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Independent review of the Kiwifruit Vine Health "Pathway standard for the movement of *Actinidia* plant material into Exclusion Regions"

Hood, I.A.[1], Bulman, L.S.[1]

Te Ara (https://teara.govt.nz/en/photograph/3706/kiwifruit-orchard-bay-of-plenty)

[1] The authors of this review declare that they have no competing interests. They are specialists in the scientific methodology of research into forest and tree diseases and as such have been selected for their independence and impartiality.

Report information sheet

Report title	Independent review of the Kiwifruit Vine Health "Pathway standard for the movement of *Actinidia* plant material into Exclusion Regions"
Authors	I.A. Hood and L.S. Bulman
Client	Kiwifruit Vine Health (KVH)
Client contract number	0000-00
MBIE contract number	
SIDNEY output number	61312
Signed off by	
Date	17 September 2018
Confidentiality requirement	Confidential (for client use only)
Intellectual property	
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Published by: Scion, 49 Sala Street, Private Bag 3020, Rotorua 3046, New Zealand. www.scionresearch.com

Executive summary

The problem

Restrictions are currently in place under the National Psa-V Pest Management Plan preventing the movement of *Actinidia* plant material from areas infested by the bacterium *Pseudomonas syringae* pv. *actinidiae* (Psa) to areas free of the pathogen. Kiwifruit vine orchardists are therefore unable to take advantage of new cultivars developed in infested regions.

Client initiatives

Kiwifruit Vine Health (KVH) has developed a draft pathway standard that will allow kiwifruit vine material to be moved outside infested areas following treatment using a protocol that will ensure it is free from the pathogen. KVH has asked Scion to undertake an independent review of the draft protocol.

The review

The draft protocol consists of three successive phases, a tissue culture screening phase based on work by Tyson et al. (2017), a quarantine greenhouse monitoring phase using as guidelines procedures specified in the post entry quarantine greenhouse section of the Ministry for Primary Industries Import Health Standard "*Actinidia* Plants for Planting", and an outdoor containment phase during which further monitoring is undertaken for signs and symptoms of Psa, before final release.

The meticulous work of Tyson et al. (2017) has shown that the addition of peptone to the culture medium enhances the growth of Psa-V to the point where it is readily detectable if present in tissue culture plantlets. By rejecting infected material and repeating culture cycles with clean plant tissue, genotype lines are obtained where the probability that Psa is present is near zero. This is then checked by polymerase chain reaction (PCR) testing. Psa free plants are further tested by monitoring them for signs and symptoms of Psa, and by sampling and laboratory testing for the pathogen, in a quarantine greenhouse over a six month period. Finally, plants are monitored and sampled in outside containment over an eight month period before release.

Despite the thoroughness of the protocol, more testing is underway to enhance the rigour of the procedure, because of the importance of preventing the spread of Psa outside the present infested area. A number of suggestions are offered by the reviewers regarding aspects that could be included in such tests. These include carrying out tests using other Psa isolates and kiwifruit genotypes, and testing other grades and rates of peptone.

Conclusion

The proposed protocol is thorough and meticulous containing multiple checks and backup reinforcement stages. It is concluded that the procedure outlined in the draft protocol reduces the risk of spreading Psa to a negligible level.

Independent review of the Kiwifruit Vine Health "Pathway standard for the movement of *Actinidia* plant material into Exclusion Regions"

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1. Background

This report is an independent review of the proposed Kiwifruit Vine Health (KVH) "Pathway standard for the movement of *Actinidia* plant material into Exclusion Regions" undertaken by Scion on request.

The purpose of the standard is outlined in detail in the draft document (App. 1) and is briefly summarised here. Restrictions are currently in place under the National Psa-V Pest Management Plan (NPMP) preventing the movement of *Actinidia* plant material into Exclusion Zones[2] in order to keep them free from the kiwifruit vine pathogen *Pseudomonas syringae* pv. *actinidiae* (including the current New Zealand strain, Psa-V or biovar 3). This regulation has the drawback of preventing orchardists in Exclusion Regions from acquiring new kiwifruit cultivars that have been developed in infested Recovery Regions.

Recent research has indicated a laboratory procedure that could be used to screen kiwifruit tissue cultures as free from Psa-V in *Actinidia* (Tyson et al. 2017). KVH, with assistance from Plant and Food Research personnel, have used this information, along with appropriate protocols from the Import Health Standard "*Actinidia* Plants for Planting" (App. 2), to form the proposed draft Pathway Standard for movement of stock within New Zealand.

KVH has the legal authority to allow movement of screened plants (App. 1, Section 2.3). However, it is clearly of fundamental importance to ensure that Psa-V is not carried beyond the present Containment and Recovery regions. Hence the request for an independent review.

2. Scope

Specifically, the reviewers were invited to:

- Review the tissue culture process outlined in the Pathway Standard against the research reported by Tyson *et al*. (2017).
- Offer an expert opinion on the probability of there being negligible risk of having Psa present in the tissue culture material that has been developed according to the standard.
- Review the proposed quarantine steps outlined in the Pathway Standard against those detailed in section 2.4 "Post entry quarantine greenhouse" of the Import Health Standard "*Actinidia* Plants for Planting" (App. 2).
- Offer an expert opinion on the probability that (a) the presence of any Psa present in plant material held in quarantine in accordance with these steps will be captured and (b) there is negligible risk of it not being detected.

3. Information base

3.1 Tissue culture research

Previous work has shown that Psa can reside in supposedly sterile *Actinidia* tissue cultures without necessarily showing disease symptoms (e.g. Minardi et al. 2015). Tyson et al. (2017) used a culture medium with a peptone (3g/L) additive to enhance the growth of the bacterium, making it much more readily detectable. Using this medium (with a non-peptone-amended medium as one control) they tested the growth of Psa alone (i.e. in the absence of plant material) and after inoculating it into *in vitro* cultured plantlets. The addition of peptone substantially enhanced the growth and detectability of Psa under all situations. In a more elaborate study they tested portions taken from three positions

[2] Under the NPMP, Psa-V regions as established are defined as follows. Exclusion Regions: those free of the pathogen within and beyond 10 km from their boundary; Containment Regions: those with an "infection rate" averaging < 35% of the orchard area; Recovery Regions: those with an "infection rate" averaging ≥ 35% of the orchard area. http://legislation.govt.nz/regulation/public/2013/0139/13.0/whole.html#DLM5179506.

(top, middle, base) on shoots of plants 3.5 cm tall of three varieties (resistant, tolerant and susceptible) held for four time periods (up to 14 days) after inoculating the base with five concentrations (between 0 and10^8 Psa-culture forming units, cfu, per mL) using five replications. Control shoots remained free of Psa while inoculated shoots were rapidly occupied by the bacteria, generally at high rates, after 14 days at all concentrations in all three plant varieties, without showing symptoms of disease. Rate of internal spread was slowest at the lowest concentration (10^2 cfu/mL) and in the most resistant variety.

Tyson et al. (2017) then conducted a series of 10 runs with up to 5 replications to determine the detection concentration threshold of Psa in the most susceptible kiwifruit variety. Leaf tissue samples from three-month-old plants were treated with a dilution series of concentrations up to 10^9 cfu/mL (depending on the run), macerated, plated onto an agar medium containing 20g/L of proteose peptone (King's B medium, King et al. 1954), and counts were made of viable bacterial cfu following incubation for 2-3 days at 20 °C. After modelling the resultant data, the authors concluded that the probability of not detecting Psa at an inoculum concentration above 30 cfu/0.1 mL was close to zero. They suggested that because of the very rapid rate of multiplication of Psa within plant tissue in their earlier experiment, any material initially testing negative would rise above this detection threshold and give a positive result in a later test.

3.2 Import Health Standard *Actinidia* Plants for Planting (13 July, 2018).

A recent Ministry for Primary Industries (MPI) import health standard prescribes the conditions for importing dormant cuttings and tissue cultures of *Actinidia* plants into New Zealand (App. 2). Of relevance here are requirements for tissue cultures if sub-cultured post-entry before being transferred to a greenhouse ("may" be in a Level 3 tissue culture laboratory; Section 2.2); and for screening of plants for planting (i.e. tissue cultures and dormant cuttings) undertaken for regulated pests (including Psa) in a post entry quarantine greenhouse of prescribed standard at a security level specified on the import permit (App. 2, Sections 2.3 and 2.4). The regulations set down a precise glasshouse screening regime of 20 months simulating all four seasons over two equivalent growing periods during which 10 inspections are to be conducted as specified. During the first "spring" petioles and midribs from young and old leaf samples are to be tested for various organisms, including Psa, by polymerase chain reaction (PCR). No further testing specifically for Psa is prescribed, but the subsequent inspections required for signs and symptoms of all regulated pests include Psa (App. 2, Subsection 2.3.3), and inspectors must forward any such material found to an MPI approved diagnostic facility for testing (Subsection 2.3.2).

4. Proposed pathway standard for moving *Actinidia* plant material into Exclusion Regions

The proposed protocol, given in detail in App. 1 (Section 3), may be summarised as follows.

4.1 Preparation of *Actinidia* plants for quarantine greenhouse

- Subculture shoots of established *in vitro* cultures onto growth medium supplemented with peptone (3g/L) for ≥ 1 week.
- Transfer shoots free of bacteria (mother plants) to new culture vessels for further propagation.
- Treat vegetative progeny of clean mother plants as having a Psa-free status.
- Discard contaminated cultures.
- Conduct ≥ three 4-6 week long culture cycles.
- During this process undertake one or more screens for bacterial contamination.

- Subject tissue samples from the base of *in vitro* plants visually examined for bacteria to additional screening for Psa using PCR for added assurance.
- Inspect plants for signs of bacteria at all stages.
- Plants from a given mother plant which have all remained free from Psa during the above procedure may be transferred to a Psa quarantine greenhouse.
- To be so transferred, *in vitro* plants must be of stage 3 status (rooted and hardened-off).
- When transferring plants to the quarantine greenhouse, convey a sample of the same plants to Plant and Food Research Te Puke facilities to be monitored there for Psa symptom expression.
- Full records to be maintained for auditing by KVH or its representative.
- KVH or its representative to review the quality assurance program of the laboratory undertaking this procedure.

4.2 Quarantine greenhouse protocol prior to release to outdoor containment

- Complete audit and obtain authorisation from KVH for movement to greenhouse.
- Quarantine greenhouse to be equivalent to Post Entry Quarantine (PEQ) Level 2 standard, outside of kiwifruit growing regions and KVH approved.
- Hold tissue culture plants after removing from containers (deflasking) in the quarantine facility for ≥ 6 months of active growth after its commencement.
- Plants to be monitored for Psa symptoms 14 days after deflasking and monthly thereafter during the quarantine period by a KVH-approved person.
- Report any symptomatic material ≤ 24 hours after detection to KVH for sampling and testing.
- Keep full records for auditing.
- Do not undertake any pruning without KVH authorisation.
- Within the last 21 days of the active growing period sample foliage from at least three positions (including a young leaf, an old leaf, and any leaf showing any form of disease symptoms).
- These leaf samples to be tested for Psa-V using the KVH authorised test method and laboratory.
- All *Actinidia* plants in the quarantine facility to be destroyed in the event of a positive Psa-V test.
- Release to a pre-approved outdoor containment location to be granted only if the above requirements have been met and endorsed by KVH.

4.3 Growth and propagation in outdoor containment

- Plants to be grown in a KVH-approved, outdoor containment location outside current kiwifruit growing regions and ≥ 20 km from known kiwifruit material.
- Plants to be held actively growing for ≥ 8 months.
- Plants to be monitored for Psa symptoms monthly during the containment period by a KVH-approved person.
- Report any symptomatic material ≤ 24 hours after detection to KVH for sampling and testing.
- Keep full records for auditing.
- All *Actinidia* plants in the containment location to be destroyed in the event of a positive Psa-V test.
- Within the last 21 days of the active growing period before release from outdoor containment sample for Psa testing using PCR. Sampling is to follow the Kiwifruit Plant Certification Scheme 600 leaf sample[3], designed to provide a 95% level of confidence of detecting Psa if it is present.

[3] E.g. "The New Zealand Ministry of Agriculture and Forestry requires that the….organisation sample and inspect….for all visually detectable regulated pests....with a 95% confidence level, that not more than 0.5% of the units in the consignment are

- These leaf samples to be tested for Psa-V using the KVH authorised test method and laboratory.
- All *Actinidia* plants in the containment site to be destroyed in the event of a positive Psa-V test.
- Release to an Exclusion or Containment Region to be permitted by KVH only if the above steps have been followed and Psa test results have all been negative.

5. Appraisal of proposed pathway

5.1 In relation to the work of Tyson et al. (2017).

Tyson et al. (2017) clearly established in their studies that the use of peptone as an amendment to the culture medium promotes the growth of Psa-V to the point that it is readily detectable when present in cultured plantlet tissue. Their detailed determination of the minimum threshold of detection together with the demonstration that the test isolate multiplied rapidly while being cultured within shoot tissue confirms that by using multiple propagation cycles the risk of not detecting the pathogen is close to zero. The proposed standard carefully incorporates the features of the Tyson et al. (2017) studies, including the use of a peptone additive and multiple culture cycles of adequate duration, into the protocol.

As a technical point, the standard appears to prescribe a minimum of just one other screening for bacteria during the whole subsequent propagation period of at least three 4-6 week culture cycles, which seems insufficient. Please clarify if at least one screening per cycle is actually meant. If not, we suggest one screening per cycle is should be specified. Also, it probably needs to be specified more precisely how the cycle length is to be "genotype dependent" (or how this aspect is to be covered in the prescription).

5.2 The probability of negligible risk – tissue culture phase.

The thoroughness of the standard and its adherence to the findings of Tyson et al. (2017) in all its steps gives great assurance that the risk of not detecting Psa-V in tissue cultured plantlets treated according to the proposed protocol is negligible.

However, despite its rigour and meticulousness, the detection threshold research undertaken by Tyson et al. (2017) was specific. In view of the importance of minimising the regional spread of Psa-V it is suggested that consideration be given to broadening the evaluation by undertaking one or several additional tests in order to increase the confidence in the proposed standard and confirm its generic nature.

It is therefore suggested that studies be conducted to test the draft protocol:

- against several other isolates of Psa.
  Only one isolate of Psa-V was used in the studies by Tyson et al. (2017). Although this isolate was highly pathogenic, vigour and virulence may not correspond. Is it possible that there may be virulent but less easily detectable isolates?

- using several more kiwifruit vine genotypes.
  Similarly, in the research to determine the threshold of detectability, only one, highly susceptible kiwifruit vine variety was used. Are there kiwifruit genotypes in which the growth and detectability of Psa may be lower than was found in these studies? Although still

infested (this equates to an acceptance level of zero units infested by quarantine pests in a sample size of 600 units)". Import Health Standard Commodity Sub-class: fresh Fruit/Vegetables, Kiwifruit, *Actinidia deliciosa* from Italy, 22 December, 1999.

vigorous in the earlier, within-plantlet test, the bacterium proliferated slightly less readily in the resistant *A*. *polygama* than in the other two varieties (even if this is a different host species).

- supplementing the medium with different grades of peptone.
  Commercially available peptone appears to be a heterogeneous product produced in varied ways. King et al. (1954) tested peptone from several sources in their research (though this was for a different purpose). Is the source of peptone likely to affect the results? Should the protocol define the peptone used more specifically?

- adding peptone to the medium at the rate prescribed in the draft standard (3g/L).
  Although Tyson et al. (2017) conducted their preliminary studies using peptone at a concentration of 3g/L (the rate proposed in the standard's protocol), their studies to determine the threshold of detection used a medium (King's B) which has a higher level of peptone (20g/L; King et al. 1954). Consideration might alternatively be given to increasing the draft peptone prescription rate to 20 g/L. Tyson et al. (2017) found no harmful effects to plant tissues at the lower and presumably also the higher rate.

These points may be viewed as overly fastidious, especially in view of the rapidity with which the Psa isolate proliferated and became readily detectable within the plantlet tissues in the work of Tyson et al. (2017). The points raised may also vary in their importance. However, in view of the concerns about the possible regional spread of the pathogen their consideration is justified. Tyson et al. (2017) took pains over their work to determine the threshold of detectability and noted particularly that because variation occurred in the different runs, averaging of curves was not an appropriate procedure and that caution in prediction was needed. They also noted the necessity of adequate testing if plantlets were grown for a period without peptone to allow for healing after wounding. Several statements in the draft protocol document suggest that it is not yet complete and that further testing is already underway[4].

5.3 In relation to the post entry quarantine aspect of the Import Health Standard 'Actinidia Plants for Planting" (App. 2).

The quarantine conditions in the draft standard are similar to those in Section 2.4 of the Import Health Standard, but there are differences. The draft standard specifies that the greenhouse be of Level 2 security, whereas the import health standard is not specific, except that compliance must be to the level indicated on the import permit. More significantly, the draft standard prescribes a period in quarantine of not less than six months in an actively growing state, while the import health standard requires a minimum period of 20 months covering two 9-month equivalent growing seasons. The draft protocol requires a monthly monitoring regime specifically for signs and symptoms of Psa induced disease whereas the import health standard prescribes a program of ten inspections and three samplings at specified growth phases, Psa being specifically named near the beginning (though included within the overall screening for regulated organisms throughout the full quarantine period). However, the draft protocol requires a carefully prescribed sampling and testing of every plant before release from the facility. In contrast to the import health standard, the draft standard places a restriction on pruning.

[4] E.g. "The protocol described has been developed using plants inoculated with Psa in the lab. rather than field infested plants as these are difficult to establish in culture. There is research underway to try and establish *in vitro* cultures of Psa directly from field plants infected with Psa.
"This screening protocol has been used to screen *in vitro* cultures of six genotypes initiated from Psa regions that cleared PEQ in the European Union (EU) and are now being grown in trials in the EU. Repeat screening with this protocol will provide a high level of confidence that material is Psa free, however it is proposed that additional measures are included in this pathway to provide even greater confidence and not rely on a single measure" (referring to the additional testing by PCR).

5.4 The probability of negligible risk – quarantine greenhouse phase.

It is not possible to answer this fully because complete details are not provided in the proposed standard. The standard states that testing will be conducted "using the KVH authorised test method and laboratory", without providing details, but it is taken that this will be of an acceptable standard. Presumably samples will be subjected to both culture isolation and PCR (Rees-George et al. 2010) procedures, as suggested by Tyson et al. (2017). However, a major contrast between the two protocols is that the plants subjected to the quarantine greenhouse conditions under the proposed standard have already undergone rigorous screening using the peptone amended growing medium at the tissue culture phase. They therefore enter the greenhouse at a stage where the risk of Psa presence is already minimal. The addition of a third, outdoor containment stage greatly increases confidence in the protocol's effectiveness.

6. Discussion and conclusion

Under the National Pest Management Plan (NPMP) KVH has the legal authority to move kiwifruit vine plant material out of a controlled area into an area in which *Pseudomonas syringae* pv. *actinidiae* (Psa) is absent. Such a procedure will allow all orchardists access to improved kiwifruit cultivars developed in Psa-infested areas.

However, material that is moved in this way must be free from Psa in order to avoid spreading the pathogen. KVH has therefore developed a draft standard protocol to ensure that this is so. Scion has been asked to undertake an independent review of the draft standard.

Given the results of the meticulous work by Tyson et al. (2017), the protocol in the draft pathway standard is considered acceptable. These authors showed that Psa proliferated rapidly in kiwifruit shoot tissue on a peptone supplemented medium, increasing substantially the probability of detection. They then demonstrated that repeated sub-culturing and selection on a similar medium will produce kiwifruit lines free from Psa with a risk of non-detection near to zero, The protocol rests on these findings and its rigour is reinforced through supplementary PCR testing, and additionally by visual inspection, sampling and testing of plants over a significant active-growth period while in a quarantine greenhouse prior to a further monitoring period in outdoor containment before final release. The quarantine greenhouse is operated to a standard equivalent to post entry Level 2 quarantine security and the greenhouse protocol is based on the Import Health Standard "*Actinidia* Plants for Planting". All this gives the proposed standard a strong basis for confidence in its effectiveness.

The proposed draft standard document indicates that additional testing is underway to improve the protocol even more and a number of suggestions are submitted here for possible inclusion in those tests. While the protocol is already effective the importance of containing Psa dictates vigilance at all stages.

It should be noted, however, that the spread of Psa to other parts of New Zealand will be restrained but may not ultimately be prevented by these measures. In forestry, for instance, spread of dothistroma needle blight, caused by the radiata pine needle fungus *Dothistroma septosporum*, occurred throughout most of the country over several decades despite counter measures designed to constrain it. It is understood that Psa spreads naturally by wind, rain and through pollination by bees (for which, however, KVH has strict regulations). This, and the potential risk of inadvertent human dispersal must also be borne in mind when making the final decision regarding the implementation of the proposed standard. The likelihood of Psa spread via authorised movement of plant material **and** inadvertent movement must be considered when comparing the commercial benefits of allowing movement under the standard with the risk. However, in view of its thorough and rigorous nature it is concluded that the proposed standard as outlined reduces the risk of spread of Psa to an acceptable level.

7. References

King, E.O.; Ward, M.K.; Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine 44*: 301–307.

Minardi, P.; Ardizzi, S.; Lucchese, C.; Bertaccini, A. 2015. A reliable method for *Pseudomonas syringae* pv. *actinidiae* detection in asymptomatic kiwifruit plants three years after pathogen inoculation in micropropagated shoots and buds. II International Symposium on Bacterial Canker of Kiwifruit (Psa).

Rees-George, J.; Vanneste, J.L.; Cornish, D.A.; Pushparajah, I.P.S.; Yu, J.; Templeton, M.D.; Everett, K.R. 2010. Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S–23S rDNA intertranscribed spacer region and comparison with PCR primers based on other gene regions.
Plant Pathology 59: 453–464. DOI: 10.1111/j.1365-3059.2010.02259.x.

Tyson, J.L.; Vergara, M.J.; Butler, R.C.; Seelye, J.F.; Morgan, E.R. (2017). Survival, growth and detection of *Pseudomonas syringae* pv. *actinidiae in vitro* cultures. New Zealand Journal of Crop and Horticultural Science, DOI:10.1080/01140671.2017.1414064.

Appendix 1

Pathway standard for the movement of *Actinidia* plant material into Exclusion Regions

1 Purpose

To provide a pathway for kiwifruit growing regions classified as Exclusion and Containment under the National Psa-V Pest Management Plan (NPMP), to access *Actinidia* plant material while minimising the risk of introducing *Pseudomonas syringae* pv. *actinidiae* (Psa).

2 Background

The kiwifruit industry currently has movement controls in place to prevent the spread of Psa into regions where this pathogen is not present or in limited distribution. For growers in these regions these movement controls are an effective means of protection that has contributed to their orchards remaining free of Psa, however they have also had the unintended consequence of restricting access to new kiwifruit cultivars (as these have been developed primarily in Recovery regions where Psa is widespread).
It is currently prohibited to move plant material from a Recovery region to a Containment or Exclusion region as this presents an unacceptable risk. This paper proposes a pathway to manage this risk to a negligible level to enable growers to access these new kiwifruit cultivars.

2.1 Benefits from the movement of *Actinidia* plant material

Plant breeders are actively working to develop improved *Actinidia* cultivars that offer additional commercial benefits for growers. Allowing the movement of these new cultivars would provide growers in Exclusion and Containment regions with the opportunity for the same commercial advantage from new cultivars that is available to growers in other regions.

2.2 Importance of restricting plant movement

Actinidia propagation material is considered the main pathway for long-distance spread of Psa, between countries, but also between growing regions within New Zealand. Restricting the movement of *Actinidia* plant movement into Exclusion and Containment regions is one of the key control factors that has helped slow the spread of the pathogen into these regions, and why we still have regions without Psa today eight years after it was first detected in New Zealand.

2.3 Legal basis for controls

Kiwifruit Vine Health Incorporated (KVH) is the management agency responsible for implementing the National Psa-V Pest Management Plan. Section 131 of the Biosecurity Act 1993 enables KVH to institute movement controls to:

- limit the spread;
- limit damage caused; and
- protect any area from the incursion of Psa-V.

KVH has declared areas of New Zealand to be controlled areas, enabled by s.131(2) of the Act; and Movement Control Notices, enabled by s. 131(3) of the Act.
The movement of risk goods (such as any kiwifruit plant material) into, within or from any Controlled Area is restricted (or regulated or prohibited) subject to the conditions of the Controlled Area and Movement Control Notices. The risk goods within the controlled area may also be subject to treatment and procedures specified in the Notice.
Section 134(1)(b) of the Act states: No person shall move, or direct or arrange the movement of, any organism, organic material, risk goods, or other goods in contravention of a notice under section 131 (3), unless permitted by an inspector or authorised person.
Accordingly, a KVH authorised person may issue a permission to move risk goods (i.e. *Actinidia* plant material) out of a controlled area. This document outlines the requirements that must be met in order to possibly allow the movement of *Actinidia* plant material from a controlled area to another area of New Zealand with negligible increase in risk.

2.4 Summary of proposed measures

The proposed pathway incorporates elements from the Import Health Standard *Actinidia* Plants for Planting (2018) and doesn't rely on a single mitigation measure to manage risk, these are summarised in more detail in the following pages and outlined below.

a) Non-destructive screening for Psa

Psa can be present at low levels in both asymptomatic mother plants and derived tissue culture explants on standard media, making detection of contaminating organisms difficult. Therefore, the proposed pathway utilises a Psa screening protocol specifically developed for *in vitro* plants, as described in Tyson et al. (2017), which incorporates peptone in the growing media to promote Psa growth and thereby provides a rapid and non-destructive visual indicator of Psa presence. This screening technique can be repeated multiple times to achieve a high level of confidence. By increasing the bacteria levels present, this method will also increase the reliability of detection with molecular techniques.

The study by Tyson *et al.* (2017) demonstrated that even if Psa is present at extremely low levels in the mother plant and happened to give a false negative result initially, the rate at which Psa multiples *in vitro* plant material overtime would result in subsequent returning positive results (Table 1). Once the inoculum level rises above c. 30 cfu / plant sample, the probability of getting a false negative result is close to zero (Tyson *et al.* 2017).

Table 1. Concentration of Psa within plant tissue following Psa inoculation (reproduced from Tyson *et al.* 2017).

		A. chinensis var. *chinensis* 'Hort16A'				*A. deliciosa* 'Hayward'				*A. polygama*			
inoculum conc. (cfu/mL)	plant section	cfu/0.1 mL (means of 5 plants)				cfu/0.1 mL (means of 5 plants)				cfu/0.1 mL (means of 5 plants)			
		day 0	day 2	day 7	day 14	day 0	day 2	day 7	day 14	day 0	day 2	day 7	day 14
BS control	Top	-	0	0	0	-	0	0	0	-	0	0	0
	Middle	-	0	0	0	-	0	0	0	-	0	0	0
	Base	0	0	0	0	0	0	0	0	0	0	0	0
10^2	Top	-	11	3201	1830	-	4	2141	800	-	0	0	3
	Middle	-	3	3200	2401	-	0	2438	800	-	0	0	343
	Base	0	3	3200	2403	0	1	1777	800	0	0	64	576
10^4	Top	-	802	4000	4000	-	1	1673	3200	-	0	911	1349
	Middle	-	179	4000	4000	-	186	1849	3200	-	3	1077	1984
	Base	35	2083	4000	4000	13	125	2430	4000	1	800	3423	4000
10^6	Top	-	1343	4000	4000	-	1607	585	3832	-	2	14	346
	Middle	-	2351	4000	4000	-	2834	2070	3994	-	6	857	2128
	Base	1172	4000	4000	4000	494	2814	4000	4000	213	1759	4000	4000
10^8	Top	-	4000	4000	4000	-	2104	4000	4000	-	68	2664	364
	Middle	-	4000	4000	4000	-	4000	4000	4000	-	355	3312	2610
	Base	4000	4000	4000	4000	4000	4000	4000	4000	4000	4000	4000	4000

The protocol described has been developed using plants inoculated with Psa in the lab rather than field infested plants as these are difficult to establish in culture. These is research underway to try and establish in vitro cultures of Psa directly from field plants infected with Psa.

This screening protocol has been used to screen *in vitro* cultures of six genotypes initiated from Psa regions that cleared PEQ in the European Union (EU) and are now being grown in trials in the EU. Repeat screening with this protocol will provide a high level of confidence that material is Psa free, however it is proposed that additional measures are included in this pathway to provide even greater confidence and not rely on a single measure. These include;

b) PCR testing
c) Growth season monitoring in containment

3 Specific Requirements

The proposed pathway for the movement of *Actinidia* plant material into an Exclusion or Containment region is outlined below and summarised in the diagram in Figure 1.

3.1 Qualifying plant material

For *Actinidia* plant material to be eligible for movement it must be at stage 3 in vitro cultures that have been prepared as follows:

- Shoots of established in vitro cultures are transferred to peptone supplemented medium (3g/L) and grown on this medium for at least a week. If shoots are contaminated with Psa (and some other bacteria) there will be rapid growth of bacteria onto the growing medium. Shoots that show no contamination can be separated from other shoots, transferred to fresh medium and propagated further; clean shoots can become the mother plants from which all other plant material is derived. The mother plant(s) is moved as a single plant to a new culture vessel. Shoots showing contamination are discarded.
- Details of all plants subsequently propagated from the mother plant are to be recorded such that every individual shoot can be traced back to its mother plant. If a mother plant is identified as Psa-free at this step, all of its progeny are assigned a Psa-free status.
- Plants will need to be propagated in vitro for at least three 4-6 week culture cycles (cycle length may be genotype dependent) during which they will be rescreened at least one further time.
- For additional confidence in the Psa screening, samples from the base of tissue culture propagated plants are screened by PCR after each peptone screening.
- After the designated number of screening cycles, in which ALL plants derived from a given mother plant are found free of Psa these plants may be transferred to a Psa quarantine greenhouse

3.1.1 Inspection of qualifying material

In vitro cultures and plant material must be inspected for any visual signs of Psa bacteria at each stage of the pathway process and a record of each inspection maintained for auditing by KVH or its representative.
In order to identify symptoms of Psa on plants, especially as different genotypes may express different symptoms, at the same time that plants are transferred to the Psa quarantine greenhouse some plants will be deflasked at PFR Te Puke and monitored for symptom expression.

3.1.2 Laboratory

The laboratory to be used for the preparation of the qualifying plant material as outlined above must provide a copy of its quality assurance programme for tissue culture for review by KVH or its representative.

3.2 Psa Quarantine

3.2.1 Movement to Psa quarantine greenhouse

- Qualifying tissue culture plant material may only be moved from the tissue culture lab once an audit is complete and authorisation has been obtained from KVH.
- Tissue culture plants must be moved to a KVH approved greenhouse that is operated to a standard equivalent to PEQ level 2 quarantine, and outside of kiwifruit growing regions.

3.2.2 Quarantine period

- The quarantine period will commence once the tissue culture material has been deflasked and started active growth
- Plant material must be held in the quarantine facility in an active growing state for a minimum of six months.

3.3.3 Monitoring

Plants must be monitored for Psa symptoms by a KVH approved person 14 days after deflasking and then monthly during the quarantine period. Details of each monitor round must be recorded and retained for auditing.

Any symptomatic plant material must immediately (within 24 hours) be reported to KVH. KVH will arrange sampling and testing for Psa.

3.3.4 Pruning

No plant material may be pruned from the plants in quarantine without KVH authorisation.

3.3.5 Sample collection

Within the last 21 days of the active growing period in the quarantine facility leaf samples are to be collected from at least three positions on each plant, including:
(a) A young fully extended leaf at the top of the stem
(b) An older leaf from a midway position for testing.
(c) Any leaf showing any form of disease symptom

3.3.6 Testing
The leaf material to be tested for Psa-V using the KVH authorised test method and laboratory.

A positive Psa-V test results will require the immediate destruction of all Actinidia plant material held in the Psa quarantine facility.

3.3.7 Release from Psa Quarantine

Authorisation for release from the quarantine facility will only be granted if all the requirements outlined above have been meet to KVH satisfaction.

Release will only be granted for movement to a preapproved outdoor containment location

3.3 Outdoor containment requirements

At this point plants may be grown and propagated outdoors, in a containment location that is preapproved by KVH, outside of current kiwifruit growing regions and at least 20km from any known kiwifruit material.

Plants must be held in an active growing state on the containment site for a minimum period of eight months.

Plants must be monitored for Psa symptoms by a KVH approved person monthly during the containment period. Details of each monitor round must be recorded and retained for auditing.

Any symptomatic plant material must immediately (within 24 hours) be reported to KVH. KVH will arrange sampling and testing for Psa.
A positive Psa test result will require the immediate destruction of all Actinidia plant material held in the containment location.

3.3.1 Sample collection

Leaf samples are to be collected within the last 21 days of the active growing period in the outdoor containment location before release for Psa testing with PCR. Sampling will follow the Kiwifruit Plant Certification Scheme 600 leaf sample, which is designed to provide a 95% level of confidence of detecting Psa should it be present in the plants. This provides the final layer of mitigation measures to reduce risk to very low levels.

3.3.2 Testing

The leaf material to be tested for Psa-V using the KVH authorised test method and laboratory.

A positive Psa-V test result will require the immediate destruction of all Actinidia plant material held on the containment site

3.4 Release from outdoor containment to Exclusion regions

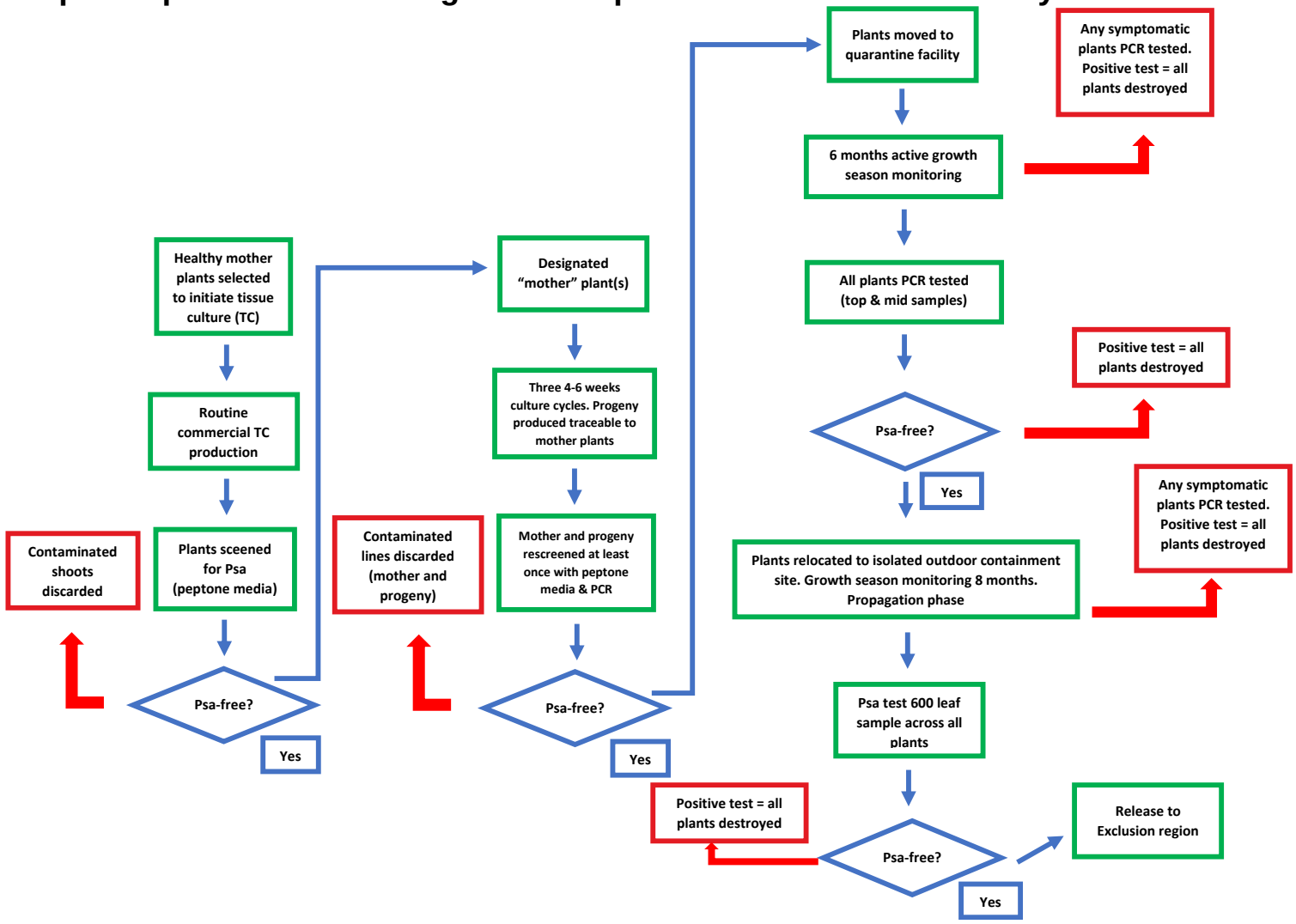
For permission to be granted by KVH for the *Actinidia* plant material to be released from the containment location for distribution into an Exclusion or Containment region the above pathway steps must be followed with all audits passed and Psa test results confirmed negative.

References

MPI (2018) Import Health Standard *Actinidia* Plants for Planting
https://www.mpi.govt.nz/dmsdocument/27879/loggedIn

Joy L. Tyson, Michelle J. Vergara, Ruth C. Butler, John F. Seelye & Ed R. Morgan (2017): Survival, growth and detection of *Pseudomonas syringae* pv. *Actinidiae* in *Actinidia* in vitro cultures, New Zealand Journal of Crop and Horticultural Science, DOI: 10.1080/01140671.2017.1414064

Proposed process for moving *Actinidia* plant material from Recovery to Exclusion or Containment regions



Appendix 2

Extracts from Import Health Standard *Actinidia* Plants for Planting (13 July, 2018).

(https://www.mpi.govt.nz/law-and-policy/requirements/import-health-standards/)

Part 2: Specific requirements

(1) All dormant cuttings must meet all requirements described in Part 2.1.

(2) All tissue cultures must meet all requirements described in Part 2.2.

(3) All *Actinidia* plants for planting must be screened for each regulated pest listed in Appendix 3, as described in Part 2.3, unless:

a) phytosanitary measures in relation to a regulated pest have been applied in accordance with an agreed *Export Plan* or at an MPI approved offshore facility. In this case the import permit will identify the regulated pests for which phytosanitary measures must be applied on arrival in New Zealand.

(4) All *Actinidia* plants for planting that require phytosanitary measures to be applied on arrival in New Zealand must be held in a post entry quarantine facility approved to the MPI Facility Standard: Post Entry Quarantine for Plants as described in Part 2.4.

2.1 Dormant cuttings

(1) All dormant cuttings must be:

a) imported to generate tissue cultures which will undergo screening for regulated pests as described in Part 2.3;
b) free from soil and other regulated articles;
c) accompanied by a phytosanitary certificate as described in Part 3:;
d) treated for insects and mites prior to export using one of the treatment options listed in Appendix 1 and Appendix 2, respectively. Insect and mite treatments must be applied a maximum of 48 hours prior to shipment;
e) imported into a Level 3 tissue culture laboratory approved to the MPI Facility Standard: Post Entry Quarantine for Plants;
f) dipped in 1% sodium hypochlorite for a minimum period of 2 minutes on arrival at the tissue culture facility;
g) destroyed in the quarantine waste after tissue culture plants have been generated.

(2) If dormant cuttings are sprouted to generate explant material, this must be done according to one of the following options:

a) cuttings must be held in a Level 3B post entry quarantine facility;
b) cuttings must be held in a sealed vessel in a growth chamber within a Level 3 tissue culture facility. The sealed vessel may only be opened in a biological safety cabinet.

(3) Each tissue culture that is generated from a dormant cutting will be considered as an individual tissue culture plantlet.

(4) Stage 1 tissue cultures must not be deflasked directly into the greenhouse. All plants must enter the stage 2 (multiplication) phase prior to hardening off and deflasking.

(5) If tissue cultures are sub-cultured before they are transferred to the greenhouse, the process must be done as described in clause 2.2(3).

2.2 Tissue cultures

(1) All tissue cultures must be:

a) derived from aerial plant parts;
b) grown in a pest proof and transparent vessel, with a maximum of one plant per vessel;
c) grown in a medium free from fungicides, antibiotics and charcoal;

d) grown in the vessel in which they will be exported for at least 14 days prior to shipment;
e) free from visible fungal or bacterial contamination;
f) in the stage 2 (multiplication) or stage 3 (rooting) phase;
g) accompanied by a phytosanitary certificate as described in Part 3:.

(2) Tissue cultures may be imported directly into a Level 3 tissue culture laboratory approved to the MPI Facility Standard: Post Entry Quarantine for Plants for sub-culturing before they are transferred to the greenhouse.

(3) If tissue cultures are sub-cultured before they are transferred to a greenhouse, the following requirements must be met:

a) at least one sub-culture from each imported stage 2 or stage 3 tissue culture plant must be developed to the stage where it can be screened for regulated pests after it is deflasked into the greenhouse (see Parts 2.3 and 2.4):

(i) this sub-culture should be taken during the first round of multiplication;
(ii) if only one plant is obtained during the first round of multiplication, further rounds of multiplication may be undertaken. In this case, a sub-culture for transfer to the greenhouse must be taken from the first round of multiplication where more than one plant is obtained.

b) surplus sub-cultures that are produced during the round of multiplication used to generate the plant which is transferred to the greenhouse may be retained at the Level 3 tissue culture laboratory throughout the quarantine period as follows:

(i) these plants may be sub-cultured and multiplied during the post entry quarantine period;
(ii) these plants may also be eligible for biosecurity clearance provided that traceability is maintained as described below.

c) clear records of traceability must be retained throughout the quarantine period;
d) only sub-cultures that can be directly traced back to both the original imported tissue culture plant, and the plant that has been transferred to the greenhouse, will be eligible for clearance.

2.3 Screening for regulated pests

(1) To ensure freedom from regulated pests all *Actinidia* plants for planting must be screened for each regulated pest listed in Appendix 3, on arrival in New Zealand as described in this Part unless:

a) phytosanitary measures for a particular pest have been applied as described under an agreed *Export Plan* or, at an MPI approved offshore facility. In this case, the import permit will identify the requirements of Part 2.3 that must be applied in New Zealand.

2.3.1 Environmental conditions

(1) Specific environmental conditions must be applied in the first and the second growing seasons, as follows:

a) a continuous three month period of spring-like conditions. The daytime temperature range must be between 18°C and 21°C, with a night time temperature range between 15°C and 18°C;
b) a continuous four month period of summer-like conditions. The daytime temperature range must be between 21°C and 25°C, and a night time temperature range between 18°C and 21°C (apart from when additional conditions described in clause 2.3.1(3) are applied);
c) a continuous two month period of autumn-like conditions, with a daytime temperature range between 15°C and 18°C. Lower temperatures may be applied at night.

(2) Plants must be held dormant at around 4°C for at least two months between the first and second growing season.

(3) The following additional environmental conditions must be incorporated into the four month period of summer-like conditions in the first growing season:

a) a continuous 28 day period at a minimum relative humidity of 75% (±5%), which includes two 48 hour periods of continuous misting. There must be a minimum period of at least two weeks between each misting period;
b) a continuous 28 day period with a daytime temperature of 25°C to 30°C and a night time temperature above 20°C . A minimum relative humidity of 75% (±5%) must be maintained during this time.

(4) The operating manual for the post entry quarantine facility must describe the environmental conditions that will be applied during each growing season, and how these will be monitored, maintained and recorded.

2.3.2 Testing

(1) All testing must be done at a facility approved to the MPI Standard 155.04.03: A standard for diagnostic facilities which undertake the identification of new organisms, excluding animal pathogens.

2.3.2.1 Diagnostic testing

(1) If a pest is found, or signs or symptoms of a pest are observed during inspections by the facility operator or by the MPI Inspector, samples must be sent for diagnostic testing as described in Part 3.7 of the MPI Facility Standard: Post Entry Quarantine for Plants.

2.3.2.2 Pre-determined testing

(1) Pre-determined testing is required for all regulated pests listed in Table 1.

(2) All samples for pre-determined testing must be collected during the first growing season according to the schedule shown in Table 1.

(3) The unit for pre-determined testing is an individual greenhouse plant. Each plant must be labelled individually and tested separately, with the following exception:

a) for polymerase chain reaction (PCR) testing, samples taken from up to five plants of the same species can be combined to form a single composite sample for pre-determined testing.

2.3.3 Inspection

(1) All plants must be inspected for signs and symptoms of regulated pests by the facility operator as described in Part 3.6.1 of the MPI Facility Standard: Post Entry Quarantine for Plants.

(2) All plants must be inspected for signs and symptoms of regulated pests by the MPI Inspector according to the schedule shown in Table 1. A total of ten inspections must be done by the MPI Inspector.

(3) The operator of the post entry quarantine facility must ensure that the MPI Inspector is notified:

a) when plants are deflasked into a greenhouse;
b) when deflasked plants start active growth;
c) before the environmental conditions described in clause 2.3.1(3) are applied;
d) when plants start active growth at the start of the second growing season.

2.4 Post entry quarantine greenhouse

(1) For all *Actinidia* plants for planting, all requirements must be applied as described in this Part, unless:

a) phytosanitary measures for a particular pest have been applied as described under an agreed *Export Plan* or at an MPI approved offshore facility. In this case, the import permit will identify the requirements of Part 2.4 that must be applied in New Zealand;

(2) Individual tissue culture plants must be deflasked into a post entry quarantine greenhouse approved to the MPI Facility Standard: Post Entry Quarantine for Plants. The level of greenhouse will be specified on the import permit.

(3) The total quarantine period will:

a) begin after tissue cultures have been deflasked and started active growth;
b) be a minimum of 20 months;
c) include two distinct growing seasons, each of at least nine months long, with a two month dormancy period in between the first and second growing seasons.

Table 1: Schedule of inspections by the MPI Inspector and pre-determined testing requirements

Season		Timing of inspection by MPI Inspector	Pre-determined testing requirements			
			Timing of sample collection	Tissue type	Organism	Test
First growing season	'Spring-like' conditions for three months as described in clause 2.3.1(1)a)	**Inspection 1** Within the first 14 to 28 days of plants being deflasked and starting active growth in the greenhouse. **Inspection 2** Within the last 14 days of the spring-like growth period.	**Sample set 1** Within the last 28 days of the spring-like growth period.	**Leaf material samples** Collected from at least two positions on each stem, including: • A young fully expanded leaf at the top of the stem • An older leaf from a midway position Leaf petioles and mid veins to be used for testing.	• *Actinidia chlorotic ringspot-associated virus*	PCR
					• *Apple stem grooving virus* [*Actinidia*-infecting strain	PCR
					• *Citrus leaf blotch virus* [*Actinidia*-infecting strain]	PCR
					• *Pelargonium zonate spot virus*	PCR and herbaceous indexing using the indicators *Chenopodium quinoa, Nicotiana benthamiana, N. glutinosa* and *N. tabacum*
					• Tobacco necrotic spot associated virus	PCR
					• *Pseudomonas syringae pv. actinidiae*	PCR
	'Summer-like' conditions for four months as described in clause 2.3.1(1)b)	**Inspection 3** Within the final 14 days of growth between 21°C to 25°C at 75% (±5%)	**Sample set 2** After at least 28 days growth in the summer period, but before temperatures exceed 25°C.	**Stem samples** Collected from at least two positions on each stem, including:	• *Verticillium nonalfalfae*	PCR or culture based identification method

Season		Timing of inspection by MPI Inspector	Pre-determined testing requirements			
			Timing of sample collection	Tissue type	Organism	Test
		relative humidity, and after at least one 48 hour misting period, see clause 2.3.1(3)a). **Inspection 4** Within the final 7 days of growth at 25°C to 30 °C, or within 7 days following the completion of this period, see clause 2.3.1(3)b).		• One shoot at the base of the stem • One shoot in the middle section of the stem If possible, the minimum length of stem taken from each shoot should be 10 cm.		
			Sample set 3 Within 14 days of completing growth between 25°C and 30°C.	**Leaf material samples** Collected from at least 2 positions on each stem, including: • A young fully expanded leaf at the top of the stem • An older leaf from a midway position Leaf petioles and mid veins to be used for testing.	• All phytoplasmas	PCR
					• *Pectobacterium carotovorum* subsp. *actinidiae*	PCR or culture based identification method
				Stem samples Collected from at least 2 positions on each stem, including: • One shoot at the base of the stem • One shoot in the middle section of the stem If possible, the minimum length of stem taken from each shoot should be 10 cm.	• *Ceratocystis fimbriata*	PCR using primers that target the Latin American Clade (LAC)
					• *Phytophthora drechsleri*	PCR or culture based identification method
					• *Phytophthora palmivora*	PCR or culture based identification method
					• *Phytopythium helicoides*	PCR or culture based identification method

Season		Timing of inspection by MPI Inspector	Pre-determined testing requirements			
			Timing of sample collection	Tissue type	Organism	Test
	'Autumn-like' conditions for two months as described in clause 2.3.1(1)c).	**Inspection 5** Within the last 28 days of the period of autumn-like conditions.				
Two month dormancy as described in clause 2.3.1(2)						
Second growing season	**'Spring-like' conditions** as described in clause 2.3.1(1)a).	**Inspection 6** Within the first 14 to 28 days of plants coming out of dormancy. **Inspection 7** Within the last 14 days of the spring growth period.				
	'Summer-like' conditions as described in clause 2.3.1(1)b)	**Inspection 8** Within the first 14 to 28 days of the summer growth period. **Inspection 9** Within the last 28 days of the summer growth period.	Pre-determined testing to be repeated for *C. fimbriata, P. carotovorum* subsp. *actinidiae* and *V. nonalfalfae* within the last 28 days of the summer growth period. The same test methods and sampling regimes used in the first growing season must be used.			
	'Autumn-like' conditions as described in clause 2.3.1(1)c)	**Inspection 10** Within the last 28 days of the autumn growth period,				