Sweetpotato sample collection and virus diagnostic protocol

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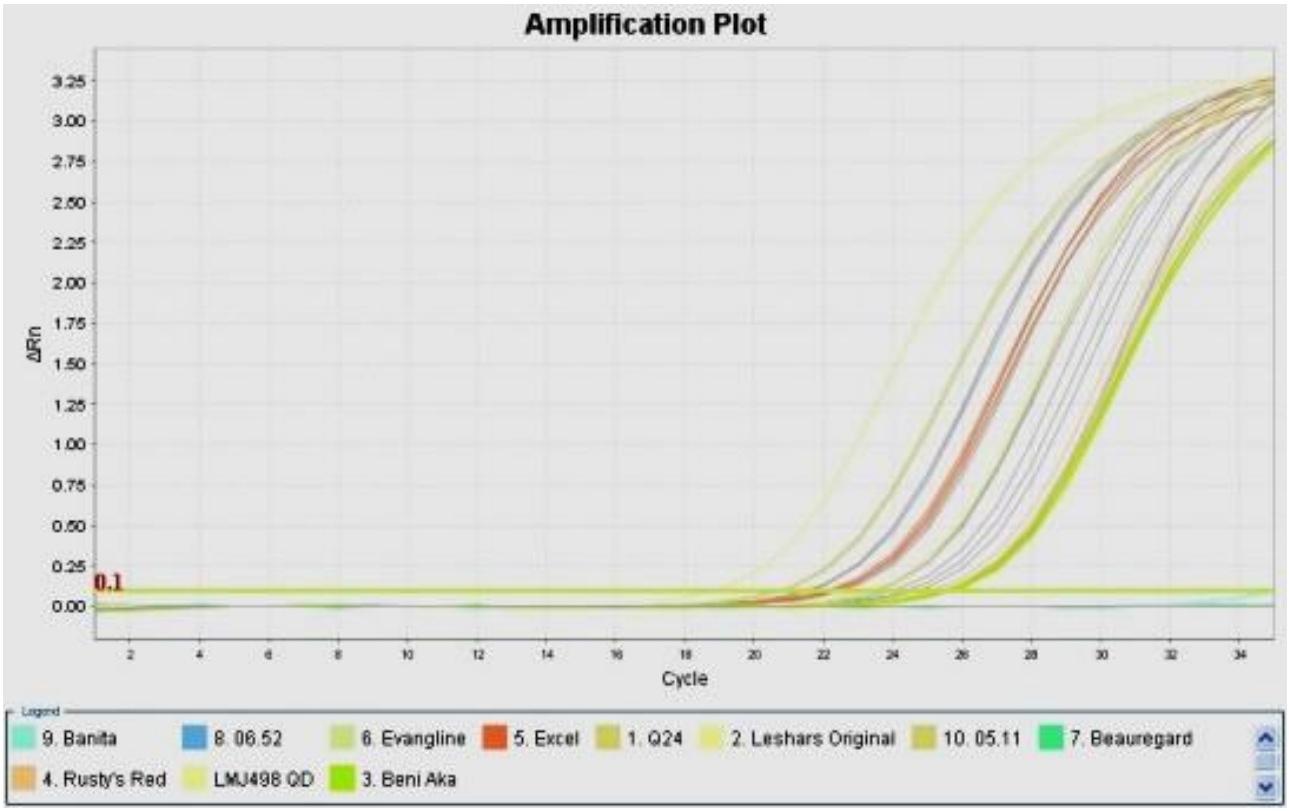
For Horticulture Innovation Australia (Hort Innovation) Ltd
Project VG13004 – Innovating new virus diagnostics and plant bed management in the Australian sweetpotato industry



Horticulture Innovation Australia

Australian Sweetpotato Growers Inc

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Introduction

The ability to detect and identify current and potentially new viruses is essential capacity for the Australian sweetpotato industry. This priority was emphasised by the establishment of the Horticulture Innovation Australia (Hort Innovation) project VG13004 'Innovating new virus diagnostics and planting bed management in the Australian Sweetpotato Industry'. This project, a collaboration of (and co-funded by) the Australian sweetpotato industry, represented by Australian Sweetpotato Growers Inc., Hort Innovation and the Queensland Government, demonstrates an ongoing commitment to Australian sweetpotato industry productivity and biosecurity.

This protocol outlines the standardised methodology currently used by the VG13004 project team to: sample sweetpotato growing areas; maintain sample and long-term diagnostic integrity; and the various diagnostic techniques currently employed.

This protocol forms the basis for inducting new and casual staff into the diagnostic process, as well as providing an updatable document for recording and improving sweetpotato industry diagnostic procedures. It builds on the methodology detailed in Dennien *et al.* (2013); in the VG13004 Virus management in Australia Literature Review (2015), and the experiences of other virus diagnosticians throughout Oceania and the rest of the world.

Ms Dennien is particularly indebted to the advice and assistance of Segundo Fuentes from the International Center for Potato Research (CIP), Dr Chris Clark from Louisiana State University, Mr Amit Sukal from the Secretariat of the Pacific Community (SPC), and staff from the Queensland Government Plant Virology section.

Sample collection

Samples of sweetpotato vines and/or storage roots are collected at random from plant beds, established commercial plantings, regrowth from previous season's crops and weeds from surrounding headlands, roads and sheds etc. Samples are collected according to either a systematic or random sampling strategy, making sure to document which system is used.

Each cutting sample is stored in a zip-lock bags (one sample per bag) and labelled with the grower's name, the area collected and the date. Storage roots are also placed into individual zip-lock plastic bags and labelled as for cuttings. Samples need to be kept cool and if more than two days away from the diagnostic facility, cuttings are wrapped with moistened paper towel, before being placed into the zip-lock bags. Bags containing roots ARE NOT to be sealed.

All samples collected are entered into the sweetpotato virus survey registry, used to create a grower sample database to track symptoms and virus testing results. The registry records growers' names, date collected, location of farm, type of sample (plant bed, commercial crop, weed or regrowth), description of symptoms if present, cropping season and an allocated accession number.

Sample preservation and maintenance

Because the herbaceous indicator species currently used has a restricted growing period, it is currently required that cutting samples be maintained as living plants, until herbaceous indexing can take place. Usually mid-late spring is the earliest this indexing can be undertaken at the Gatton Research Facility.

Samples (vine cuttings and storage roots) are potted in 100 ml pots containing modified pasteurised UC mix (one-third washed river sand, one-third peat and one-third perlite, with the addition of blood and bone, dolomite, hydrated lime, potassium nitrate, potassium sulphate, superphosphate and trace elements as per Dennien *et al.* (2013); The potted samples are labelled with the grower's name and the accession number. They are then placed into an insect-screened 'quarantine' bench, within an insect-proof glasshouse, and regularly hand watered as required.

Quarantined plants are sprayed with insecticides and miticides at fortnightly intervals. Application is done in the late evenings, so as not to cause foliage burn. A 'fogger' producing fine droplets is used to further guard against any possible spray damage to *Ipomoea setosa* leaves. After establishment (4-6 weeks), the quarantined plants are relocated to an insect-proof screened bench, within a larger insect proof igloo, and base-watered using an automatic micro-irrigation system. Precautionary sprays for mite and insect control are continued at fortnightly intervals.

Current diagnostic techniques

Herbaceous indexing method

Current sweetpotato virus diagnostic methods involve the use of the biological indicator plant, *Ipomoea setosa* (Brazilian morning glory). Herbaceous indexing commences at the Gatton Research Facility during September, when increasing daylight and warmer night time temperatures are experienced. Optimal temperature for *Ipomoea setosa* production is 25°C (Fuentes pers. comm.). Sweetpotato infecting viruses increase titre rapidly in *I. setosa,* enabling more accurate detection of viruses through symptom observation, than observation of sweetpotato plants alone. Viruses are often present in sweetpotato plants in low titres, while symptom expression and severity can vary markedly within cultivars, depending on the viruses involved. The use of *Ipomoea setosa* gives a "level playing field" to observe symptoms.

Seeds of *I. setosa* plants for use in sweetpotato virus indexing are produced annually at Gatton Research Facility, in a quarantine-meshed, insect-proof igloo, dedicated to indicator-plant seed production. Single seeds of *I. setosa* are planted into pots using the potting mix described for sweetpotato sample maintenance. *Ipomoea setosa* seeds readily germinate in Queensland and therefore pre-germination seed treatments are not required. Single *I. setosa* seeds are placed into 100 mm pots filled with the above potting mix.

Pots are seeded within a screened area, inside an insect-proof glasshouse. They are placed under automatic micro-sprinklers, set to water for five minutes once a day. This irrigation intensity is increased to five minutes twice a day as daytime temperatures increase during summer. Germination is usually evident after two to seven days, depending on temperature. After germination, Osmocote®, a granular, slow-release fertiliser, is applied at the rate of five grams (one teaspoon) per 100 ml pot, as per the product label. Access to this area is limited to staff working on the project, to prevent possible insect incursions within the glasshouse.

I. setosa plants are large enough to graft when the first two true leaves are fully opened, at three to five weeks after germination (depending on temperature). Two *I. setosa* plants are grafted, unlike Dennien *et al.* (2013), where five *I. setosa* plants are recommended to be grafted (to check for presence of virus in plants undergoing pathogen testing to enter a "clean" seed system). For survey purposes, the project team is confident grafting each accession onto two I. *setosa* plants is sufficient, and greatly reduces requirements for labour, materials and the cost of herbaceous indexing.

Vine sections containing at least five nodes are removed from the accessions of previously-potted cuttings and/or sprouting storage roots. Two grafts are performed on each *I. setosa* plant, one end-cleft graft, and one side-veneer graft. Two *I. setosa* plants are grafted (each with an end-cleft and side-veneer graft) with cuttings (single node) from each collected sample. Numerous proven positive controls for a range of viruses previously detected in Australia, as well as pathogen-tested negative controls, are also grafted to a pair of *I. setosa* plants, during each round of virus indexing, for symptom comparisons.

Freshly-grafted plants in the pots are covered with plastic bags. The pots are placed into large, shallow trays lined with plastic sheeting and filled with water to a depth of 10 mm, within an insect-proof glasshouse. Large trays are placed on the concrete floor of the glasshouse, where the plastic-covered plants are out of direct sunlight and exposed to cooler temperatures. Twice-weekly checks are performed, to ensure plants have sufficient water, but are not waterlogged.

Plastic bags are lifted slightly after five days, depending on the current temperature. During the warmest summer months (December to January), bags are lifted at three to four days after grafting. All plastic bags are totally removed one week after grafting. Pots containing the grafted plants are placed into individual 200 mm pot trays and arranged onto weld-mesh benches. We construct plastic trellises into a cylinder shape (using cable ties as fasteners), roughly 150-200 mm wide and one metre high, and position them adjacent to the *I. setosa* plants. Each pair of *I. setosa* plants (for each sample to be virus indexed) are placed side by side, sharing one trellis. Eight pairs of plants in pots are positioned on each bench.

A foliar fertiliser (Aquasol®), at the rate of 16 grams in 10 litres of water, is applied directly into the pot trays, using a watering can. Plants are observed every two days and watered when necessary for the next 6-8 weeks. Foliar fertiliser application is repeated every two to three weeks for optimum growth of the grafted *I. setosa* plants. As maximum temperatures in late November into December can regularly exceed 40°°C, shade cloth covers fitted to the glasshouse are dropped down, to reduce daytime temperatures within the glasshouse. Preventative insecticide and miticide sprays are applied every fortnight.

Grafted plants are observed twice weekly at watering. Symptoms are recorded between two and three weeks after grafting, with observations repeated between four and five weeks after grafting. Regular monitoring is essential to capture early leaf symptoms, especially those of potyviruses, which can often develop rapidly, and then disappear just as rapidly. At the time of symptom recording, each pair of *I. setosa* plants is photographed in full, with any apparent symptoms additionally photographed. Symptom data collection sheets are designed to encompass a wide range of possible symptoms, including mottling, vein-clearing, chlorosis, chlorotic spots, chlorotic flecks, necrosis, leaf cupping or dishing, leaf roll, rugosity, stunting, leaf deformation, leaf balling and flowering. The area of the plants in which symptoms occurred is recorded, as this can also indicate which viruses may be involved.

Survey samples with suspected phytoplasma infections undergoing indexing are observed for eight to ten weeks. Others are totally removed at six to eight weeks after grafting. In both cases, once indexing is completed, all plant material is placed into freezer bags and frozen for several days, before placing into a domestic rubbish skip. Pots are emptied, with soil placed into pallet-sized tubs and stockpiled as waste, not to be re-used for virus indexing. Pot trays are washed in detergent and left to air dry, trellises are wiped down with 70% alcohol. Benches and trellises are then left empty for one to seven days. The above process is repeated for each sequential group of samples to be tested.

NCM ELISA

Serology is done using the NCM ELISA (nitrocellulose membrane enzyme-linked immunosorbent assay) kit supplied by the International Potato Centre (CIP). The kit contains antibodies for 10 known sweetpotato viruses: Sweetpotato Feathery Mottle Virus (SPFMV); Sweetpotato Mild Mottle Virus (SPMMV); Sweetpotato Mild Speckling Virus (SPMSV), Sweetpotato Chlorotic Stunt Virus (SPCSV), Sweetpotato Collusive Virus (SPCV); Sweetpotato C-6 virus (SPC-6); Sweetpotato Chlorotic Fleck Virus (SPCFV); Sweetpotato Virus G (SPVG); Sweetpotato Latent Virus (SPLV); and Cucumber Mosaic Virus (CMV).

At two to three weeks after the grafting, three leaves are collected from each pair of grafted *I. setosa* plants, early in the morning. Leaves from the grafted *I. setosa* plants are used, rather than leaves from the sweetpotato sample plants, because the grafted indicator plants are more likely to have sufficient virus titres if viruses are present. Leaves showing symptoms are collected for preference, if no symptoms are observed on each pair of *I. setosa* plants asymptomatic leaves are sampled. Each group of three leaves is placed into a new zip-lock bag, and labelled with the same information as on the labels of the respective potted plants. Collected leaves are taken to the sweetpotato diagnostic laboratory for NCM ELISA testing. NCM ELISA is carried out as per kit instructions (CIP). Leaf sampling and NCM ELISA testing is repeated at between four and five weeks after grafting.

New detection methods

qPCR (currently for SPLCV detection)

At two to four weeks after grafting, three leaves from each pair of *Ipomoea setosa* grafted plants are sampled for DNA extraction, to test for Sweetpotato leaf curl virus using qPCR, as per Barkley (2011). Positive controls GRF 1 (93-93-Q24), GRF 300 (Lester Loader's original *Beauregard*), GRF 340 (*Excel*) and GRF 341 (*Regal*), which consistently produced leaf cupping and curling symptoms in *I. setosa,* have been previously tested positive to Begomovirus by Lee McMichaels at DAF laboratories, Indooroopilly in 2009, using primers SPG1 and SPG2. The PCR protocol they used was recommended by Segundo Fuentes from the International Centre (CIP) for SPLCV detection in sweetpotato.

Whole leaves are removed from plants, as per the above ELISA sampling procedure, placed into new labelled zip-lock bags and taken to the laboratory. Due to sensitivity of qPCR method, gloves are worn when samples are collected, and throughout the DNA extraction process. Three leaf discs, weighing up to a maximum of 100 mg, are cut from the mid-rib section of each leaf, using the large end of a disposable 1 ml pipette tip. A new pipette tip is used for each plant sample.

Discs cut out from the leaf samples are placed into a 2 ml Eppendorf ® safe-lock tube and ground with 2 to 3 mm stainless steel beads, using a Bullet Blender Storm® tissue homogeniser (Next Advance). DNA extraction was performed using the Dneasy® plant mini kit from Qiagen, as per the manufacturer's protocol. DNA was quantified using a Qubit 2.0 ® fluorometer (Life Technologies). For the qPCR assay, a primer and probe set for SPLCV as per Barkley (2011) was purchased from Applied Biosystems. The total reaction volume of 20 ul comprised of: TaqMan Gene Expression Master Mix® including ROX, 2X, 10 ul; SPLCV Probe primer set (Barkley 2011), 20X 1 ul; Sample 1 ul; and Nuclease free water 8 ul. Cycling conditions were: 1 cycle 60°C for 30 sec, 1 cycle 50°C for 2 min, 1 cycle 95°C for 10 min, 35 cycles 95°C for 15 sec, 60°C for 1 min, 60°C for 30 sec (Barkley 2011).

The assay was performed on an Applied Biosystems StepOne Plus real time PCR system, using optical 96 well plates, including no template controls.

Preservation of plant tissue in RNA Later

To preserve samples for later analysis, 6 to 8 leaf discs are cut from the mid-rib section of the grafted *Ipomoea setosa* leaves, using a 1 ml disposable pipette tip. These are placed into individually-labelled 2 ml Eppindorf tubes. One ml of RNA later solution is added, making sure that all plant tissue is fully submerged in the solution. Tubes are placed into a refrigerator overnight. The next day RNA later solution is removed (tipped out), leaving the plant tissue in the tube. These samples are then frozen for future RT qPCR testing.

Bibliography

Barkley, N., Pinnow, D., Wang, M., Ling, K.S., Jarret, R., 2011. Detection and classification of SPLCV isolates in the US sweetpotato germplasm collection via a real-time PCR assay and phylogenetic analysis. *Plant Disease* **95**, 1385-1391.

Dennien, S., Homare, D., Hughes, M., Lovatt, J., Coleman, E., Jackson, G., 2013. Growing healthy sweetpotato: best practices for producing planting material, ACIAR Monograph No. 153. Australian Centre for International Agricultural Research, Canberra.

Dennien, S. 2015. Sweetpotato viruses and phytoplasmas found in Australia, worldwide, and their current methods of detection: Literature review March 2015. ASPG website link.