axillary) stem segments (~2.0–2.5 cm) were collected from two branches of each plant and placed *in vitro*. Fourteen days later, shoot tips (1.2–1.5 mm) were excised from the stem segments and *in vitro* plants were regenerated. Post-treatment leaf samples were taken from regenerated *in vitro* plants and analyzed by multiplex PCR for the viruses.

The accessions recovered from shoot tips showed a genotype-specific response (Annex 1, Figure 4), with *in vitro* plants derived from axillary buds showing a significantly higher recovery rate at 19 weeks than those from apical buds (79.3% vs. 56.7%) (Annex 1, Figure 5, Table 7). Shoot tips derived from plants treated with antiviral sprays showed a similar full-plant recovery rate as the controls, confirming no measurable phytotoxic effect of salicylic acid or lactoferrin at the concentrations sprayed on sweetpotato greenhouse plants (Annex 1, Figure 6, Table 7).

CIP 420169 was found not to contain any viruses, and in three of the accessions some virus-free plants were detected. The accession originally infected with only SPCSV (CIP 440693) had the highest percentage of virus-free plants (56.5%). Interestingly, two accessions (CIP 441541 and CIP 443681) had viruses post-treatment (SPCV, SPVCV) that were not originally present (Annex 1, Table 8).The appearance of these novel viruses was thought to have been due to either undetectable titer levels in the pretreatment plants, cross contamination in the greenhouse, contamination in handling and RNA extraction, or contamination in the PCR process. Nevertheless, the detection of some virus-free samples provided hope that the method could work. In the end, faulty PCR machines were determined to be the cause. But owing to COVID-19, it was not possible to get the PCR machines repaired and samples rerun in time before the project ended.

In a larger experiment, greenhouse plants from 18 sweetpotato accessions were exposed to (1) thermotherapy (36°C), (2) spraying with salicylic acid (280 ppm), (3) a combination of both (36°C + 280 ppm of salicylic acid), and (4) a control treatment (28°C), to assess the treatment's efficacy in virus elimination. Virus diagnostic was done on a sample of greenhouse plants prior to treatment (baseline) and on *in vitro* plants recovered from shoot tips (post-treatment).A preliminary experiment with six sweetpotato accessions identified a salicylic acid concentration of 280 ppm as the highest concentration that could be applied with minimal phytotoxic effects (Annex 1, Tables 9 and 10).

Eight weeks after shoot tip excision, 17 of the 18 accessions showed high full-plant recovery rates (74.4–100%).The one accession (CIP 401114) with a significantly lower recovery rate (40.6%) experienced stress conditions in the greenhouse that could have accounted for this lower recovery rate. At this point in time (eight weeks), no statistical differences were

observed for the recovery rate of the different treatments (Annex 1, Table 11). PCR virus analysis are ongoing.

Cassava

Lactoferrin (Apolactoferrin, Life Extension, Quality supplements and Vitamins, Inc., Florida, USA) was used to assess the antiviral effect on cassava infected with ACMV and EACMV. Three genotypes of cassava were tested, two of which were naturally infected with ACMV (TMe1962) and EACMV (TMe4217), and one virus-free cultivar TMe168. Three concentrations (250, 500, and 1000 mg/L) of lactoferrin solution were applied to the root zones of individual plantlets; untreated plantlets were used as the control (Annex 2, Figure 14). The treatment was repeated at weekly intervals for four weeks. Plants were assessed every two weeks for foliar symptoms of cassava mosaic disease (CMD). Data were collected on plant height, CMD incidence, and severity every two weeks for the next six weeks post-transplanting in the screenhouse. Samples were indexed for ACMV and EACMV by PCR. Plants of TMe1962, infected by ACMV, recovered after treatment as plants were asymptomatic and the virus was undetected in PCR (Annex 2, Table 9, Figures 15 and 16). However, the EACMV-infected genotype, TMe4217, remained symptomatic and plants tested positive to EACMV in PCR.

In a subsequent experiment, Actigard (acibenzolar-s-methyl, Syngenta) was tested for enhancing host defense response. Actigard (Syngenta) is a commercial product reported to stimulate systemic acquired resistance against bacteria in plants and was tested on CMD-affected cassava (cv NR8082) to evaluate its effects on virus/symptom suppression. The stem cuttings were treated by a 10-min soaking in 250 mg/L of Actigard solution, dried at room temperature for about 8–12 h, and then planted in pots under screenhouse conditions. A general stem treatment method of treating stem cuttings with an aqueous solution of Karate (4 ml/L) + Mancozeb (7 g/L) in water, which is used for phytosanitation of bacteria and acarid and arthropod pests, was included as a second treatment. Plants were monitored for CMD symptom severity and growth by measuring plant height. Leaf samples were tested by PCR for ACMV and EACMV. Twelve weeks after planting, plants in all treatments were sprayed with three concentrations of Actigard (i.e., 50, 150, and 250 mg/L), with distilled water as a control. Stem treatments had no effect on CMD incidence or severity. Whereas foliar sprays of Actigard resulted in yellowing of sprayed plants, the newly emerging leaves on several plants showed recovery from CMD symptoms, including those of the control (Annex 2, Table 10, Figure 17). Recovered leaves, when tested with PCR, were negative for ACMV, but so were several leaves of the control treatment.

The efficacy of *in vitro* applied antivirals

Sweetpotato

Six virus-infected sweetpotato *in vitro* accessions were propagated on modified MS medium supplemented with two antiviral compounds. Treatments included (1) solid modified MS medium + salicylic acid (5 mg/L), (2) solid modified MS medium + ribavirin (20 mg/L), (3) solid modified MS medium without antivirals (Control 1), (4) liquid modified MS medium + 500 mg/L lactoferrin, and (5) liquid modified MS medium without antivirals (Control 2). Survival, regrowth, rooting rates, and leaf number were recorded 2, 4, 7, 11, 15, 19, 23, and 27 weeks after shoot tip excision.

The salicylic acid treatment resulted in a lower total number of regenerated plants (79) and the lactoferrin treatment the highest (152) (Annex 1, Figure 7, Table 12).

PCR analysis for viruses again gave spurious results with viruses (SPFMV, SPVC, SPCSV, SPVCV) being detected that were not detected in the control pretreatment samples (Annex 1, Table 13). As in the preliminary experiment, however, virus-free plants were obtained in five of the six accessions, providing some confirmation that the application of antivirals *in vitro* could be a viable option for virus eradication.

It has been determined that there were issues with the PCR machines. But because of COVID-19, it was not possible to get the PCR machine repaired or the samples rerun in time before the project ended.

Yam

In vitro yam plants from 13 accessions (*D. rotundata* [TDr 4611, TDr 4474, TDr 4375, TDr 3521, TDr 3517, TDr 3410, TDr 3148, and TDr 1918] and *D. alata* [TDa 4550, TDa 3228, TDa 1467, TDa 1190, and TDa 1056]) were tested by Real Time (RT)-PCR for yam mosaic virus (YMV), yam mild mosaic virus (YMMV), and yam virus Y (a betaflexivirus detected by sRSA which appears to be cryptic in yam), and PCR for the detection of yam badnaviruses. Treatments included three antiviral compounds at varying concentrations: Ribavirin (0, 10, 20, and 25 mg/L), Lactoferrin (0, 100, 200, 500, and 1000 mg/L), and Salicylic acid (0, 5, 10, 20, and 50 mg/L).

In vitro nodal cuttings were maintained for four weeks on medium supplemented with the antiviral agents, after which leaf samples were tested for virus. The *in vitro* plantlets were then transferred to a semi-solid multiplication medium and again tested for viruses after eight weeks, after which plants were transferred to *ex vitro* peat substrate and again tested

for viruses after 16 weeks (Annex 2, Figure 18). Incidences of viruses were recorded based on the virus-indexing results and used for estimating the percent recovery after antiviral agents.

The second trial of this study was affected by lockdown imposed due to the COVID-19 pandemic. IITA closed all its activities between April and July, allowing only essential work to be carried on with minimal staff. This affected multiplication and plant establishment activities, and loss of plants in some treatments. As a consequence, the second trial was repeated, which concluded in the first week of February 2021.

The analysis of data collected from this second experiment is congoing. In this report, a summary of virus-indexing data is presented as a simple means of percent virus infection (Annex 2, Figures 19–21). These preliminary data show the incidence of YMV, YMMV, and YVY to be 0–~20% for all treatments as well as the control. The reduction in the virus in the control to the same level as treated plants implies spontaneous elimination of viruses or other factors (also observed in sweetpotato, see below section on sRSA). We do not have a clear explanation, but ongoing analyses are expected to help clarify these unexpected findings. The incidence of badnavirus detection was higher, mainly due to the detection of integrated sequences.

sRSA

Validation of sRSA

Sweetpotato

To validate that sRSA would work for the detection of all sweetpotato viruses, sRSA was tested on plants infected with as broad a range of viruses as possible. Historical data from virus indexing were used to select a set of 30 *in vitro* accessions infected with the range of known sweetpotato viruses which were retested by PCR to confirm the historical results. From these, a subset of 14 accessions were selected for repeated standard indexing and parallel processing of sRSA. Eight of these sweetpotato accessions were also used for sensitivity and specificity analysis by sRNA sequencing. Of the 30 virus-infected accessions initially selected, 16 had virus infection status fully or at least partially confirmed (Annex 3, Table 1). It is interesting that only ~50% of the historical virus infection statuses could be reconfirmed. It is also intriguing to speculate whether this was due to low virus titers in *in vitro* material or the fact that decades of *in vitro* culture could select for tissue free of viruses.

Comparison of six extraction methods for virus detection in sweetpotato

To test sRNA extraction and library preparation protocols, and to evaluate cheaper and less laborious methods, six protocol variations were tested for the detection of six sweetpotato viruses (SPFMV, SPCSV, begomovirus, SPCFV, SPLV, and SPMSV) in composite samples. Because five samples were combined, the effect was that the test started with a fivefold dilution of each virus-infected sample as compared with the case where a single plant would be sampled. The following protocols were performed each in triplicate:

protocol 1: Previous standard extraction method with Trizol and cutting and extracting the siRNAs band from a gel prior to library preparation according to the previous standard procedure

protocol 2: Extraction using the E.Z.N.A miRNA kit and library prep according to the previous standard procedure

protocol 3: Extraction with CTAB Buffer, LiCl precipitation, siRNA isolation from gel, and library prep without using RNaseOUT, and replacing reverse transcription and polymerase enzymes with **M-MLV/ Protoscript** & GoTaq

protocol 4: CTAB buffer, LiCl precipitation, but NO siRNA isolation from gel no RNaseOUT, **M-MLV/Protoscript** & GoTaq

protocol 5: CTAB buffer, NO LiCl precipitation or siRNA isolation from gel, no RNaseOUT, **M-MLV/Protoscript** & GoTaq

protocol 6: Previous standard extraction method with Trizol, but without cutting siRNA isolation from gel

Sequences obtained from the different libraries were then cleaned using VirusDetect. Results of the cleaning process and siRNA size distribution were compared (Annex 3, Figures 1 and 2). Sequenced libraries prepared with the different protocols were analyzed by VirusDetect v1.6, and detection of different viruses were compared based on average genome coverage and normalized sequence depth (Figure 3). In addition, subsamples of 1, 3, 5, and 7 million reads were extracted from each library and the effect on virus detection analyzed. This analysis revealed that the EZNA kit protocol provided better genome coverage for most viruses at lower read numbers than the other protocols, whereas there was little difference in average sequencing depth.

In summary, results revealed that all extraction methods work, although we observed the following:

- CTAB reduces genome coverage.

- Virus genome detection and coverage are good with all methods; however, extraction with the EZNA kit performed the best across the six viruses analyzed.
- Traces of a carlavirus related to SPCFV were detected.

The EZNA miRNA kit was chosen as the standardized method for further RNA extractions due to most consistent results across viruses, the reduced extraction time, and fewer operational steps, thereby lessening the opportunities for contaminations to occur.

During this experiment, and all following experiments, badnaviruses related to sweet potato pakakuy virus (SPPV) were identified in nearly all accessions and sequences corresponding to sweetpotato symptomless mastrevirus were also detected in many of the accessions. These viruses have not been shown to affect sweetpotato in any way, occur in extremely low concentrations, and seem to be unaffected by meristem tip culture (Kreuze et al. 2020[1]). Thus, to reduce complexity of results, these two viruses were not considered in most of the analysis below.

Comparison of small RNA vs large RNA for virus detection in sweetpotato

The EZNA miRNA kit enables isolation of the large RNA fraction and thus permits the sequencing of the large RNAs. This was used to confirm whether large RNA sequencing would be more or less sensitive for virus detection or could complement small RNA sequencing. Ribosome-depleted large RNA sequence library preparation was outsourced to Fasteris and results were analyzed with VirusDetect v1.6, including subsamples of 1, 2, 3, 5, 7, and 9 million reads and compared with results obtained by small RNA sequencing. The comparison showed that although small RNA sequencing could detect all viruses in all the subsamples, large RNA sequencing only resulted in the detection of SPCSV in all subsamples and SPPV from 7 million reads or more. None of the other viruses, including SPFMV, begomovirus, and the mastrevirus, were detected even with 9 million reads. Thus, small RNA sequencing is more sensitive and reliable than large RNA sequencing in the case of sweetpotato viruses. However, SPCSV, which generates rather low amounts of siRNAs, was more easily detected (greater genome coverage with lower read numbers) by l-RNA than by siRNA. We will (beyond the end of this project) test if the same might hold true for SPCFV based on results described in the two sections below.

Determination of the detection limit of siRNA sequencing of sweetpotato viruses

The sensitivity of sRSA was tested through a dilution series of eight accessions infected with the most important sweetpotato viruses (Annex 3, Tables 1 and 2). Because this experiment was performed before the use of the control virus for estimating contamination was implemented, we used the average contamination rate observed in later experiments to set a best-guess cutoff for contamination in this experiment (2.5%). Results showed that some viruses were identifiable through a 128-time dilution; sensitivity for SPCSV detection, however, was lost at the 16- to 32fold dilutions in two of the repetitions, whereas potyvirus (single infection) detection was lost at the 32- to 64fold dilutions in those same replicates. On the other hand, in a few samples a begomovirus reads were above the estimated contamination levels, leading to false positive detections. This could have been a result of applying an erroneous threshold, as we observed these could vary significantly between sequenced libraries.

On the basis of loss of detection of SPCSV, SPV2, and SPCV, a theoretical minimum number of unique reads for each virus can be proposed. Dividing the number of unique reads of each library by the dilution factor gives the theoretical number of reads of each sample in the mixture after dilution. This would suggest that the minimum number of unique reads would be ~40,000 for SPCSV, ~20,000 for SPV2, and ~5,000 for SPCV, although all of them were detected in one repetition at 5,500 unique reads. Significantly, SPCFV was not detected even without dilution, yet this virus was confirmed through standard indexing (Annex 3, Table 1), and was only just detectable from *in vitro* plants in the experiment described below. This suggests that SPCFV hovers at the detection limit for siRNAs at the read depth targeted in sweetpotato. Begomovirus was also detected clearly by sRSA in all experiments (Annex 3, Tables 2 and 3), whereas it was missed by standard indexing. Despite the theoretical minimum read numbers calculated above, we recommend the generation of 1–4 million reads for reliable virus detection.

Comparison of standard indexing with sRSA in sweetpotato using in-vitro plants and greenhouse plants at two time points

Fourteen accessions (Annex 3, Table 1) were tested by standard indexing and also tested at three different stages by

[1] Kreuze, J.F., Perez, A., Gargurevich, M.G., and Cuellar, W.J. 2020. Badnaviruses of Sweet Potato: Symptomless Coinhabitants on a Global Scale. *Frontiers in plant science* 11: page 313.

sRSA: (1) directly from the *in vitro* plants, (2) after plants had been transferred, and (3) established in a greenhouse and once again after the greenhouse plants had been cut back and left to re-sprout. This was done to (1) compare sRSA to standard indexing procedures, (2) understand if there were any differences between sRSA from *in vitro* or greenhouse plants, and (3) determine if the age and other environmental conditions in the greenhouse could affect sRSA results. VirusDetect was used to identify viruses and to compare sRSA with standard indexing (Annex 3, Table 3). Whereas some virus were not detected in some samples by sRSA, in all but one case (SPVCV in *in vitro* plants in library 2 [accession 400780]), this could be ascribed to the poor library quality and low read number. Even in accession 400780, the result remains unexplained as the same *in vitro* accession was used for the detection limit experiment described in 2.1.1.4 above. SPVCV was readily detected up to the 128x dilution in all three repetitions. One explanation is that a spontaneous loss of the virus occurred during *in vitro* multiplication, as was hypothesized in comparison of historic and current virus testing results above (Annex 3, Table 1).

Development of contamination and library quality control for sweetpotato

Several experiments were carried out to build quality control measures into the library preparation process:

- Nine artificial, small RNA spike-in sequences were designed, ordered, and included in the libraries as an internal quality control for library preparation. The spike-in sequences were designed to show no similarity with any known sweetpotato-infecting virus or sweetpotato genome sequences. They were 21 nt in length, with one 24 nt in sequence. The last four nucleotides on each end were random to avoid any ligation bias due to nucleotide type at the siRNA extremes. Spike-in sequences were added at eight different concentrations, with a fivefold difference between each of them in five different ranges, from 0.002–1.25 to 1.25–97656 amol (10–18 moles) per library prep. Results showed that far fewer spike-in sequences were recovered by sequencing than expected, due likely to losses during RNA extraction as the spike was added to the buffer prior to extraction as a control of the whole process. On the basis of these results, the minimum amount of spike-in sequence needed to obtain reliable recovery of at least one spike-in sequence is ~500–1,000 amol.
- Illumina sequencing is prone to 'virtual' contamination between barcoded (indexed) samples run together in a single lane (referred to as index-hopping or bleeding), and can lead to false detection of viruses if the contamination is large, due to a virus having large amount of reads in one of the combined samples. To address this issue, and be able to determine if a detection is due to contamination, a sample of Capsicum baccatum infected with an endornavirus was included as one of the 48 samples as a control for contamination between samples. Using alignment of reads from each of the samples in a library to the control virus, the contamination rate in each sample can be determined. By taking the average contamination rate and adding twice the standard deviation thereof, a contamination cutoff value could be determined. This value was then applied for each virus detected in the library, selecting the sample with the highest sequencing depth to determine a threshold sequencing depth value for each virus. If a virus was detected in any sample with a sequencing depth below that threshold, it would then be considered a contamination. This worked almost perfectly to remove all false positive detections, particularly when further adjusting to other library quality parameters such as number of unique reads or 21–24 nt reads.
- Options were included in the new VDW software to automatically analyze and visualize spike-in sequence counts for each sample, and to analyze contamination rate(s) using the control sequence to provide a cutoff rate for contamination in each library.

Cassava and yam

Prior to the NextGen phytosanitation project, IITA outsourced sRSA library preparation and sequencing. With the initiation of the project, IITA adopted the sRSA protocol (Annex 4, Table 1, sRSA Protocol v1) established for potato and sweetpotato virus indexing at CIP. The VirusDetect software was used for sequence assembly and virus detection. Technology transfer started with the training of five members of IITA's Virology and Diagnostics Unit (VMD) in a hands-on training workshop (20 February–3 March 2017) at VMD and continued in 2018 with a visit by two technicians to CIP-Lima.

Three sets of 48 sRSA libraries were generated from 39 cassava samples sourced from TC (N = 23), semi-autotrophic hydroponics (SAH) (N = 5), and screenhouse (N=11) and 101 yam samples, comprising TC (N = 66), SAH (7), and screenhouse (N = 28). The small RNA from source tissues were extracted using the EZNA MicroRNA kit (Omega-BioTek, USA) and the conventional extraction method using TRIzol reagent. Seventy samples were extracted using TRIzol, and 72 were extracted with the EZNA kit. In addition, 15 samples were extracted using both methods.

Sequencing resulted in reads between 500 and 35.5 million/library, with an average sequence read of about 8 million/ library (Annex 4, Table 2). However, clean reads (libraries with inserts) were very low, ranging 14–250,000, with a mean

of 48,671 (Annex 4, Figure 1). Overall, ~70% of the libraries constructed during the learning period failed. The analysis of available sequence using the VirusDetect software indicated the presence of ACMV, EACMV, and Ivorian bacilliform cassava virus in the cassava and YMV, badnaviruses, and a few cases of flexiviruses in yam. Owing to the low number of reads, data generated from the first three sets of 48 libraries were not considered for further analyses. Nonetheless, lessons learned from this exercise helped in the establishment of sRSA technology at IITA.

Development of SOPs for sweetpotato indexing with sRSA

Two standard operating procedures (SOPs) were developed, one for siRNA library preparation based on the library preparation protocol using EZNA columns (Annex library prep SOP), and one on data management storage, sequence analysis, and interpretation and documentation of results (Annex 7, CIP-SOPABC, CIP-SOPXXX). In parallel, IITA developed an SOP for the process in their laboratory (Annex 8). These SOPs will be compared to improve both and harmonize them as much as possible beyond the end of this project, and further shared with other CGIAR germplasm health units as a standard SOP.

Standardization of sRSA for cassava and yam

Trouble-shooting of libraries Lib-001 to Lib-003 prepared during 2017–2018 indicated a high number of empty libraries pointing to the loss of dsRNA during LiCl precipitation or gel elution of sRNA. Improvements to the protocol were made by the elimination of the LiCl precipitation and gel elution steps (Annex 4, Table 1, sRSA Protocol v2). Two staff from VMD (Ms. Patricia Ogunsanya and Mr Masood Lateef) were further trained in sRSA and VirusDetect in CIP-Lima in March 2018. Modifications in protocol and sample processing resulted in an increase in enriched library concentration by at least 8× (1.5–12.8 ng/µl) and sRNA libraries that yielded a large number of clean reads (average 2.1 million per sRSA library). The modified protocol also reduced the time to generate sequence-ready libraries from eight days to five working days. The sRSA Protocol v2 was used for the construction of 4× 48 libraries in 2019–2020 (Annex 4, Table 2).

In 2020, the NGS sequencing provider, FASTERIS, replaced Illumina HISeq-4000 with the Illumina NovoSeq-6000, as the former had been phased out by the manufacturer. Fortunately, the protocol established for HiSeq sequencing library preparation, including adaptors, worked well for NovaSeq sequencing. However, we observed depletion in the number of sRNA reads of 21–24 nts. Troubleshooting revealed an increase in the yield of < 20 bp reads. Consultation with FASTERIS revealed the NovaSeq sequencer bias toward amplification of short reads. To help overcome this bias, gel separation and elution of sRNAs of about 21–25 nt bands from 2% agarose gel was reintroduced and a second elution to extract 20–25 bp nucleotides from the gel using Zymoclean Gel DNA Recovery Kit (Zymo, California, USA) (Annex 4, Figure 2). This version of the updated protocol, referred to as sRSA Protocol v3 (Annex 4, Table 1), resulted in an average of ~4.3 million clean reads per library. Although this protocol extended the library preparation time to eight days, sequencing data from the libraries generated confirmed enrichment of >15 nt reads and a reduction in empty reads (Annex 4, Table 2). The sRSA protocol was validated on all the crops routinely used for virus indexing at IITA (Annex 4, Table 3). Libraries at CIP, however, were not affected in the same way by switching to NovaSeq platform, which indicates the problems with the first two libraries of 2020 from IITA were perhaps caused by other factors and they could revert to sRSA Protocol v2 to save time and money. This will be reviewed further in detail.

Comparison of sRSA with standard indexing

Sweetpotato

Two different types of material were used to compare sRSA with standard indexing:

- One-hundred sweetpotato accessions of unknown health status being introduced into *in vitro*. The assumption is that these would have a relatively high number of plants infected with viruses that would ensure a good number of virus-positive samples.
- A total of 170 *in vitro* accessions from the genebank collection which had either an unknown health status or were considered infected based on historic data. Because these accessions had been maintained in *in vitro* for many years, and some had gone through meristem culture, the number of infected materials in this group was expected to be low. Results from these two groups are described separately below.

Standard indexing and sRSA of sweetpotato accessions being introduced to *in vitro*

One-hundred accessions were processed in two different groups of 50. The first was processed from greenhouse plants before including a control virus to assess contamination between libraries was implemented, whereas the second group of greenhouse plants was processed after introduction of the control virus. The same greenhouse plants were processed by standard indexing and the results were directly compared. The accessions were then introduced into *in vitro* using

standard procedures and, after establishment, were sampled again by sRSA. Results from the sRSA analysis of the *in vitro* plants are still pending due to slow growth of introduced plants and impacts of COVID-19 restrictions on staffing during 2020 (shown diagrammatically Annex 3, Figure 4).

The first group of 50 greenhouse accessions were tested between February and March 2018, and libraries were prepared and sent for sequencing at the end of June 2018.The resulting libraries were of poor quality, with unexpected size distributions and high levels of read duplications, resulting in a large number of samples with unique reads below the 1 million minimum cutoff for reliable detection. Virus detection by sRSA nevertheless revealed many of the plants were infected with viruses, some with extremely high numbers of reads that led to suspected cases of contamination in other samples, particularly for begomoviruses (reads detected in nearly all samples). To address this, the average contamination rates from other libraries (second group of 50 and 170 accessions described in 2.1.2.2) were used to apply a contamination threshold. When compared with the standard virus-indexing process done in parallel to the preparation of libraries, the two methods gave the same results for the vast number of accessions; but unfortunately not all (Annex 3, Figure 5).

The second group of 50 greenhouse accessions was tested in December 2018 and sent for sequencing at the end of March 2019. Unlike the first group, in these samples an external control from capsicum infected with an endornavirus was included as one of the samples in the pooled library to estimate contamination rates due to index hopping (a term used for the observation that in Illumina sequencing runs index-sequences are mis-assigned due to processes inherent to the sequencing technology) in the library. Thus, exact contamination rates in the library could be determined (as described in section 2.1.1.6 above) and applied to samples to determine cutoff rates for contaminants based on the calculation of the average contamination rate of the endornavirus siRNAs between samples. This value was then used to calculate the maximum average sequencing depth that could be expected from contamination for each virus based on the sample with the maximum sequencing depth found in the library for that virus. The use of the external control clearly gave cleaner and more expected results where there was strong congruence between standard indexing and sRSA with only two accessions having viruses detected by sRSA and none with standard indexing only (Annex 3, Figure 6).

Standard indexing and sRSA of in-vitro maintained sweetpotato samples from the genebank with unknown infection status

The 170 accessions of *in vitro* plants with historical virus infections or unknown health status (Annex 3, Figure 7) were prepared as four sequencing libraries of 47, 47, 47, and 29 accessions each sent for sequencing in January 2019. All samples included the capsicum control for estimating cross-sample contamination. The initial plan was to use plants that had recently been indexed for viruses by the standard methods to be able to compare these results with sRSA. But owing to logistical and cost issues, this did not occur and plants were submitted to virus elimination therapy and only indexed afterwards. As a result, only 90 out of the 170 accessions provided information that could be compared. The results showed congruence in all but five cases, where indexing showed a positive result and sRSA a negative result. All these cases were due to unidentified symptoms in indicator plants, whereas in one case the quality of the sRSA library also was low and thus not reliable. Upon re-indexing these plants in 2020, four plants resulted negative, whereas one showed mild chlorosis in indicator plants; sRSA was not repeated on these plants (Annex 3, Figure 8).

Conclusions of sRSA in sweetpotato

In general, the comparison of sRSA and standard indexing showed a high degree of congruence, although there were a number of unexplained results where the same accessions tested positive for certain viruses in one experiment and negative in another, or there was disagreement between sRSA and standard indexing. Fortunately, false positives by sRSA were the most frequent discrepancy and can be confirmed by follow up PCR testing. Whereas repeat experiments are still ongoing to resolve some of these results, in all cases where this was done for potato in a complementary project, the differences were finally resolved. This probably reflects the inherent biological variability found in plants; indeed, accessions that have been indexed by standard methods over the years surprisingly often give conflicting results and, in that sense, seem to be no different to sRSA. Nevertheless, there have been extremely rare cases of escapes with that system showing it is highly robust. An approach including repeat testing by sRSA or combination of sRSA and RNAseq, combined with PCR testing and enzyme-linked immunosorbent assay to compensate for any error, is recommended for final implementation of sRSA.

Separate from the technical performance of sRSA, the fact that sequencing is outsourced for cost-efficiency has presented a challenge to get the procedure accredited for ISO17025 by UKAS, who have insisted all processes must be performed in-house. On the other hand, UKAS accreditation is currently being reconsidered at CIP due to cost. Other quality management systems could be considered that are not that restrictive.

Cassava and yam

Viruses detected in yam included YMV, YMMV, yam badnaviruses, and YVY. The new viruses detected which were not reported previously in West Africa included yam necrosis-associated virus, *Dioscorea* mosaic-associated virus, and *D. nummularia*-associated virus. All these viruses were recently reported in the Caribbean yam collection sequenced using NGS by CIRAD and were not associated with any symptoms. Samples that previously tested negative to viruses remained virus free, except for some contigs matching several flexiviruses (ampelovirus, foveavirus, carlavirus, and potexvirus). Trace levels of YMV were detected in several libraries made from various crops, including cassava. Extensive retesting to confirm YMV in the original leaf and total nucleic acid samples using standard RT-PCR gave negative results. We concluded that YMV detected in other samples was due to contamination in the sequencing flow-cell. A control (i.e., sRNA from a fungus) in each multiplex library is planned in the future as a check to identify contamination in the flow-cell.

In cassava, the viruses detected were ACMV and EACMV, CBSV, and Ugandan cassava brown streak virus (only in leaf tissues fixed in glycerol received from East Africa). Cassava Ivorian bacilliform virus and CMV C were detected in one sample. The latter two viruses are new records.

The results of sRSA and RT-PCR are similar for the known viruses (i.e., YMV, ACMV, and CBSV) for which PCR or RT-PCR could be used for confirmation. Very few samples that tested negative in PCR where positive in sRSA (a cassava negative in RT-PCR tested positive to CSBV in sRSA; a cassava negative to EACMV in PCR tested positive in sRSA; a yam sample tested positive to YMMV in sRSA was RT-PCR negative for the same virus). In summary, of the 240 sRNA samples tested, results of 55% of the samples were similar for known viruses in RT-PCR and sRSA; 8% of the samples tested positive by sRSA to known viruses but negative in RT-PCR; 22% of the samples were positive in RT-PCR but negative in sRSA, and 15% of the samples were tested by only one method and therefore comparison was not possible (Annex 4, Figure 3). The discrepancies between the two methods could be due to either false positives in the RT-PCR or because the badnaviruses detected by RT-PCR are integrated into the yam genome not expressing sRNAs or inducing sRNAs. Further work will focus on understanding the reasons for these differences. In a meeting between the CIP and IITA teams in February 2021, CIP's experience with contamination control approaches were shared, and it was agreed to re-analyze IITA's data, taking into consideration these learnings, which will likely improve consistency of results.

RNase3 INHIBITORS

High throughput screening for chemical inhibitors of SPCSV RNase3

The mechanism of the synergism between SPCSV and other viruses was shown to be linked to a highly conserved RNase III encoded by SPCSV (named hereafter CSR3) that acts as an RNA silencing suppressor and cleaves small interfering RNAs (siRNAs), thus interrupting the endogenous plant antiviral response for RNA silencing. Therefore, the possibility of interfering with CSR3 activity was seen as a promising approach to the control of SPVD.

Previous studies showed that CSR3 belongs to class 1 RNase III enzymes. An amino acid sequence analysis (Annex 5, Figure 1a) confirms its similarity with three prototypical class 1 RNase III enzymes from *E. coli* (EcR3), *A. aeolicus* (AaR3), and *T. maritima* (TmR3). To generate a CSR3 structural model, I-TASSER was used to identify the template's structure using the LOMETS server and then to select and score the templates with the highest significance in the threading alignments, which were used to simulate a pool of protein structure decoys. Finally, the top five models were identified according to pairwise structure similarity using the program SPICKER. Top identified template structures consisted of the PDB structures 1O0W, 5B16, 3C4T, 2EB1, 2A11, 2FFI, 3O2R, 2NUG, 1YYK, and 4CE4.

Similar to other class 1 RNase III enzyme, the highest ranked CSR3 model was composed of an endonuclease domain (endoND) and a dsRNA-binding domain (dsRBD) connected by a flexible linker (Annex 5, Figure 1b). Based on previous structural and functional analysis in EcR3, AaR3, and TmR3, the catalytic site of CSR3 was determined to be composed of the four amino acids: 40E, 44D, 126N, and 129E (Annex 5, Figure 1a, black arrows). As depicted in the superimposed image of the catalytic sites of CSR3 and AaR3 (PDB 2NUG, 1.7 Å) shown in Annex 5, Figure 1b, 107D in AaR3 corresponds to 126N in CSR3.

The highest ranked model was selected for Glide-docking analysis and processed with the Protein Preparation Wizard of Schrödinger, whereas structures of 136,353 small molecules were prepared with the LigPreg function of Schrödinger using the default setup conditions. The active site residues of CSR3 (40E, 44D, 126N, and 129E) were selected as the center of the Glide-Grid box, and docking was performed using SP and XP scoring modes using the OPLS3 force field. By taking into consideration possible inaccuracy of the predicted model, the top 6,620 compounds were selected according

to the GlideScore rank order.

In parallel, His-tagged CSR3 (GenBank: ADQ42569.1) and its double-mutant CSR3-A (D37A, D44A) cloned into pET11d vector was expressed in *E. coli* and purified with a nickel-nitrilotriacetic acid (Ni-NTA) affinity column. The size of recombinant CSR3 and its mutant were ~26 kDa (Annex 5, Figure2A), and native western blots demonstrate that purified CSR3 and CSR3-A were able to form a monomer, dimer, and tetramer (Annex 5, Figure 2B). Further characterization of CSR3 oligomerization was done by size-exclusion chromatography with detection using multi-angle light scattering (Annex 5, Figure 2C). On agarose gel, substrate (200bp dsRNA) was completely cleaved in the presence of purified CSR3 but remained intact in the presence of CRS3-A and without any endoribonuclease enzyme (Ctl) (Annex 5, Figure 2D), indicating the successful purification of the active form of CSR3.

The large number of candidates selected by Glide-Docking (6,620) had diverse structures with no common scaffold, preventing efficient chemical improvement of *in vitro* testing. Therefore, a fluorescence resonance energy transfer (FRET)-based high throughput (HTS) assay suitable for large numbers of candidates was developed based on the assumption that CSR3 could cleave a labeled siRNA to generate a fluorescent signal (Annex 5, Figure 3A, 3B).

To set up the FRET-based HTS assay, real-time relative fluorescence units (RFU) of a negative control condition, represented by the absence of a FRET induced by CSR3 activity (Annex 5, Figure 3A) and two positive control conditions, represented by the presence of a FRET carried out either with the catalytically inactive CSR3-A or in the absence of any added enzyme (Annex 5, Figure 3B) was measured. A clear increase of fluorescence in the absence of FRET (i.e., negative controls) was observed, while fluorescence remained stable over time in the presence of FRET (i.e., positive controls) (Annex 5, Figure 3C). Moreover, these two control conditions were validated by analysis of the reaction products with 2% agarose gel electrophoresis (Annex 5, Figure 3D).

To optimize the FRET-based HTS assay, a CRS3 titration was conducted that demonstrated that the higher concentrations of CSR3 led to a rapid increase and higher fluorescence at the beginning of the kinetic measurement (Annex 5, Figure 4A). Slopes between all neighboring detection cycles (Annex 5, Figure, 4B), representing fluorescence changes over each cycle and depicting the reaction rate of CSR3, were used to select an optimal detection time for each CSR3 concentration and evaluate suitability for the HTS.

This suitability was estimated with the coefficient Z prime (Z'), measuring statistical effect size by considering means of positive and negative slope values and their respective standard deviation, Z' value above 0.5 is generally the considered threshold for an excellent assay.

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} + \mu_{c-}|} Eq.A$$

Mean of positive control (μ_{c+}) and negative control (μ_{c-}). Standard deviation of positive control (σ_{c+}) and negative control (σ_{c-}).

To validate homogeneity within and consistency between plates for the FRET-based HTS assay, whole-plate validation assays were conducted on replicate plates containing only positive and negative reactions (Figure 5A) which could easily be distinguished (Figure 5B). Slope values of positive and negative calculated and differed significantly (analysis of variance; $p < 0.001$; Figure 5C). Calculated Z' value of all plates were above 0.5 (0.77 ±0.08), signal-to-background ratio [μc- / μc+] was 9.84 ±0.75 and signal-to-noise ratio [(μc- – μc+) / σc+] was 43.17 ±8.72 confirming homogeneity of the HTS assay.

Primary screening of the 6,620 small molecules selected by Glide-Docking was conducted using compounds provided by the Institute for Molecular Medicine Finland (FIMM). Both negative (with CSR3) and positive (without CSR3) controls were included in all screening plates. The slopes of raw fluorescence between the negative and positive controls were clearly separated (Annex 5, Figure 6a). The average Z' was 0.82 ±0.04 (0.5 is generally the considered threshold for an excellent assay), indicating that the kinetic-based HTS was technically successful and that the results were qualitatively and quantitatively adequate. Percentage of inhibition (PI) of each compound for CSR3 was calculated according to ***Eq. B***. For primary screening a PI threshold of 30% was used as cutoff, resulting in the selection of 109 compounds (Annex 5, Figure 6b). The 12 compounds having PI values >90% had diverse structures, and no common scaffold was readily apparent (Annex 5, Figure 7).

$$PI = 100\,' \left[1 - \frac{(S_s - S_{c+})}{(S_{C-} - S_{c+})}\right]\% \; Eq.B$$

Slope mean of the positive control (Sc+), Slope mean of the negative control (Sc-), and Slope mean of samples (Ss).

To assess the FRET-based HTS method for other class 1 RNase III, and to determine the specificity of identified inhibitors, the aforementioned 109 compounds were screened for inhibition of *E. coli* RNase III (EcR3) activity. FRET-based HTS yielded a Z' value of 0.82, clearly confirming the suitability of the method for other class 1 RNase III enzymes. However, average PI values of EcR3 were only 17.1%, which significantly differed from the 54.4% average PI value obtained with CRS3 (analysis of variance; $p < 0.001$, Annex 5, Figure 8). In addition, only 32 of the 109 compounds had PI ≥ 30% with EcR3. These differences demonstrate the expected specificity of identified compounds for CSR3.

To further characterize the inhibitory effects of the 109 selected compounds, a dose-response assay was carried out with compound concentrations ranging from 1.25 nM to 50 µM. All data were analyzed with Breeze software, which generates, dose-response curves, and calculates the half-maximal inhibitory concentration (IC50). Considering that IC50 alone cannot comprehensively evaluate drug sensitivity of the dose-response model in an HTS assay, we also estimated a drug-sensitivity score (DSS) that integrated five factors: IC50, the slope at IC50, minimum activity level, and top and bottom asymptotes of the dose-response model. The DSS values of the 109 compounds ranged from 0 to 19.2. Structure of the top three compounds based on DSS and dose-response curve were identified (Annex 5, Figure 7, black stars).

To confirm and decrease the number of candidates, the dose-response assay was repeated a second time with the same compounds and a third time with 99 of the 109 compounds prepared by a second supplier. By combining these results and using a DSS threshold >4, 41 compounds were selected (Annex 5, Figure 9A, red circle).To assess a compound's binding affinity toward CSR3 and exclude false-positive results from FRET-based HTS assays, several binding affinity assays were conducted on the 99 commercial compounds with CSR3. A microscale thermophoresis (MST) was used and which resulted in 36 compounds of interest being identified (Annex 5, Figure 9B, MST). After obtaining results from the FRET-based HST dose responses and MST assay, two additional binding affinity steps were conducted by surface plasmon resonance (SPR). From the results of the FRET-based HTS and MST, the binding affinity of 56 compounds was tested by SPR. On the basis of the steady-state affinity (K_D) and the kinetics in the dose-response assay, 36 compounds of interest were identified (Annex 5, Figure 9B, SPR).By combing all assay data, 30 compounds were ultimately selected as potential CSR3 inhibitors (Annex 5, Figure 9B, red circle).

Design and synthesis of aptamers for inhibitory effects on SPCSV RNase3

The identification and synthesis of aptamers interfering with RNase3 activity were planned as an alternative to the chemical inhibition of RNase3. However, due to the promising results of the main approach **during year 1**, the necessity to develop this objective was less relevant, and resources were concentrated on the chemical inhibition objective. With the promising results from chemical inhibition in the later years of the project, this objective was never deemed as a priority and thus was not conducted.

The most efficient RNase3 inhibitors (chemicals and/or aptamers) tested for inhibition of SPCSV accumulation and SPCSV-induced viral synergism *in vitro*

To test the effects of the 30 compounds selected by kinetic-based assays on viral infection, sweetpotato (cultivar Huachano, CIP42006) co-infected with SPCSV and SPFMV were grown *in vitro* on medium supplemented with 50 µM of each compound (diluted in DMSO; final DMSO concentration, 0.1%) or only 0.1% DMSO as a control. The effects of the compounds on plant growth were monitored by imaging the plants once a week over 28 days (see representative picture in Annex 5, Figure 10B). At the end of the experiment, SPFMV and SPCSV accumulation was quantified by measuring relative expression of their respective viral coat proteins by qRT-qPCR.

Three of the 30 compounds caused plant stress symptoms and were excluded due to their potential toxicity in plants. The effect of the other 27 individual compounds on virus accumulation were estimated by comparing viral accumulation in treated and control plants. From the 30 selected compounds—Annex 5, Figure 9C, blue circle (DSS, MST, SPR)—with a 40% reduction threshold for SPFMV (Annex 5, Figure 9C, yellow circle [SPFMV<0.6]) and SPCSV (Annex 5, Figure 9C, green circle, [SPCSV<0.6]) accumulation, seven and 11 compounds were selected, respectively (Annex 5, Figure 9C). Among them, five reduced the accumulation of both viruses by at least 40% (Annex 5, Figure 9C, red circle). Specifically, SPCSV accumulation was reduced ~eightfold by two compounds (FIMM022230 and FIMM005536), fourfold by two compounds (FIMM051696 and FIMM000096), and twofold by the compound FIMM031755. SPFMV accumulation was reduced almost fourfold by three compounds (FIMM022230, FIMM005536, and FIMM051696) and twofold by two compounds (FIMM000096 and FIMM031755) (Annex 5, Figure 10A). Overall, these five compounds reduced both SPCSV and SPFMV accumulation without any phytotoxicity effects in sweetpotato plants (Annex 5, Figure 10B).

Structural clustering of the five compounds was done with ChemBioServer using the Tanimoto coefficient, WardLinkage, and a threshold of 0.5. They clustered hierarchically into two classes, with FIMM000096 placed in class 1 and the four

other compounds (FIMM005536, FIMM031755, FIMM051696, and FIMM022230) into class 2 with highly similar structures (Annex 5, Figure 11). Among the five inhibitors, the class 1 compound FIMM000096 has been approved as a powerful emetic. It has also been used in the treatment of Parkinsonism, but with adverse effects. The four other compounds in class 2 have not been reported either in the Drugbank database or for the treatment of viral diseases. However, they have been included in inhibitor screens for human enzymes or bacterial proteins according to PubChem identifier: CID: 2948389 (FIMM022230), CID: 7114450 (FIMM031755), CID: 2857906 (FIMM005536), and CID: 4240943 (FIMM051696). They were all inactive in these studies except for FIMM031755, which affected the activity of chain B of the human cytokine/receptor binary complex.

In parallel, to determine relevant morphological or physiological parameters to assess the effect of the selected compounds on viral infection, single- and co-infection of SPCSV and SPFMV were phenotyped in a non-destructive assay by using an imaging-based high-throughput plant phenotyping platform (NaPPI) evaluating six conditions (Annex 5, Table 1).

Sweetpotato plants were propagated from *in vitro* into soil and grown in the NaPPI. Measurements were obtained every second day over 29 days, including visible top-view (RGB), chlorophyll fluorescence (ChlF), and thermal infrared (TIR) imaging. In addition, side view imaging was done manually after seven, 14, 21, and 28 days post-transplanting (dpt). After 31 dpt, detached leaves were imaged and samples were taken for viral titer and biomass measurements.

A representative picture of the six conditions at 31 dpt can be seen in Annex 5, Figure 12A. No differences in plant height (Annex 5, Figure 12B), biomass (Annex 5, Figure 12C), or leaf surface area (Annex 5, Figure 12) were detected between non-infected and SPFMV single-infected (Wt-H, R3-H, and Wt-F). Whereas all other conditions (Wt-C, Wt-FC, and R3-F) depicted significant reductions compare with those three treatments. No significant differences in shoot/root ratios from the six conditions, except for RNase3 transgenic plants infected with SPFMV, were detected.

Leaf symptoms (Annex 5, Figure 13A) and viral distribution (Annex 5, Figure 13B) between single- and co-infected plants differed. SPCSV accumulation was higher in older leaves for both Wt-C and Wt-FC, whereas SPFMV was slightly higher in younger leaves in Wt-FC and stable in Wt-F (Annex 5, Figure 13B). Data indicated that Wt-FC SPFMV and SPCSV distribution were opposed (SPFMV in younger leaves and SPCSV in older leaves) with a cross-over point around leaf five (Annex 5, Figure 13B, black arrow). Moreover, SPCSV was higher in Wt-C than Wt-FC while the opposite was observed with SPFMV (Annex 5, Figure 13B). Finally, viral accumulation measured in the first fully developed leaves in all conditions showed that relative expression of exogenous RNase3 in R3-F and R3-H was insignificant compared with expression of SPCSV and SPFMV coat protein, SPCSV accumulation was not different between Wt-C and Wt-FC and SPFMV accumulation increased drastically in Wt-FC and R3-F as expected (Annex 5, Figure 13C).

Plant physiological performance, focusing on photosystem II (PSII)-related parameters, was monitored over 29 days. Five parameters—that is, effective quantum yield of PSII (ΦPSII), photochemical quenching (qP), maximum quantum yield (QY_max), non-photochemical quenching (NPQ), and leaf temperature—were closely analyzed.

According to Tukey's HSD test, the six conditions grouped into four and five subsets for ΦPSII and qP, respectively (Annex 5, Figure 14A and B). With ΦPSII, single-infections grouped with R3-H while all other conditions were in their own group. Similar results were observed with qP, except that R-H could be distinguished from WT-F. The gradual decrease of these two parameters from Wt-H, R-H, single-infected, co-infected until R3-F demonstrated that PSII photochemistry was correlated with the severity of disease symptoms.

QY max indicating the maximum efficiency at which light absorbed by PSII is used for reduction of the quinone is usually a good indicator of plant stress. The six conditions were grouped in only three subsets with this parameter (Annex 5, Figure 14C). No difference could be estimated between healthy plants and single-infected or between single-and co-infected. NPQ, estimating the constant rate of heat-loss for PSII, correlated with changes in heat dissipation. The six conditions grouped in two subsets where co-infected and R3-F grouped together with an important heat-loss while the other subset included the remaining conditions (Annex 5, Figure 14D). Finally, thermal imaging monitoring of the leaf surface temperature grouped the six conditions in four subsets with significant differences among healthy plants, Wt-F, Wt-FC, and R3-F showing that higher temperature correlated with symptom severity (Annex 5, Figure 14E). Top-view images of healthy and co-infected plants representing ΦPSII, qP, and leaf temperature (TIR) parameters from day 3 to 17 could be found in Annex 5, Figure 14F.

To confirm the PSII efficiency reduction observed by imaging-base phenotyping, expression of several genes involved either in photosynthetic or Calvin cycle pathways (Annex 5, Table 2) were compared in single- and co-infected plants. All fold changes were calculated using gene expression of Wt-H as control. All genes related to PSI and PSII (PsaA, PsbA, PsbC, and PsbN) were upregulated in Wt-C, while only *PsaA* and *PsbC* were upregulated in Wt-F. For Calvin cycle

pathways genes, *RbcL* was upregulated in both single-infected plant, *FBA5,* and *RbcS1* was upregulated only in Wt-F and *Rca* underwent an abnormally strong downregulation in Wt-C, but was stable in Wt-F. In co-infected plants, expression of all PSI and PSII complex genes were downregulated, while expression of only two Calvin cycle pathway genes (*FBA5* and *Rca*) were downregulated **(**Annex 5, Figure 15). In summary, most of the studied genes were upregulated in single-infected plants, whereas most of these genes were downregulated in co-infected plants (Annex 5, Figure 15). Thus, viral synergism leads to a clear dysfunction in photosynthesis, glycolysis, and gluconeogenesis pathways. In addition, the single-infected plant with mild symptoms depicted an upregulation of those pathways, which is possibly due to the interaction between plants and viruses.

Taken together, these results validate the use of ChlF- and TIR-based imaging systems to distinguish virus disease severity related to SPFMV and SPCSV in sweetpotato and demonstrate that the ΦPSII and qP are sensitive parameters for the quantification of virus effects.

Unfortunately, only four of the five inhibitors (FIMM000096, FIMM031755, FIMM022230, and FIMM005536) were available in sufficient amounts for *in planta* treatments. Co-infected sweetpotatoes were grown in the NaPPI as described above. After one week, plants were treated by foliar spray either with individual compounds diluted in water at 10 µM or water (control/mock) twice a week over one month. NaPPI measurements were taken twice a week, two and three days after each treatment. After the last treatment, three additional measurements were taken.

At the morphological level, no difference between each treatment and control could be observed in terms of plant height (Annex 5, Figure 16A), total leaf surface (Annex 5, Figure 16B), plant area, total number of leaves, or biomass. At the physiological level, leaf temperature (Annex 5, Figure 16C) was similar for all conditions, whereas the quantum yield of PSII (ΦPSII) was significantly higher in all four treatments compared with the control, reflecting improved photosynthetic performance in the treatments relative to the control (Annex 5, Figure 16D). In addition, top-view images of all treated plants representing the ΦPSII parameter after one month of treatment (Annex 5, Figure 16E) showed signs of plant stress, as represented by visible light (RGB) imaging.

After 41 days of treatments, both SPFMV and SPCSV accumulation was quantified by measuring relative expression of their respective viral coat proteins by qRT-qPCR. The effect of the four compounds on virus accumulation was estimated by using the fold change (FC) of their expression compared with controls (Mock). Accumulation of SPCSV was reduced in three out of the four compounds (FIMM000096, FIMM022230, and FIMM005536), but high variability of values prevented the ability to draw a clear conclusion regarding SPCSV (Annex 5, Figure 17, lower panel). In contrast, SPFMV accumulation was significantly reduced by all four treatments: reduction ranged from 40% to 60% (Annex 5, Figure 17, upper panel). Taken together, these results demonstrate that the four compounds had a negative effect on SPFMV accumulation and a positive effect on photosynthetic performance of co-infected sweetpotato plants.

In further analyses, three concentrations of the compounds were applied by foliar spray over a one-month period. NaPPI measurements were done as described above. No morphological differences could be detected. Interestingly, the highest concentration (100 µM) failed to increase ΦPSII values, whereas treatment with 10 µM and 1 µM significantly improved ΦPSII parameters, with the highest value occurring with the lowest concentration for all four compounds (Annex 5, Figure 18). These results confirmed the improvement of photosynthetic performance by all four compounds and point out that excess of inhibitors could limit its effect on photosynthetic performance.

To validate that the four compounds could interfere with RNA silencing by suppression of SPCSV RNase3, co-suppression experiments were initiated using transgenic *N. benthamiana* 16c. Several *Agrobacterium tumefaciens* (GV3850) strains (Annex 5, Table 3) previously generated by our laboratory were used.

Infiltration of GFP/GUS (negative control) and GFP/p22 (positive control) lead to no fluorescence and strong fluorescence signals respectively, as expected in all experiments, confirming success of infiltration (Annex 5, Figure 19). But the infiltration of GFP with any SPCSV RNase3 (Ug, KML33b or SOR71 isolates) failed to trigger silencing suppression, showing only background signal (Annex 5, Figure 19). Different parameters (*A. tumefaciens* OD, mixing ratio and acetosyringone concentration) were adjusted leading to similar results. By increasing the number of infiltrated plants we finally observed weak silencing suppression (Annex 5, Figure 19; lower panel, left leaf) with SPCSV RNase3 (Ug strain) but only in one infiltration spot over 24 (repeated twice with same yield). Owing to the COVID-19 situation, further fine-tuning of the experiment became more and more difficult and results to date were not sufficient to continue the testing of the four compounds on suppression of RNase3 silencing.

Taking a different tact, to test if the four selected compounds could prevent SPCSV RNase3 cleavage of vsiRNA, we generated and sequenced small RNA libraries of treated/untreated sweetpotatoes. Total RNA of treated sweetpotato

generated for NaPPI measurements (foliar sprayed twice a week for one month either with 10 µM of each compound or water) was isolated. The proportion of SPCSV and SPFMV vsiRNA ranging from 20 to 24 bp were compared between the five treatments and the control condition (Mock). Normalized counts of SPFMV vsiRNA in all treatments were similar to the controls (Annex 5, Figure 20A). However, SPCSV vsiRNA normalized counts were increased in all four treatments, but only two compounds (FIMM022230 and FIMM03175) showed a significant shift. However, importantly, variability in the control condition prevented the drawing of a clear conclusion (Annex 5, Figure 20B). Altogether, the increased pattern observed in SPCSV vsiRNA seems promising as they could correlate with the expected effect of the compounds. Unfortunately, these effects remained minor in our experiment, preventing a clear conclusion, and would require further experimentation to draw a definitive conclusion.

In parallel, based on the structural clustering of the five inhibitors (Annex 5, Figure 11), an *in silico* similarity search approach was used to identify new compounds structurally similar to the two classes identified. This similarity search approach led to the selection of 314 compounds. To test their inhibition potential on SPCSV RNase3 activity, one concentration of the FRET-based HTS was conducted using compounds provided by FIMM on 310 out of 314 compounds. Fifty-nine compounds with PI values above 50% were selected for dose-response FRET-based HTS. Dose-response screening was conducted twice with those 59 compounds. Together, these experiments allowed the identification of a wider list of alternative inhibitors for SPCSV RNase3; nonetheless, none of them were validated *in planta* for their phytotoxicity or effects on viral accumulation. Thus, they could be used as a reservoir of potential inhibitors but would require further validation step *in planta*.

All in all, identification of CSR3 inhibitors conducted during this project could be summarized in five major phases (Annex 5, Figure 21). During phase one, the structure of CSR3 was modeled and virtual screening using Glide-docking was performed targeting the active site of CSR3. In phase two, compound screening in the laboratory was first performed with a new kinetic-based HTS method using FRET, and then the binding affinity between CSR3 and the compounds was characterized using two complementary assays, MST and SPR. Phase three involved an *in vitro* screening assay using co-infected (SPCSV + SPFMV) sweetpotato plants grown in TC medium. The effects of the inhibitors on viral accumulation were monitored by RT-qPCR. Phase four consisted of a posterior cluster study of the hits based on the compound structures. Finally, phase five involved validation assays *in planta* of co-infected sweetpotato plants grown in soil using imaging-based methods.

Considering all screening steps, five compounds (FIMM000096, FIMM022230, FIMM051696, FIMM031755, and FIMM005536) belonging to two structural classes (Annex 5, Figure 11) were shown to inhibit the catalytic activity of SPCSV RNase3 and reduce significantly virus accumulation *in vivo*. Four of them (FIMM000096, FIMM031755, FIMM022230, and FIMM005536) further validated *in planta* depicted a significant improvement of photosynthetic performance correlating with a significant reduction of SPFMV accumulation. Altogether, these five steps resulted in a final hit rate of 0.0029% (four compounds out of 136,353). The hit summary of all assay steps is shown in Annex 5, Table 4. Additionally, preliminary results indicate that all four compounds could prevent cleaving of SPCSV vsiRNA, and based on structure similarity search and FRET-based HTS assays, a wider reservoir of potential inhibitors was defined.

VIRUSDETECT

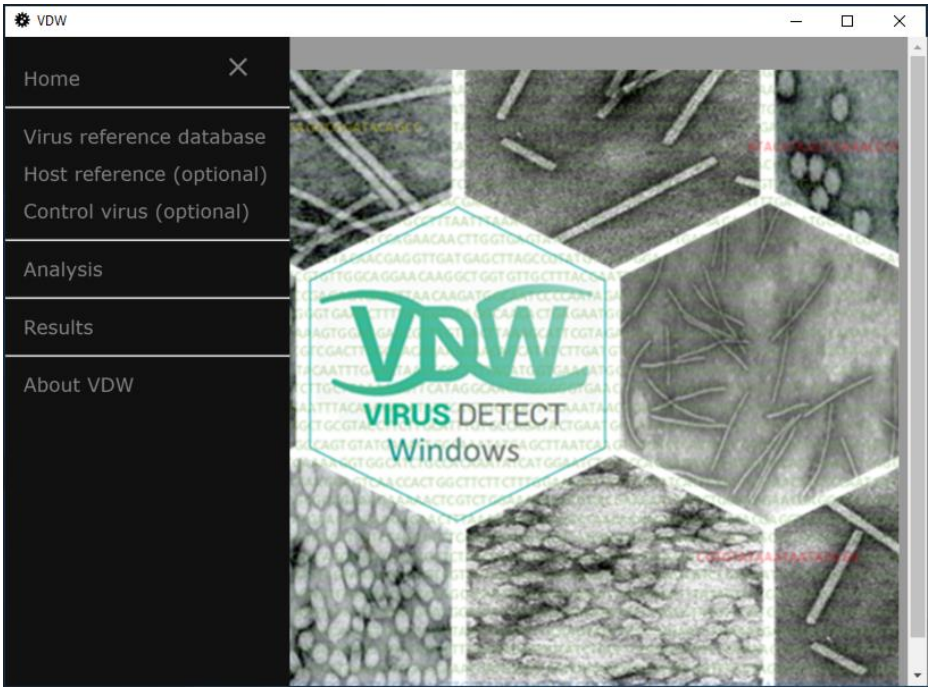
Initially, the plan with the VirusDetect platform was to integrate it with the Chipster platform of CSC–IT Center for Science (https://chipster.csc.fi/news_archive.shtml) and training was provided on how to run VirusDetect on the Chipster platform (https://www.youtube.com/watch?v=Z-zxzeQxRkQ&list=PLjiXAZO27elCYwsme8hPqdw0zCTd8mXs6&index=4). The objective was to make VirusDetect more user-friendly and capable of running on laptops with enhanced capacity and functionality. The move to Chipster enabled the incorporation of many user-friendly elements (simplified algorithms, less guesswork in calling sequence matches), but the combined platform was still a relatively heavy program (requiring ~ 1 Tb of hard disk space) with many elements not needed for virus detection. Hence, although greatly simplified, VirusDetect was still complicated to download, particularly in areas where bandwidth is low (such as Africa). Additionally, even with these improvements, certain functions still needed to be added while other functions needed to be developed to enable the characterization of whole genomes from partially characterized viruses or new and novel viruses. The Chipster Platform did not facilitate the straightforward implementation of these functions as was envisioned. It was therefore decided that development of a new Windows standalone version of VirusDetect was needed.

To address the limitations of the VirusDetect/Chipster integrated platforms, the VirusDetect software was completely reformatted into a Windows (VDW) compatible platform which made VirusDetect much more user friendly. To achieve this, individual VirusDetect requirements and source code were reformatted for adaptability and integration with

Windows. The source code of each of the requirements was checked and compiled according the original programming language. As a result, executables for Windows are available: Hisat2. One limitation of the Windows environment for this purpose is that it does not have Perl language installed by default. Therefore, a portable Perl component was included by the development of a customized version of a portable Perl that included BioPerl libraries used by VirusDetect.

The first version, which could process both large and small RNA datasets, perform sequence trimming and cleaning and fastQC (quality analysis), was presented in June 2019 and has since been used by CIP and IITA for analysis of sRSA data. CIP took over further VirusDetect development, and a second version was released that provides the ability to include optional spike-in and contamination control sequences, an option for de-duplicating reads and the ability of setting maximum as well as minimum size of small RNA reads. If spike-in and control sequence options are chosen, the software now reports data on the total and normalized number of spike-in sequences that are detected in each sample to serve as a quality control step for the library and to provide a percentage value for contamination occurring relative to the control sample that can used to determine thresholds for positive samples and virus presence. New individual charts were also created to report sRNA reads by size and per sample as well as to display results of read cleaning. The summary report gathers all information corresponding to the analysis of samples analyzed in a run (usually a set of 48 barcoded samples including controls): Cleaning reports, size distribution of reads, control report, as well as blastx and blastn reports. Several versions with iterative bug-fixes have been produced, and further improvements are still likely as bugs are discovered with further use.

The current VDW v0.94 can be downloaded from: https://research.cip.cgiar.org/virusdetect/ or http://potpathodiv.org/vd_download.html. Testing continues and a manuscript is in development for publication in a scientific journal. Further plans are to release the VDW version officially at a scientific conference as well as to provide training workshops, but have been delayed due to the COVID-19 pandemic.



QUICKCHIP

With the UW-M departing the project in 2019, the PI there felt that the QuickChip technology and reader were almost ready for testing. At this time (May 2019), the work done at UW-M included the following:

- The pre-identification of relevant viruses and the development of recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP) assays, or other isothermal assays.
- Testing both LAMP and RPA assays in the QuickChip format, demonstrating that it can reproducibly achieve S/N ratios of two to ten.
- Testing the viruses related to this project (Annex 6, Table 1), the RPA assays worked very well on the chip, with low to moderate S/N ratios for LAMP assays.

The testing consisted of (1) testing synthetic blocks of DNA *in vitro* using primers and probes designed specifically for

detecting the viral sequences; (2) testing extracted viral RNA or DNA isolated from plant leaves in the lab using a laser-based fluorescence imager (Typhoon Imager, GE); (3) testing extracted viral RNA or DNA isolated from plant leaves in the lab using a bench-top reader that is an early prototype of the portable readers; and (4) testing leaves from infected plants using the portable readers.

CIP and Dr. Douglas Weibel from UW-M discussed in the first half of 2019 how the QuickChip phase of the project could be finished. In the end, the university shipped all the equipment and consumables on hand necessary to produce the chips, know-how to design new chip layouts, know-how to run the devices and interpret the results, and two prototype readers to CIP-Lima in hopes this would enable CIP to finish the testing of the QuickChip. Dr. John Crooks from UW-M traveled to CIP-Lima in mid-2019 to help with capacity building at CIP to run the assays and test the QuickChips on material in the field. The readers were tested with sweetpotato leaf extracts and purified RNA extracted from sweetpotato plants infected with SPFMV, SPCSV, and begomovirus. Additionally, Dr. Crooks worked with CIP staff to work out the hurdles with low S/N ratios for the LAMP assays.

Although the lab testing of the QuickChip was completed, the reporting interface for the QuickChip was insufficient to enable field testing. Nor did CIP have the technical expertise to make the necessary adjustments. One additional challenge is that the QuickChip reader needs to be attached to a computer, and there appears to be additional programing needed to enhance the functionality of the app associated with the QuickChip. CIP initiated a student project with UTEC in Peru to try and address the interface issue; however, the COVID-19 pandemic prevented its advancement. Nevertheless, CIP will try and continue to advance the QuickChip development with UTEC in the future if able.

2. Deliverables not met or completed

- The preliminary test to determine the effect of dark periods for meristem growth on the regrowth of meristems had only a single repetition in sweetpotato with a limited number of five accessions, which did not show encouraging results. Therefore, priority was given to other activities as the COVID-19 pandemic created staff limitations. Thus the focus was on the completion of other activities.
- The original experimental design included an experiment to test the effect of the addition of proline into the culture medium to stimulate meristem growth. The concept was that proline was reported to stimulate *in vitro* growth of embryogenic systems and to induce the production of plant secondary metabolites. Thus, we hypothesized that it could also stimulate meristem growth. However, when this was further investigated, we discovered that commercially available proline needed to be derivatized prior to use *in vitro*, and this process involved some purification and quality control steps for the derivatized product that were beyond the means of this project.
- Because of slow growth of *in vitro* plants and the COVID-19 situation, there was a shortage of plant material which necessitated the prioritization of available plant material for priority experiments. Thus, this experiment to test the use of axillary buds for meristem excision was cancelled to allow increased repetitions of other experiments.
- The identification, generation, and testing of aptamers with potential to prevent dsRNA cleavage by SPCSV RNase3 was kept aside to focus resources on the chemical inhibition task. With the promising results and advancement of chemical inhibition along the whole project, there was neither enough time nor little relevance to spend resources on this deliverable, so it was not conducted and no aptamers were identified or tested. Instead, we identified and validated, *in vitro* and *in planta*, five chemical inhibitors for SPCSV RNase3. In addition, a list of alternative inhibitors validated only *in vitro* was generated.
- With the UW-M withdrawing late in the project period, the deliverables this partner was accountable for (development of the QuickChip reader and high throughput screening of novel antiviral compounds) were not completed due to the highly specialized, nature of these deliverables.

3. Changes made to the deliverables in the course of the project

Slow growth of *in vitro* plants limited the inventories available for multiple experiments. This, together with the recent COVID-19 crisis, resulted in the cancellation of some of the planned activities.

A COVID-19 lock-down started at CIP-Lima in mid-March 2020, where only limited number of staff could enter CIP and thus work needed to focus the most crucial conservation activities, principally to maintain our *in vitro* collections (~ 15,000 accessions). During the first few months of the pandemic, the present project was completely on stand-by, and when fully restarted in July 2020, focus was exclusively on the experiments involving antiviral spraying and thermotherapy of greenhouse plant. In March 2020, repetition 1 of this experiment in sweetpotato had to be abandoned and started over from the scratch using greenhouse cuttings as propagation material.

In the early phases of the project, the focus was on multiple preliminary experiments in parallel (with the same set of accessions), which forced the multiplication and growth rate of the *in vitro* plants to the limit. It was noted that accelerated multiplication, with short periods between each multiplication cycle, reduced the growth potential of the plants, thus further delaying the replication of experiments.

Fortunately, the project enabled the development of a liquid *in vitro* multiplication step in sweetpotato. This increased the time for conversion of plants from meristems by 33%, which in the future will significantly help generate larger numbers of plant materials for subsequent experimentation. In cassava, the addition of abscisic acid was found to be beneficial for meristem regrowth, and the addition of charcoal to the medium enhanced regrowth from yam meristems.

For the RNase inhibitor screening, due to the high numbers of candidates selected from *in silico* screening (6,620; Glide-Docking), traditional *in vitro* testing of the compounds could not be achieved. Instead, a high-throughput screening method (FRET-based HTS) suitable for testing a large numbers of candidates *in vitro* was developed. The development and achievement of our high-throughput screening approach, and further essential validation (MST and SPR) of selected candidates, delayed the *in vitro* testing. In addition, because of the large number of candidates, we could not conduct chemical modifications of promising inhibitors. Since the most promising inhibitors were shown to be effective *in planta* without any obvious side effects, no chemical improvement was deemed to be required within the project's timeframe.

Finally, as reported in the last annual report, UW-M withdrew from the project after the PI took another job. As this occurred late in the project, and the experiments being done (development of the QuickChip reader and high throughput screening of novel antiviral compounds) were highly specialized, despite efforts to transfer the QuickChip technology to CIP-Lima to continue this phase of the project, the technologies were not developed adequately to a successful transfer.

4. Capacity building activities done in conjunction with the project

Topic	Date(s)	Location	Participants	Summary of activity
sRSA, VirusDetect and virus therapy training	2/20-3/3, 2017	IITA (Ibadan, Nigeria)	5	Training in the analysis of a diverse set of yam and cassava tissues (fresh samples for established plants grown under screen house and field conditions; samples from in vitro plantlets, dehydrated specimens, and samples from yam tubers).
Project Awareness Workshop	3/2/17	IITA (Ibadan, Nigeria)	20+	A 3-hour mini-workshop was held for staff from IITA and the Nigerian Agricultural Quarantine Services, in addition to a few students from the University of Ibadan to present an overview of the science of the proposed research, as well as the expected impact and potential outcome of the project. The presentations were followed by a Q&A session where the PIs from the two Centers engaged actively with IITA staff.
International Tropical Root Crops (ISTRC) Meeting	3/5-3/10, 2017	Dar es Salaam, Tanzania		Poster presentation promoting the NextGen Phytosanitary Cleaning Project presented at the ISTRC meeting (Appendix 2). Ellis, D., Gueye, B., Kreuze, J., Kumar, L., Valkonen, J., Weibel, D.2017.NextGen Phytosanitation: Rapid Elimination of Viruses from RTB Plants for Crop Improvement and Seed System
NextGen Phytosanitary Cleaning Project Initiation Workshop (ISTRC Meeting)	3/7/17	Dar es Salaam, Tanzania	50+	A special open evening session for the introduction of NextGen Phytosanitation project with all project PIs participating. Session introduced key project themes, opportunities for collaboration and capacity development. A full-page flyer on the project was designed and distributed to the participants.
Training on use of VirusDetect	6/16/2017	Dar es Salaam, Tanzania	22	Workshop on the use of the VirusDetect software.
High Throughput Screening (HTS) of chemical inhibitors of SPCSV RNase3.	18/09/17	U of Helsinki, Helsinki, Finland	8-9	NextGen Phyto annual meetings reporting project progress done by Linping Wang.
sRSA and VirusDetect workshop	3/18	CIP, Lima, Peru		Workshop to transfer technology of sRSA and VirusDetect – attended by 2 IITA staff + a student from U of Ibadan
VDW course	4/2018	Lima Peru	14	A course on VDW for Latin American phytosanitary agencies using technology and knowhow partially from the NextGen Project
sRSA and Virus use for clonal crop virus indexing Detect Training	18-22/6/2018	CRI, Kumasi, Ghana	13	The courses offered hands-on training in sRSA library preparation and data analysis using VirusDetect software. Participants were allowed to use their own samples for processing and model data

Workshop				sets were given for virus detection using VirusDetect
sRSA and VirusDetect for virus indexing Training Workshop	25-29/6/2018	ICRAF, Nairobi Kenya	10+	The courses offered hands-on training in sRSA library preparation and data analysis using VirusDetect software. Participants were allowed to use their own samples for processing and model data sets were given for virus detection using VirusDetect
4th Global Cassava Partnerships in the 21st Contrary (GCP21) International Conference	11-15/7/18	Cotonou, Benin	N/A	Poster on NextGen phytosanitation project outputs at the sRSA and VirusDetect
VDW course	7/2018	Helsinki, Finland		A course to launch VDW using technology and knowhow partially from the NextGen Project
Study of chemical inhibitors of SPCSV RNase3	8/09/18	Amsterdam, Netherlands	8-9	NextGen Phyto annual meetings reporting project progress done by Linping Wang
Weblink presentation on high throughput sequencing for virus detection	8/2019	Lima. Peru	~30	Presentation at the "Taller REGIONAL DE LA CONVENCIÓN INTERNACIONAL DE PROTECCIÓN FITOSANITARIA (CIPF) PARA AMÉRICA LATINA"
RTB-ISTRC	10/2018	Cali, Columbia	~50	Update on research results from project presented
30th Technical Consultation among Regional Plant Protection Organizations (RPPOs)	10/2018	Lima, Peru	20	Update on research results from project presented
Outreach activities	Throughout 2018		N/A	Distribution of flyers and incorporation of sRSA virus indexing as part of the seminars on clean seed production and virus indexing
VDW course	9/2018	Nairobi, Kenya	6	Library preparation and data analysis (using VDW) course at was given at KEPHIS, Muguga, Kenya using technology and knowhow partially from the NextGen Project
VDW course	11/2018	Gembloux, Belgium	20+	A course on VDW for CGIAR genebank GHUs and European NPPOs using technology and knowhow partially from the NextGen Project
Phenotyping viral infection in plant using chlorophyll fluorescence and thermal imaging.	20-24/07/19	Minneapolis, MN. United State	N/A	Poster presentation at The American Society for Virology's 38th Annual meeting.
Identification of chemical inhibitors for RNase3 of SPCSV	26/05/20	Helsinki, Finland	60	PhD defense of Linping Wang.
RTB Webinar	28/05/2020	From IITA Ibadan, Nigeria	Online	Webinar for working group and SweetGains project on the 'Rapid propagation of virus-free cassava and yam using stem cuttings
23rd IARSAF Symposium	14/09/2020	Online from IITA, Ibadan, Nigeria	Online	Talk entitled "If Covid-19 is a plant infecting virus? Lessons from the pandemic for viral disease control"
BBSRC CONNECTED Annual Meeting	29/9-2/10/2020	Online from IITA, Ibadan, Nigeria	Online	Talk entitled "Sample Preservation and Field Diagnostics Kits"
International E-Conference on "Multidisciplinary Approaches for Plant Disease Management in Achieving Sustainability in Agriculture	6-9/10/2020	Online from IITA, Ibadan, Nigeria	Online	Talk entitled "The interplay of diagnostics, phytosanitation, and regulations in surveillance and control of transboundary plant diseases"
AU-Inter-African Phytosanitary Council virtual workshop for the Member States Capacity Building and Implementation of IPM Strategies and Migratory/ Trans-Boundary Pest	15/10/2020	Online from IITA, Ibadan, Nigeria	Online	Talk on "IPM Innovations and Technologies for Surveillance and Control of Transboundary Pests"

Management Workshop				
AU-Inter-African Phytosanitary Council virtual workshop for the Member States Capacities on Invasive Alien Plant Species, Risk Assessment and Management	23/11/20202	Online from IITA, Ibadan, Nigeria	On-line	Talk entitled "IITA initiatives on diagnosis and control of invasive plants pests in Africa"
Latin American workshop "applied-bioinformatics, molecular epidemiology, and sars-cov-2 pandemic"	1-4 December	Online from Colombia	30	Training in the use of VDW for SARS-CoV-2 genome assembly

- 2017: two face-to-face meetings (Dar es Salaam + Helsinki) + four PI Skype calls
- 2018: face-to-Face, Amsterdam + meetings at workshops + four PI Skype calls
- 2019: no formal face-to-face although many bi & tri lateral meetings in various venues + four PI Skype
- 2020: no formal face-to-face PI meetings due to COVID-19 – nine PI Skype calls

5. Activities related to the project that will continue to provide impact from the research after the project ends.

CIP

It was shown for sweetpotato that reducing the macro-nutrient concentration of the meristem culture medium from full-MS to ½ or ¼-strength or removing NH_4 from the full-strength MS medium (and replacing it by KNO_3) significantly improved the recovery and rooting rates, as well as the number of leaves formed per plantlet. These results will now be tested on a wider range of genotypes (20–50 genotypes) to confirm the results prior to making a change in our protocol.

Using agitated liquid medium for meristem culture (after five weeks on solid medium), a three-times higher recovery rate can be realized and the time required to obtain complete *in vitro* plants can be reduced from a maximum period of 27 (routine) to 18 weeks . Placing the meristems earlier in liquid medium (e.g., after three weeks on solid medium instead of five weeks) may further reduce the process time and increase efficiency. As this experiment was repeated three times with 10 accessions, we believe that the results could be directly applied for meristem culture and used by CIP projects in the regions (Africa, Asia) or other institutes. A paper on these results is in preparation.

It was shown that it is feasible to introduce shoot tips into *in vitro* directly from greenhouse plants (instead of stem segments), and that prior treatment of the greenhouse plants with antivirals (salicylic acid), thermotherapy, or a combination of both may help speed virus elimination in sweetpotato. Additional experimentation is needed to further develop and fine-tune a protocol, but the results from these experiments are very promising.

As a side effect, it is possible to grow two sweetpotato plants per 4.5-in. pot (instead of one plant/8-in. pot), which can contribute to a more efficient use of space in future experiments or operations (some four times more plants per area).

Further confirmation of the sRSA results versus conventional phytosanitary cleaning by PCR is ongoing now that the PCR machines at CIP have been fixed; additional analysis of the sRSA spiked experiments are also still under analysis. After this work is done, CIP will compile the data in a way that will meet ISO certification standards to replace the biological indicator plant indexing with sRSA. This will reduce the time needed to obtain a certified phytosanitary clean sweetpotato *in vitro* plant by at least 60% (savings of at least a year). We will start implementing this in 2021.

IITA

The *in vitro* meristem regrowth media preparation method was updated to incorporate ascorbic acid in the cassava meristem regrowth media and activated charcoal in the yam meristem regrowth media to enhance the regrowth rate of genebank and breeder's germplasm. Further experiments will be conducted by combining various combinations tested in this project for identification of most effective protocol for enhancing meristem regrowth. The optimized protocol will be published as a methods manual for use by other stakeholders.

Optimized protocol for virus elimination on cassava and yam will be tested on a larger number of accessions and scaled-up for enhancing availability of clean plant material faster to the breeding, production, and germplasm conservation (cryobanking and *in vitro* genebank) programs.

The lack of a significant antiviral effect of three agents used was unexpected. However, a high percent of virus-negative plants was observed in these experiments, suggesting an unknown mechanism contributing to auto-recovery from virus infection. Treated and untreated plants of these trials are being maintained to continue observations and plan further studies to understand this phenomenon and to assess its potential for exploitation to generate virus-free clean plants.

The sRSA protocol established for clonal crop indexing at IITA is a major advancement that is expected to improve confidence on certification of breeder and genebank planting materials. Moreover, it is expected to fast-track international germplasm distribution within and outside Africa.

University of Helsinki

This project successfully identified four inhibitors of SPCSV RNase3 interfering with viral accumulation *in planta*. Moreover, based on their common structures, a list of alternative inhibitors was also generated. These inhibitors or closely related compounds could be used as new tools for the generation of virus-free materials and would also serve as a starting point for the development of novel methods in the management of SPVD under field conditions. A patent application covering the use of the five inhibitors and their derivative formulas in the suppression of SPVD was filled by the University of Helsinki in order to protect their use by public entities and ensure their affordable and meaningful access for later use. This patent was filled on 17 April 2020 (Application number 20205392) and is still under review to assess whether the invention is patentable (six to nine months).

In this project we developed a novel FRET-based HTS method that could be used to identify broad-spectrum inhibitors of other class 1 RNase III.

6. Publications, presentations, and publicity that resulted from the project research

Accepted publications:

Wang, L., Poque, S., and Valkonen, J.P.T. 2019. Phenotyping viral infection in sweetpotato using a high-throughput chlorophyll fluorescence and thermal imaging platform. *Plant Methods* **15**:116.

Wang, L., Saarela, J., Poque, S., and Valkonen, J.P.T. 2020. Development of FRET-based high-throughput screening for viral RNase III inhibitors. *Mol. Plant Pathol.*, mpp.12942.

Submitted publications:

Kutnjak, D., Tamisier, L., Adams, I., Boonham, N., Candresse, T., Chiumenti, M., De Jonghe, L., Kreuze, J.F., Lefebvre, M., Silva, G., Malapi-Wight, M., Margaria, P., Pleško, I.M., McGreig, S., Miozzi, L., Remenant, B., Reynard, J.S., Rollin, J., Rott, M., Schumpp, O., Massart, S., and Haegeman, A. 2021. A primer on the analysis of high-throughput sequencing data for detection of plant viruses. Microorganisms, submitted.

Wang, L., Poque, S., Laamanen, K., Saarela, J., Poso, A., Laitinen, T., and Valkonen J.P.T. 2020. Identification of chemical inhibitors for viral RNase III to eliminate Sweet potato virus disease (SPVD). PLOS Pathogens, under review.

Papers in prep:

- Research article on *in vitro* activities from this project highlighting the use of liquid medium for sweetpotato meristem grown is planned for submission to a peer-reviewed journal during the first quarter of 2021.
- Research article on VirusDetect for Windows, including method optimizations and validations conducted in this project for sweetpotato, cassava and yam, and potato from a complementary project.
- In collaboration with Valitest EU consortium: Guidelines for the selection, development, validation and routine use of high-throughput sequencing analysis in plant health diagnostic laboratories.

2. Geographic Areas to Be Served

Provide the final list of countries and sub-regions/states that have benefitted from this work and associated dollar amounts. If areas to be served include the United States, indicate city and state. Add more rows as needed. More information about Geographic Areas to Be Served can be found here.

Location	Foundation Funding (U.S.$)
Nigeria	700,000
Ghana	50,000
Benin	50,000
Uganda	100,000
Tanzania	100,000
Kenya	100,000
Mozambique	50,000
Other countries W. Africa	200,000
Other countries in SSA	200,000
Global	835,906

3. Geographic Location of Work

Provide the final list of countries and sub-regions/states where this work has been performed and associated dollar amounts. If location of work includes the United States, indicate city and state. Add more rows as needed. More information about Geographic Location of Work can be found here.

Location	Foundation Funding (U.S.$)
CIP, Peru—Lead organization	1,129,731
IITA, Nigeria	668,529
University of Helsinki, Finland	272,920
University of Wisconsin–Madison, Madison, WI	316,333

4. Lessons Learned

Describe the top one to three takeaways or lessons learned from this project.

- In working with a broad multinational research team (Peru, US, Nigeria, Finland), people management and flexibility in research planning are key. In this project, we had one partner (UW-M) drop out when the PI left the university. This resulted in almost half a year of reorganization in trying to recover lost deliverables from this partner. In the end it would have saved time and allowed better planning to have changed the deliverables in a timelier manner. Working with the diverse partners required management to maintain focus on the big picture. Finally, the COVID-19 pandemic demonstrated the need for absolute flexibility, as ongoing research could not be completed and restarting experiments to still deliver on time (despite a no-cost extension) were a challenge.
- Large-scale *in vitro* multiplication of plant material takes time, and research on optimization of multiplication is needed prior to committing to experiments needing large amounts of *in vitro* material. If the first year were focused on multiplication and the second two years on the experiments, this would have enabled a smoother path when approaching the virus-cleaning experiments.
- High-throughput sequencing is a powerful but very sensitive tool. Our goal of using it on *in vitro* and/or greenhouse sweetpotato plants presented us with the challenge that all our control viruses were maintained in greenhouse indicator plants. The identification of appropriate virus-infected *in vitro* material to use as control delayed initiation of experiments for over a year as PCR results did not coincide with historically reported viruses. Similarly, validation experiments suffered from the same issue of historic and current virus-indexing data not concurring, leading to a lot of uncertainty and necessity to repeat experiments. Also, sRSA analytical methods existed but had never been used at this scale or been used to answer the sensitivity versus contamination questions that arose during experimentation. This would definitely have benefited by greater a priori awareness of the challenges and thus the early development of solutions prior to the challenge becoming a limitation. A good example is the rapid changing sequencing technology, which makes evolution of techniques a necessity.

5. Feedback for the Foundation

Provide one to three ways the foundation successfully enabled your work during this project. Provide one to three ways the foundation can improve.

Enabled

- We are extremely grateful to the Foundation for their flexibility throughout the project. With four main partners, all having unique challenges, acknowledgment of these challenges was critical for success. The project requested and was granted two no-cost extensions. These differed by partner but enabled the project to meet deliverables even during the unpredictability of daily operations due to the COVID-19 pandemic. A further example of our gratitude in adaptability was in the redirection of the project when one partner (25% of the project) dropped out, and we were able to redirect the funds to help achieve bigger results with the other deliverables.
- Our program officer, Jim Lorenzen, was an active participant in our project, often sitting in on our monthly PI meetings. This provided invaluable assistance, guidance, and expertise in real-time when difficult problems arose such as the need to redirect focus or expand existing plans to accommodate the overall goal.

Improve

- The project would have benefited from a longer ramp-up time, from notification of the award to initiation of the project. For the execution of future projects containing *in vitro* activities, we need significantly more time to propagate subsets of the accessions used in advance of the official start date of the project

6. Global Access and Intellectual Property

If your funding agreement is subject to Intellectual Property Reporting, please click the following link to complete an Intellectual Property (IP) Report.

If not, please acknowledge by typing "N/A": _N/A_____

To delegate permissions to another member of your project team or for any questions regarding the Intellectual Property Report, please contact GlobalAccess@gatesfoundation.org.

7. Regulated Activities

Do you represent that all Regulated Activities[1] related to your project are in compliance with all applicable safety, regulatory, ethical and legal requirements? Please mark with an "X":

_NA____ N/A (no Regulated Activities in project)

_____ Yes

_____ No (if no, please explain below)

1 Regulated Activities include but are not limited to: clinical trials; research involving human subjects; provision of diagnostic, prophylactic, medical or health services; experimental medicine; the use of human tissue, animals, radioactive isotopes, pathogenic organisms, genetically modified organisms, recombinant nucleic acids, Select Agents or Toxins (www.selectagents.gov), Dual Use technology (http://export.gov/regulation/eg_main_018229.asp), or any substance, organism, or material that is toxic or hazardous; as well as the approvals, records, data, specimens, and materials related to any of the forgoing.

Financial Update

The purpose of this section is to help the foundation understand how programmatic performance affects actual expenditures over the life of the investment.

Feel free to reach out to your foundation contact for support with these progress reporting requirements.

Note: Budget template and financial narrative instructions can be found here. If you are using an older version of the budget template, this information could be in a different location in your template.

1. Latest Period Variance:

"Latest period variance" compares expenditures that occurred in the reporting period against the most recent forecast. See "Financial Summary & Reporting" sheet in the foundation budget template for calculated variance (for example, column AD, starting on row 29 for period 1). Note that the allowable variance is defined in your grant agreement.

Latest Period Variance:

- Did the project spend more-or-less than anticipated in comparison to the most recent forecast? Please explain the primary drivers and their causes of the overall variance for the latest period (for example – programmatic changes, delays in recruitment).
- Please provide a detailed explanation for any expense category in which the variance was greater than 10%. This should include an explanation of programmatic decisions affecting expenditure amounts and/or how actual costs differed from prior assumptions.

Financial Summary: Period 4.

Financial Summary : Period 4					
Budget Category	**Approved Budget($)**	**Total Expenditure($)**	**Burn Rate**	**Budget Variance($)**	**% Variance**
Personnel	122,379	111,070	91	11,309	9
Travel	-	-	-	-	-
Consultants	18,500	20,329	110	(1,829)	(10)
Capital Equipment	-	-	-	-	-
Other Direct costs	84,800	112,826	133	(28,026)	(33)
Sub-Awards	418,075	400,859	96	17,217	4
Indirect Costs	96,563	96,839	100	(276)	(0)
Total Approved Budget	**740,317**	**741,923**	**100**	**(1,605)**	**(0)**
Interest Earned	1,606	-	-	1,606	100
Grand Total Budget	**741,923**	**741,923**	**100**	**-**	**-**

The summary above represents the financial implementation status in period 4 against the projection submitted in our previous report. In addition to the total forecast for the period (US$ 740,317), we also credited interests earned for the project use (US$ 1,606). These funds were allocated into the project in period 4 to finance activities under other direct costs. The available funds, therefore, increased to US$ 741,923.

All the project activities in period 4, were implemented and a total expenditure of US$ 741,923 was recorded with an implementation rate of 100%. There was an over expenditure in Other Direct Costs of 33%, but this only represents 13% variation on the total amount for the whole Life of Project, which is explained below.

Consolidated Financial Summary: Period 1-4.

Consolidated Financial Summary : Period 1-4					
Budget Category	**Approved Budget($)**	**Accumulated Expenditure($)**	**Burn Rate**	**Budget Variance($)**	**% Variance**
Personnel	527,681	516,373	98	11,308	2
Travel	14,425	14,425	100	-	-
Consultants	37,000	38,829	105	(1,829)	(5)
Capital Equipment	-	-	-	-	-
Other Direct costs	220,598	248,624	113	(28,026)	(13)
Sub total	**799,704**	**818,251**	**102**	**(18,547)**	**(2)**
Sub-Awards					
IITA	673,428	668,529	99	4,899	1
University of Helsinki	285,237	272,920	96	12,317	4
University of Winconsin	316,333	316,333	100	-	-
Sub total	**1,274,997**	**1,257,782**	**99**	**17,215**	**1**
Indirect Costs	311,205	311,479	100	(274)	(0)
Total Approved Budget	**2,385,906**	**2,387,512**	**100**	**(1,604)**	**(0)**
Interest Earned	1,606	-	-	1,606	100
Grand Total Budget	**2,387,512**	**2,387,512**	**100**	**-**	**-**

As per the summary above, the total available funds including interest earned during the project life was $2,387,512.

We implemented all the project activities according to the agreed plans and the accumulated expenditure stood at $2,385,512 with an overall execution rate of 100%.

A Nil overall variance was recorded with some variances, beyond the required limit of 10% on other direct costs category.

On Personnel, the execution rate was 98%. Some project staff left the services of the organization in period 4 of the project which led to the 2% underutilization of this budget line.

The Variance on Consultants category was attributed to more time which was dedicated to the project by the project consultant, David Ellis, to review partners' reports and support the final project report preparation during the close-out period.

Other Direct Costs category had an implementation rate of 113% in overall. This is because, as previously informed by our PI, CIP had to redistribute activities initially proposed for University of Wisconsin (QuickChip) and, in addition, we also had to carry out extra activities originally planned for University of Helsinki (VirusDetect Windows), which they weren't unable to complete.

Unforeseen additional costs were also incurred on computing service from Amazon cloud to enable us perform data analysis more rapidly, which were initially intended to be performed on a local server, the service of which was however discontinued.

The Sub- Awards category realized a burn rate of 99% with an overall variance of 1%, within the allowable limit. Although the University of Helsinki and IITA reported an underutilization variance of 4% and 1% respectively, they implemented all the activities as per their updated work plans.

In conclusion, the project was successfully implemented with all the key deliverables achieved within the approved budget.

2. Sub-awards (if applicable)

This sub-award section provides visibility to an often-critical component of the grant spending where the budget template provides limited insight. The total of actual disbursements for this reporting period should equal the actual sub-award expenses reported on the "Financial Summary & Reporting" sheet in the budget template for this reporting period.

Use the table below to provide the detail of all sub-grantee(s) or subcontractor(s).

Organization Name	Actual Disbursement for this Reporting Period (U.S.$)	Total Disbursed from Primary Awardee to Sub to Date (U.S.$)	Total Sub-Awardee Spent to Date (U.S.$)	Total Contracted Amount (U.S.$)
IITA	229,015	668,529	668,529	**673,428**
University of Helsinki	171,845	272,920	272,920	**285,237**
University of Wisconsin	0	316,333	316,333	**316,333**
Total (ties to budget file(s))	**400,860**	**1,257,782**	**1,257,782**	**1,274,997**

For sub-awards greater than $1M, please provide explanatory detail as requested in the latest and future period sections above.

Note: It is the foundation's discretion to ask for updated sub-award budget files as part of the traditional progress report review process

3. Other Sources of Support (if applicable):

Other Sources of Support include interest earned, current foreign exchange impacts, and co-funding (in-kind and other contributions).

Other Sources of Support (if applicable): Explain any notable impacts from other sources of support.

The total interests earned during the life of the project amounted to $1,606. These funds as earlier mentioned, were used in period 4, to support the project activities under other direct costs budget category.

A summary of interest earned is as shown below:

Other sources	Period 1	Period 2	Period 3	Period 4	Total
Interest Earned ($)	657	107	487	355	**1,606**

Checklist - As you review your answers to questions in the financial update section, ensure that your report provides the following:

1. Explanation of how project expenditures differed from plan and the implications on programmatic progress to date.
2. Explanation of how future period projections differ from the original budget and previous forecasts, and the implications.
3. Explanation of other sources of support (funds) from other funders, interest earned or converting to non-USD currencies.

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For Foundation Staff to Complete

Analysis (required if PO assessment differs from grantee/vendor assessment or if there are unexpended funds)

Progress Analysis

Include analysis of significant project variances and key learnings that may inform portfolio discussions for progress against the strategic goals.

Budget and Financial Analysis

Include analysis of unexpended funds or over expenditures. Refer to the Unexpended Grant Funds Policy for options available when recommending how to handle unexpended grant funds, or reach out to your primary contact in GCM.