Report Cover Page

ACERA Project
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Title
Plant biosecurity: Detectability of arthropods in fresh produce consignments

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Summary
Current DAFF import policies state that there is a 95% confidence that less than 0.5% of units in a consignment harbour quarantine pests if no pests are detected during a 600-unit sample. However this implicitly assumes that inspectors detect 100% of the pests infesting a 600-unit sample. This ACERA project assesses the potential for a further study to test this assumption for a number of groups of pests. This study tests an experimental design intended to estimate the likelihood of detection of a variety of pests within a 600-unit sample at various infestation levels. The International Plant Protection Convention (IPPC) sets the standards and guidelines for trade under the WTO and requires that phytosanitary measures are technically justified. This ACERA project provides technical data to underpin experimental assessment of the robustness of the sampling protocol of one of the mostly commonly used phytosanitary tools.

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Plant Biosecurity: Detectability of arthropods in fresh produce consignments

ACERA 1006C

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Executive Summary

Phytosanitary inspection is one of the most commonly used phytosanitary measures to manage arthropod pests worldwide. For most pests, we do not know the threshold prevalence of a population infesting a product that would lead to reliable detection by inspectors, nor do we know if the threshold for establishment is lower than the threshold level for detection by inspection. Although substantial work has been done on the statistics of inspection (Cannon 2001, Robinson *et al.* 2009 a, b and c), little work has been done on understanding the biological or operational basis of standard practices.

The current standard inspection regime in Australia for large consignments of fresh produce is the inspection of a random sample of 600 units. The sample size is based on the statistical relationship that if all of 600 inspections fail to detect infestation then the estimated level of infestation is below 0.5% of units with 95% confidence. However, this prescription ignores imperfect detectability. Clearly, some pests are more difficult to detect than others. By acquiring relevant data on detectability, systems managers may be able to allocate their resources more effectively.

ACERA Project 1006C begins to tackle one of these components by developing a methodology for determining detection probability and the relationship between prevalence and detection probability. The aim of ACERA 1006C is to design a comprehensive detectability experiment, the purpose of which is to determine the population levels at which a range of arthropods are detected reliably in fresh produce. This project provides a framework for further work that will guide decision making about strategic investment in phytosanitary measures and inspection systems. These trials outline possibilities and provide preliminary estimates that may be used for more thorough analyses. The trials also test an experimental design to assess detectability of pests in a consignment. It is not intended to examine other issues that impact on the efficacy of an inspection such as the duration and conditions of inspection, human behavioural traits or how a particular commodity is handled. Nor is it intended to infer any consequences of the level of detection. These other important factors need to be examined with appropriate regimes developed on these findings then integrated with the current design to comprise a full protocol for inspection. The results provide a sound platform on which a comprehensive and informative study may be designed.

1.0 Context: Phytosanitary inspections

Phytosanitary inspections are one of the most frequently used phytosanitary measures to prevent the introduction of regulated plant pests worldwide. The Australian standard regime for fresh produce is to inspect a random sample of 600 units, based on the statistical relationship that if all of 600 inspections fail to detect infestation then the estimated level of infestation is below 0.5% of all units with 95% confidence: however, this does not take into account imperfect detectability. Some pests are more difficult to detect than others and may not be found with this level of confidence in a sample size of 600.

Little or no work has been done to understand the abilities of inspectors to detect pests, nor the abundance of pests that would be necessary to establish a viable population. The latter is important because the threshold level for abundance may be lower than the threshold level for detection by inspection. Hence work is needed to better understand the abundance at which pests can be detected with reasonable probability, taking into account the biology of the organism and organism-host relationship; and the biological factors that drive establishment of a pest.

We designed an experiment in which some fresh produce is artificially infested at various prevalence levels with several arthropods of variable size and mobility, and test if these pests are reliably detected by the DAFF inspectors. The primary motivation for this study is to evaluate the operational details, logistical challenges, costs and time involved in each step of the experiment, providing a sound basis on which to design a more comprehensive study.

Two trials were designed to test the experimental design and were run in Brisbane in conjunction with DAFF North East Region Brisbane and Bugs for Bugs (a beneficial-insect rearing company in Mundubbera, Queensland). Our first logistics pilot trial took place in October 2011 and involved the infestation, transport and inspection of sets of 600 citrus artificially infested at low prevalence levels, to test the capacity and logistical components of the system. This first trial was followed by a second one, including 6 000 infested citrus at a greater variety of low prevalence levels in May 2012.

During our first trial, the calyces of two sets of 600 class 2 Valencia oranges were randomly infested at a 0.5% prevalence with two species of mites (one fast crawling, predatory species and one slow crawling phytophagous species) and one species of mealy bug. Two controls representing surrogate (sessile) pests were also included and used for infestation at the same prevalence level. The 12 boxes of 100 citrus were packed and sent for inspection by a team of DAFF (formerly AQIS) inspectors. The inspectors followed their usual routine for this type of commodity. The logistics trial tested the feasibility of artificially infesting fruit to a predetermined level and the infestation surviving packing and transportation. It also evaluated the logistics of packing and transportation of a relatively small quantity of fruit from a small Queensland country town to the DAFF inspection location in Brisbane and the slightly unusual pathway for introducing consignments into the standard inspection system.

The second trial focussed on the design of the experiment to ensure that the trial tests the detectability of organisms in fresh produce appropriately and with sufficient power to detect important differences in inspection performance for different types of pests and compare the detection of mobile versus static pests. Six thousand class 2 Navel oranges were used for random infestation at 0.1%, 0.5% and 5% using the same protocol, invertebrates and sessile controls as in trial 1. Detection levels were recorded by the same team of DAFF inspectors and following their usual routine for this type of commodity.

The inspection team found every infested citrus during the first trial of two sets of 600 Valencia oranges. During the first trial, inspectors detected all specimens of the two introduced mite species

and all specimens of the mealy bug species plus a resident species of scale, a dead bee and a live spider, which were not part of the deliberate infestation. Our second trial used 6 000 Navel oranges that had high infestation levels of resident invertebrate species, which provided a different biological background compared to the first trial. Our second trial demonstrated that detectability varied with the type of pest used to infest the fresh produce, and depended on the mobility of the live pests and also the high level of resident arthropods populations already present. Nevertheless, inspectors' performance remained consistent for the duration of the inspection of an unusually large number of class 2 fresh produce.

These two trials laid out the logistics and experimental design necessary to conduct the inspection of artificially infested consignments of different sizes at various prevalence levels and provide recommendation for future testing and statistical analysis.

1.1 Towards a comprehensive detectability study

The scoping trial gathered information to support the design of the second detectability experiment. The purpose of both detectability experiments was to determine the population levels at which a range of arthropods could be detected reliably in fresh produce. These two trials examine several aspects of the experiment.

1. Potential host and pest organism interactions;
2. Range of population sizes, the distribution of pests within samples, and prevalence conditions (number of elements within a load that are contaminated);
3. Methods for creating contaminated samples to test inspection systems, including the logistics of transportation of material;
4. Engagement of DAFF Operational Inspectors in the trial, holding of commodities and disposal of commodities;
5. Design and numbers of replicates required for statistically powerful results, including an assessment of the strengths and weaknesses of using surrogate "species" (inanimate models of arthropods) versus live specimens;
6. The potential for collecting and analysing leakage data to provide ongoing assessments of detectability from a range of arthropods.

1.2 Host and pest organisms

The candidate host products used for this project were commercially produced Valencia and Navel varieties of oranges. Oranges are relatively simple in surface structure and easy to handle, they can be stored for a relatively long time in cool rooms and can be readily artificially infested. Valencia oranges were used for the first trial and Navel oranges were used for the second trial.

To ensure that insecticides did not confound the outputs of this experiment and that arthropods would colonise these oranges, we used class 2 Valencia and Navel oranges grown following an IPM program for pest control. None of the oranges were waxed after picking and many were already hosting various resident arthropods populations at various levels. However, all oranges were subjected to the usual post-harvest treatment before packing, including a fungicide dip followed by a two days ethylene de-greening treatment. Washing of all oranges occurred in-line before packing.

The three candidate arthropod species used for this trial were citrus mealy bug (*Planococcus citri*) and two species of mites: a predatory mite species (*Carpoglyphus lactis*) and a mycophagous mite species (*Transeius montdorensis*) were transferred together onto the host product. These species were obtained from commercial rearing facilities (Bugs for Bugs, Mundubbera, Queensland). These

arthropods can be produced in high numbers all year round and can be readily transferred onto citrus hosts.

In addition to the live organisms, static markers (surrogate species) were also used to infest the oranges. The markers were designed to be distinctive and to mimic sessile arthropods or symptoms. They consisted of black dots (made with a marker pen) and beads (around 0.3 cm x 0.3 cm, up to 0.5 cm) of Blu-Tack. The Blu-Tack beads were placed where arthropods are more likely to be found (i.e. around the calyx and navel). The black dot was positioned randomly on the fruit surface.

1.3 Population sizes

Two trials were designed to test the experimental design and were run in Brisbane in conjunction with DAFF North East Region Brisbane and Bugs for Bugs (a beneficial insect rearing company in Mundubera, Queensland). Our first logistic pilot trial took place in October 2011 and involved the infestation, transport and inspection of sets of 600 citrus artificially infested at low prevalence level, to test the capacity and logistical components of the system. Two sets of 600 oranges were randomly infested with such live pests and static markets were introduced to 0.5% of the fruit. Boxes (1 to 12) and layers (1 to 5) within each box were selected using a random generator (table 1). Therefore three citrus per total of 600 were individually infested (see section 1.4 for infestation protocol). Oranges were unpacked, numbered, artificially infested and packed again so that each box contained 100 oranges. The 12 boxes of 100 citrus were sent for inspection by a team of three DAFF inspectors. The inspectors followed their usual routine for this type of commodity.

Table 1: Random infestation of 12 boxes of 100 Valencia oranges in trial 1.
Box and orange layers within each box were selected by using a random number generator (Random.Org, http://www.random.org/integers/).

Box number (1 to 12)	Layer number (1 to 5)	Black dot	Mealy bugs	Blu-Tack beads	2 mite species
2	5	1			
6	1	1			
1	2	1			
3	5		1		
3	5		1		
1	1		1		
11	4			1	
10	2			1	
12	5			1	
9	4				1
10	1				1
8	3				1

The second trial in May 2012 focussed on the design of the experiment to ensure that the trial tests the detectability of organisms in fresh produce appropriately and with sufficient power to detect important differences in inspection performance for different types of pests and compare the detection of mobile versus static pests. Six thousand class 2 Navel oranges were used for random

infestation at 0.1%, 0.5% and 5% using the same protocol, invertebrates and sessile controls as in trial 1. Detection levels were recorded by the same team of DAFF inspectors and following their usual routine for this type of commodity. Boxes and oranges within each box were selected randomly using a random generator (see table 2) and infested using the same protocol as previously.

Table 2: Random infestation of 60 boxes of 100 Navel oranges in trial 2.
Box and orange numbers within each box were selected using a random generator (Random.Org, http://www.random.org/integers/).
IF = Infested Fruit
* Fast and slow crawling mites were reared and used to infest oranges together.

Category	Mealy bugs	Mites*	Black dots	Blu-Tack beads
Prevalence	0.5%	0.5%	5%	0.1%
IF count	30	30	300	6
Count of infested boxes and number of IF/ box	- 10 boxes @ 1 IF/box = 10 IF; - 10 boxes @ 2 IF/box = 20 IF		-20 boxes @ 10IF/box =(200 IF); -10 boxes @ 5IF/box = (50 IF); -20 boxes @ 2 IF/box = (40 IF); -10 boxes @ 1 IF/box= (10 IF);	- 6 boxes @ 1IF/box

1.4 Methods for creating artificially contaminated samples and transport

1. Numbering of boxes and individual oranges:

For both trials, each box containing around 100 oranges, packed on 5 layers, was individually numbered both on the bottom right corner (see photo 1) on the original product label on the outside, and on the inside cardboard insert. For both trials, all boxes were unpacked, each orange was individually numbered (see photo 2), infested according to the infestation table 1 and repacked (see photo 3). The infestation of 12 boxes took less than a day for trial 1.

For the second trial, infestation of 60 boxes of 100 oranges according to tables 2 and 3, took two full days and involved a team of three at Bugs for Bugs. All boxes were unpacked and each orange was labelled including both box number (1 to 60) and orange number (1 to 100) sequentially using a water proof marker. Oranges that needed to be infested (with mealy bugs, mites or with the surrogate pest models) were set aside for infestation and the rest placed back in the box. Once the arthropods had settled, the boxes were unpacked again and the infested oranges placed in the box.

2. Live infestations

Note that specific population levels for live organisms were not set for these experiments. The invertebrate population levels were not measured before infestation and only roughly estimated (especially for the mite species). Infested oranges needed to host at least one invertebrate of each selected species. During inspections, fresh commodity (individual fruit or vegetable) found to host some pests are set aside by inspectors regardless of the level of infestation, which is not measured during inspection.

a. *Mealy bug infestations*

Between one and five mealy bugs were transferred either singly or in clusters from the rearing host (butternut pumpkins) onto numbered oranges using a soft brush. Infested oranges were left to settle for 24 to 48 hours and repacked in sequence in each numbered box (see photo 4).

b. Mite infestations

A plastic vial containing around 5g of rearing media containing both predatory (fast crawling) and mycophagous (slow crawling) mites was secured onto the navel or calyx of the oranges (see photo 5) using some Blu-Tack and sticky tape. The vial was left for 24 to 48 hours to permit settling of both mites species onto the host, then removed.

c. Surrogate pest models

Both sessile markers were placed on selected numbered oranges and packed in sequence in boxes. The Blu-Tack beads were positioned around the calyx (see photo 6) whereas the marker pen dots were randomly placed on the orange surface (see photo 7).

3. Packing, transporting and storage

Boxes were randomly stacked so as to fit on wooden pallets used for transport (see photo 8) and secured. For the first trial, the boxes were sent by road two days after infestation from the commercial rearing facilities located at Mundubbera to Brisbane DAFF Operational inspectors (a distance of around 370 km). The Valencia oranges were not cooled before or during transportation. Transport was organised so that the consignment would travel overnight and arrive early in the morning of the inspection at the DAFF facility (see photo 9). The consignment was kept at room temperature for the duration of the experiment and during the inspection for the first trial. The inspection of the first infested consignment took place immediately after delivery of the pallets.

For the second inspection of 6 000 Navel oranges, the consignment was also sent by road overnight, one day after the infestation at Bugs for Bugs. The infested consignment was kept at room temperature until it was sent to Brisbane DAFF Operational Inspectors. Again, transport was organised so that the consignment would arrive early in the morning of the inspection. Half of the boxes were placed in the cool room at 3°C whilst the first 30 boxes were inspected immediately after reception.

1.5 Inspection by DAFF Brisbane

1. Inspection

The same team of 3 experienced DAFF fresh produce inspectors undertook inspections of the two consignments. These inspectors volunteered to take part in this study. The team was asked to follow the usual DAFF guidelines for the inspection of citrus. Before the trials started, inspectors had been briefed via email and face to face. It was stressed to the team that both trials were not assessing their ability to successfully detect pests in a given commodity but the validity of the current operating procedure.

At the arrival of the first consignment, the inspectors were briefed again on what to do: they were asked to follow the normal procedure for inspection of citrus consignments, but warned that two types of controls (i.e. Blu-Tack beads and marker pen dots mimicking sessile pests) had been included in the set of boxes to inspect. Inspectors were not given any information regarding the identity of the live pests nor the prevalence levels of live pests or sessile markers.

Each inspector inspected 4 boxes of 100 Valencia oranges in trial 1 over one day and 20 boxes of 100 Navel oranges in trial 2 over two days, four days apart. Each inspection lasted three to five hours per day. Inspectors randomly selected boxes to inspect from the pallet. Every orange in each box was inspected up to orange number 100. Two ACERA team members were recording the three inspectors' findings and observations for the duration of both trials.

Overall, the inspection typically occurs in two steps: a first examination is done under an illuminated magnifying glass (a magilamp) for each fruit. Then a second, closer examination under the dissecting microscope follows on a minimum of 10% of the calyces and/or oranges.

Usually, inspectors count the fruit as they go until they reach the 100th orange in each box, even though there might be a few more fruit present in the box. In normal circumstances, they would not record comments for each fruit nor sequential fruit numbers. They were asked to do so for both these trials. Normally, inspectors would place any suspicious fruit on the side and stop when a total of 600 fruit has been inspected. At the end of the inspection, the result usually obtained is "presence or absence of any given pest". The team does not record any infestation levels nor disease prevalence levels but only the presence or absence of live pests per sample of 600 items.

At the beginning of both trials, the inspectors inverted the bottom box content into the lid so that the fruit on the top layer ended up in the bottom and were the first to be inspected. The top lid of each box was first examined, and then tapped onto a sheet of white paper to dislodge any invertebrates or contaminants. The sheet of paper was scrutinised under the magilamp (see photos 10 and 11).

Each individual fruit, starting from the top layer, was inspected under the magilamp and special care taken to check around and under the calyx of each orange and navel, if present. Under the dissecting microscope, individual calyces and any blemishes or necrotic areas were examined (see photos 12 and 13). A thorough examination was also carried out for each orange particularly when a navel was present. If navels were deep and convoluted, they were later dissected to reveal any intricate area where invertebrates could be concealing. In some cases, the navels were very deep and provided an ideal location for invertebrates.

Out of each inspected box, between 10 and 20 oranges (a minimum of 10%) were set aside for further examination of the calyx, navel and/or the whole fruit under the dissecting microscope. These fruit were not selected randomly but according to what the inspectors found under the magnifying glass (e.g. scales, blemishes etc.) during their first examination, the orange's general aspect, or the presence of unusual or suspicious invertebrates or their structures (eggs, pupae, pupal cases etc.), symptoms and/or blemishes. When these oranges were selected, both the box number and the sequential fruit number were recorded by the ACERA team together with additional comments the inspectors might make during the inspection of each fruit (see photos 14 and 15) both during the first examination under the magilamp and the second examination under the dissecting microscope.

If anything unusual was found, it was carefully removed, placed in a vial and sent to the diagnostics unit for identification. At this stage, the link between the sequential number of the orange, the box number and the pest to identify was lost (see photo 16).

In a normal inspection, if dead eggs, casings, whole invertebrates or pupae are found, they are reported as "clear". Only live pests are recorded. However, for the purpose of these trials, inspectors notified the ACERA team when they found unusual occurrences: Blu-Tack beads, black dots, cases, webbing, pupae, eggs, dead invertebrates and blemishes (soft rots and necrotic spots). They also informed ACERA team members of the result of their second examination under the dissecting microscope even though the calyces or navels might be clear and therefore not recorded.

The inspection team found every infested citrus during the first trial of two sets of 600 Valencia oranges. During the first trial, inspectors detected all specimens of the two mite species and all specimens of the mealy bug species plus a resident species of scale, a dead bee and a live spider which were not part of the deliberate infestation.

Our second trial used 6 000 Navel oranges which had high infestation levels of resident invertebrate species which provided a different biological background compared to the first trial. Our second trial demonstrated that detectability varied with the type of pest used to infest the fresh produce, and depended on the mobility of the live pests chosen for this experiment and also the high level of resident arthropods populations already present. Nevertheless, inspectors' performance remained consistent for the duration of the inspection of an unusually large number of class 2 fresh produce.

2. Leakage data

The two ACERA team members only recorded the three inspectors' comments during the examination of both consignments. It was essential to ensure that none of inspectors felt that their skills and performance were being assessed. Due to the way the inspections were performed, it was impossible for only 2 ACERA team members to carry out a second inspection of all oranges within the time scale and budget allocated for this project. One would need a second team of inspectors of equivalent expertise to carry out a leakage survey immediately after the inspection is competed and before the fruit perish.

Furthermore, the detection of live organisms mostly involved destructive investigation as unknown invertebrates, or invertebrates selected for identification, were set aside and trace back to the numbered orange was lost. Several oranges were also destroyed when calyces were removed before examination and navels dissected out to reveal concealing clusters of invertebrates.

No leakage data were therefore gathered for any of the two trials but would need to be included in further studies.

1.6 Disposal of commodities

Once inspected, all oranges were donated to a local charitable organisation.

2.0 Results

2.1 Trial1 – Inspection of 2 sets of 600 Valencia oranges

1. Additional infested fruit:

Out of the 30 Valencia oranges packed separately for trial 1, of which 27 had been infested with mealy bugs and three with the two species of mites (see photo 17), three oranges were found without mealy bugs. Some mealy bugs had moved to the cardboard box bottom and sides, others had moved to wedge themselves in between two oranges or in between a fruit and the cardboard side.

2. Trial 1: infested Valencia boxes:

All of the surrogate pest models (Blu-Tack beads and marker pen dots) were intact. The Blu-Tack beads remained around the calyx of all three Valencia oranges. The live pests settled and remained on the fruit during transport and until the inspection took place. Some of the fast crawling mites however had moved from the initial fruit to neighbouring ones (which was expected). Inspectors therefore recorded a frequency which was over 0.5% for the fast crawling mite species (more than three Valencia oranges were found with fast crawling mites in one box).

Furthermore, inspectors usually do not inspect class 2 fruit but class 1 consignments of waxed citrus. The presence of blemishes, spots, scales and mites is an unusual occurrence on imported waxed class 1 oranges. At times, it was difficult for the inspectors to differentiate between a black dot (our control made with a black marker pen) and some fungal black spots, necrotic areas or localised physical damage. All the Blu-Tack beads on the other hand, were found correctly and very quickly, even the ones that had fallen to the bottom of the box.

The mealy bugs were also found without difficulty as they are big enough to be seen with the naked eye and are easily detected under the magnifying lens. In the first set of six boxes, in addition to the mealy bugs, some resident mite populations were present and the inspectors detected them during the first examination when looking under the magnifying lens.

In the second set of Valencia boxes infested with the two types of mites (fast crawling and slow crawling) plus Blu-Tack beads as sessile pests, the inspectors found three types of mites: the ones the ACERA team infested the fruit with at Bugs for Bugs, plus a resident species which was probably present at harvest. Inspectors were able to differentiate between the three species of mites even though they could not initially identify them to the species level. Inspectors also found a dead honeybee, a spider (still alive) and a silver fish that had crawled in probably after or during the unpacking, infesting and repacking of the set of boxes at Bugs for Bugs or during transport and storage.

During standard inspection, no data are gathered on infestation levels during an inspection. Inspectors usually record the total number of infested fruit (or units), not the concentration or number of pests on each fruit or in the whole consignment. Inspectors neither count nor record infested fruit numbers or their sequence number, nor report on disease incidence or dead invertebrate numbers. They only report on the presence or absence of live pests of relevance.

2.2 Trial 2 - Inspection of 6 000 Navel oranges

This trial involved the inspection of ten times the number of oranges normally examined from a consignment. It is also important to note that these class 2 Navel oranges had high levels of resident arthropods including scales, mites and an unusually high prevalence of blemishes. A large percentage of calyces were dry. Additionally, some oranges succumbed to soft rot before they were numbered at Bugs for Bugs and a further 10% had developed some mould by the end of the inspection at the DAFF facility in Brisbane.

The 60 boxes arrived on the morning of the first day of inspection. Thirty boxes were inspected in day 1, whilst the 30 others were kept in the cool room at the DAFF facility to be inspected the following week on day 2. Navel oranges kept in the cool room were wet when the inspectors opened the boxes to start the inspection. Some of the marker points had smeared onto neighbouring oranges. Our Navel oranges hosted high level of resident invertebrates before artificial infestations were performed at Bugs for Bugs. These included scales, thrips, mites and mealy bugs already present in some of the navels. Additionally the inspectors found the following invertebrates: a dead Psocoptera nymph, *Planococcus minor* (very similar to *P. citri*), *Chrysomphalus aonidum* (black scale or Florida scale), *Karnyothrips flavipes* (a coccoid predator) and *Aeroglyphus robustus* (warty grain mite). Furthermore the invertebrates moved before the inspection was completed. That is, the infestations had not stabilised on particular oranges. However, the numbers of organisms detected were at least at the infestation rates applied to the oranges.

Statistical analysis of the second trial has been carried out for each pest species. As the inspection was spread over two days, a comparison of the two days' results was also considered.

1. Surrogate species 1: Black marker dot

Of 300 artificially infested oranges, the inspectors correctly found 215 (72%), and an additional 24 (8%) incorrectly. When one orange was infested, the inspectors identified it correctly five out of six times. When two oranges were infested per box, inspectors identified both correctly seven out of twelve times, one correctly three times, three oranges once (one incorrectly) and four oranges once (two incorrectly). It is important to note here that our use of the term 'correctly' presupposes that infestations had not moved. However, in a few instances, the marker pen had smeared onto a neighbouring orange but was not detected by inspectors as additional black dots.

A logistic regression with the number correctly found by the inspectors against the proportion of infested oranges showed little evidence of "overdispersion" in regard to number identified correctly. We can conclude that inspectors were essentially examining each orange independently rather than the box as a cluster.

The percentage of positive detection for the two days was 76% in day 1 and 74% in day 2, suggesting that inspectors remained focussed and consistent for the duration of the inspection of the 6 000 Navel oranges.

2. False and true positives

A table can be constructed for each examined box, for example, boxes 1 and 15 (table 3):

Table 3. Classification table illustrating true and false positives and negatives for boxes numbered 1 and 15.

Box 1		ACERA		
		no	yes	total
AQIS	no	94	4	98
	yes	0	2	2
	total	94	6	100

Box 15		ACERA		
		no	yes	Total
AQIS	no	87	4	91
	yes	3	6	9
	total	90	10	100

There was a weak positive correlation ($r = 0.26$, $P = 0.044$) between false positive rate and prevalence. Figure 1 illustrates the relationship.
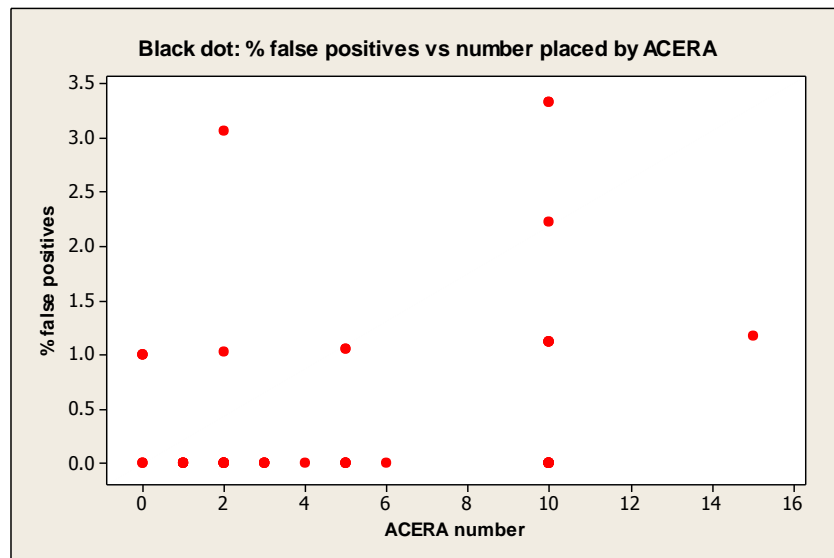

Figure 1. Percentage of false positive versus prevalence for one of the surrogate species used in trial 2 (black dots).

There was little correlation ($r = -0.21$, $P = 0.14$) between true positive rate and prevalence per box, as illustrated in figure 2.
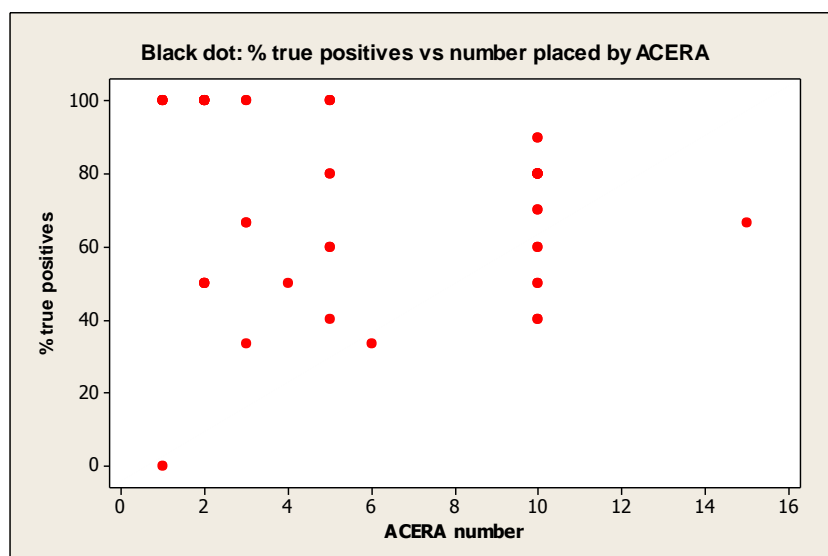

Figure 2. Percentage of true positive versus prevalence of black dots per box in trial 2.

Neither of the above relationships was statistically significant at the 0.05 level when logistic regression was used as a statistical model.

3. False and true detections

If we consider target-pest detection anywhere in the box (yes/no) rather than the number of oranges (table 2), frequencies arise (table 4):

Table 4. Classification table illustrating true and false detections of black dots in boxes.

		ACERA		
		no	yes	Total
AQIS	no	3	1	4
	yes	4	52	56
	total	7	53	60

However, there are too few boxes without black dots to draw any overall conclusions about the false detection rate. There was a weak positive relationship between false detection rate and prevalence per box. The estimated odds increased by 1.13 (95% CI (0.98, 1.30)) for each additional infested orange. Then again, the relationship between true detection rate and prevalence cannot be estimated because inspectors failed to detect only one infested box.

4. Mealy bugs

Of 31 artificially infested oranges, the DAFF inspectors detected 122 (394%), and only five (16%) correctly. However this provides little information in terms of false and true positives, because some of the mealy bugs were certainly present at the start, and can move from one fruit to another or one layer to another.

There was no significant correlation between the number of mealy bugs found by the inspectors and the number placed by the ACERA team (r = –0.20, P = 0.13). However, if we consider detection (yes/no) rather than the number, we get frequencies as shown in table 5:

Table 5. Classification table illustrating true and false detections of mealy bugs in all infested boxes.

		ACERA		
		no	yes	total
AQIS	no	8	7	15
	yes	31	14	45
	total	39	21	60

The association is not significant (X^2 = 1.20, df = 1, P = 0.27), which is consistent with the lack of correlation between the numbers of bugs. This suggests that the infestation with mealy bugs by the ACERA team has been overwhelmed and rendered largely irrelevant by a background infestation of mealy bugs and also by their spontaneous behaviour (movement and/or reproduction). It is unlikely that inspectors could have mistaken something else for mealy bugs because they are very easy to detect and identify.

5. Mites (both species together)

Of 30 inoculated oranges, the DAFF inspectors found 60 (200%), but only one (3%) was found correctly. Again, this provides little information in regard to false and true positives, because some of the mites were probably present before ACERA began its infestations, as occurred for the mealy bugs. Mites, particularly fast crawling species, can move either from one fruit to the next or from one layer to the next. Since the boxes had openings down the base, mites could potentially move from one box to the next.

There was a significant correlation between the number of mites found by the inspectors and the number placed by the ACERA team (r = 0.33, P = 0.01), but if one box with large numbers (ACERA = 2, AQIS = 8) is removed, r drops to 0.22 (P = 0.09). If we consider detection (yes/no) rather than the number of mites, we get frequencies as shown in table 6.

Table 6. Classification table illustrating true and false detections of mites in boxes.

		ACERA		
		No	yes	total
AQIS	no	19	9	28
	yes	21	11	32
	total	40	20	60

The association is not significant (X^2 = 0.03, df = 1, P = 0.86). This suggests once more that the placement of mites by the ACERA team has been overwhelmed and rendered largely irrelevant by a background infestation of mites as well as their spontaneous behaviour (movement and/or reproduction). It is also possible that inspectors could have mistaken mites for other species, which is more likely than for the mealy bugs, as they are smaller and more difficult to spot and identify, even though inspectors were able to differentiate them successfully in the smaller first trial.

6. Blu-Tack beads

Of 6 artificially infested oranges, inspectors found 6, of which 5 were correct. Two were found in one box where one had been placed by the ACERA team, and one was not found. However, the sample size is too small for any statistical analysis.

3.0 Discussion

3.1 Host and pest organisms

Both these trials have demonstrated that oranges provide a reasonable model commodity for artificial infestations. Oranges are relatively cheap, easy to handle and keep well for long periods of time. However, inspectors are usually dealing with class 1 citrus which have been treated for local pests (including diseases) and waxed. Both trials used un-waxed class 2 oranges produced in Queensland, which had been submitted to an IPM program. These were found to host unknown arthropods populations.

Both trials results were obscure by two major factors: Firstly the presence of unknown arthropods populations and secondly the very nature of the pests used in this trial. Both mites and mealy bugs were found to move from the infested host onto neighbouring oranges. Additionally, a range of resident arthropods were found during both inspections (see below), which could be mistaken for, and add to the infestation levels of the species chosen to artificially infest the host oranges (e.g. mites). These further confounded the results. In both trials, resident mites species and mealy bugs could have been already present around the calix of both orange varieties. Navel oranges used in trial 2, often possessed deep and very convoluted navels which provided ideal nesting areas for a range of arthropods. Valencia oranges do not possess a navel and are faster and easier to examine than Navel oranges as the inspectors focus on the calyces. However, navels provide an ideal location for arthropods to conceal themselves and withstand transport and storage. Then again, there is a significant risk of navel's infestation by pre-existent populations of arthropods which could interfere with artificial infestations in this cultivar. To reduce the background infestation levels, fresh, clean or disinfested class 1 Valencia oranges should be used. A small experiment should be conducted to check if waxed Valencia oranges are good hosts compared to un-waxed class 1 Valencia oranges, as the thin layer of wax on the fruit could prevent scales and mealy bugs to settle.

From our second trial, results for invertebrates, both mealy bugs and mites, are inconclusive. Inspectors found infestation levels of 400% for mealy bugs of which only 16% were found on the original orange. For mites, levels of 200% were detected of which only 3% were found on the original oranges. As mites and mealy bugs were seen to move within the infested boxes (see 2.1.1 Additional infested fruit), this is not surprising. There were also a range of arthropods already present, and indeed the inspectors found a significant number of contaminants including scales (including *Chrysomphalus aonidum*, the black scale or Florida scale), thrips, mites (including *Aeroglyphus robustus,* the warty grain mite), mealy bugs (including *Planococcus minor* which could be mistaken for *P. citri*) a silver fish, a coccoid predator, a spider and a honeybee. Furthermore, the oranges were packed in boxes presenting several openings at the base, which means that arthropods could crawl in and out, adding further to error in infestation levels. In future studies of this kind, boxes should be either completely closed or gauze should be placed in front of the openings to limit the movement of arthropods. Such issues will be minimized if the trials use clean (disinfected) class 1 oranges followed by a pre-inspection of the fruit to ensure that the resident arthropods levels are known and at appropriate levels. Oranges might need to be cleaned again if they host high levels of superfluous arthropods.

During our second trial, Navel oranges kept in the cool room were wet when the inspectors opened the boxes. This probably interfered with introduced live pest species, populations of which could have been displaced from one orange to the next with dripping condensation, or one layer of oranges to the next. It might be best to plan each inspection as soon as the consignment arrives at the DAFF facility to avoid storage in cool rooms. This means that for our current design, with three inspectors recruited for inspection, a minimum of 600 to a maximum of 3 000 oranges could be inspected at a time. If more oranges have to be inspected, then individual and recurrent infestation, transport and detection should be organised.

The percentage of correct identifications of surrogate pest species was higher than that of mobile live organisms mostly because a higher percentage than expected moved in all boxes. However, there have been instances when the black dots made with a black marker pen looked very similar to necrotic spots, blemishes or physical damage. Smearing of the black marker pen also occurred in the set of 3 000 oranges kept in the cool room, however if this might have obscured some other necrotic areas or blemishes, they were not mistaken for the original black dots by any of the inspectors. The percentages of positive detections for black dots were 76% (day 1) and 74% (day 2) in the second larger trial. These were mainly due to fungal infections already present before infestation or which had developed before inspection. In the future, a bright red, silver or gold marker pen should be used so as not to confuse the inspectors. The Blu-Tack beads were easily spotted, however, they can fall so dots are probably the best candidate to mimic an inanimate pest. Both mites and mealy bugs moved from one orange to the next, within layers and perhaps, between boxes. This influenced infestation prevalence levels too greatly for any meaningful detection study to be conducted.

In the future, scales could be used as live pests, as they are sessile, can be reared in commercial facilities, transferred onto oranges several days or even weeks in advance, and can be easily numbered and identified by inspectors. They can also be placed randomly on the surface of the oranges independently of the presence of a navel. An additional small-scale experiment should be organised to ascertain that single scales can be successfully transferred and settle on class 1 de-infested oranges.

3.2 Methods for creating artificially contaminated samples and transport

Due to the way each box is artificially infested and inspected, starting by inverting the box to examine the bottom layer first (i.e. orange number 100 becomes orange number 1), boxes of 100 oranges maximum should be used.

Individual oranges should be infested at the rearing facilities several days before infestation of boxes and with known levels of preferably sessile pests. If arthropods have reproduced in the period between infestation of individual fruit and infestation of boxes, the additional arthropods should be removed to keep the prevalence levels constant.

Transport overnight, as soon as the infestation is completed at the rearing facilitates, would void problems associated with cooling and storage. Consignments could arrive early the following morning for the first round of inspection to take place at the DAFF facility.

3.3 Population sizes and prevalence levels

Remarkably, the inspection team found every infested citrus during the first trial involving samples of 600 oranges. Inspectors detected all oranges infested with the two mite species and all oranges infested with the mealy bug species, as well as the surrogate pest species at very low prevalence levels (0.5%). Additionally, they found a resident species of scale, a dead bee and a live spider that were not part of the deliberate infestation and had crawled in either during or after packing and infestation.

The second trial of 6 000 was unusually large, as inspectors regularly examine much smaller samples of 600 items. The inspection had to be organised in two steps over two days, 4 days apart. In the second trial, the Navel oranges hosted higher levels of resident arthropods than in trial 1 and were of lower quality, another unusual occurrence for the inspection team. Even though this second trial was designed to ensure that detectability be tested appropriately and with sufficient power to reveal differences in inspection performance for different types of pests, results were not conclusive mainly because both live pests moved in the infested boxes and background infestation levels were high for

3 out of the 4 pests (i.e. mealy bugs, mites and black dots which could be mistaken for necrotic spots, blemishes or physical damage).

However, the second trial demonstrated that inspectors did remain focussed and engaged during the inspection of a much larger sample and that they still could detect the range of introduced organisms plus the set of resident species. The black dot prevalence level was unusually high in this second trial, with 300 marked fruit out of 6 000. The black dots could be easily mistaken for necrotic spots which could account for some of the mistakes. The percentage of correct positive detections for the two days was 76% in day 1 for 74% in day 2, with a slight drop in positive detection the second day.

To check the validity of each inspection, a leakage survey directly following the inspection should be considered. This would include a second inspection by ACERA members of each previously inspected fruit and any arthropods that might have been set aside for further identification. This would also ascertain the link between the orange number and the identity of the pest. Currently, inspectors tend to remove unknown organisms or organisms that require confirmation from diagnostics, at which stage the link between the numbered orange and the organism is lost. Therefore the inspection as it is currently performed results in the destruction of a percentage of the pests. If a post-inspection is organised, it will only capture the pests which are correctly and confidently identified by the inspectors, and miss information regarding the percentage of "unknown or uncertain" organisms placed in glass vials and sent to diagnostics for identification. In the future, when arthropods are set aside for confirmation or identification by the diagnostics branch, each sample should be placed in a numbered glass vial so that each diagnostics result can be traced back to the original infested orange. As for individual navel dissections, cross examinations should be carried out on the spot before the sample is discarded. In any case, because of the logistics of the inspection protocol, one person per inspector would be needed to record leakage data, before, during and after the inspection.

3.4 Human factors

This design did not allow the measurement of efficacy or fatigue. It concentrated on detectability of different prevalence levels, particularly low levels ranging from 0.1% to 5%. Human factors would need to be investigated as part of a separate experiment. However to reduce the risk of fatigue and learning, inspection of a series of 600 class 1 units, infested at known prevalence levels and in rotation, with a sessile pest such as scales or a bright marker pen dot, could be organised over several weeks or months.

3.5 Recommendations for future experiments

From the results of both trials, future trials and statistical analysis would be greatly facilitated if individual oranges are the units of analysis rather than boxes, i.e. detection can be assessed reliably for each orange. It would therefore be best to choose one set of sessile rather than mobile pests. Scales, for example, could be used. In any case, live pests should be given ample time to settle on individual host oranges before the trial starts so as to reduce the probability of packing oranges with dead scales which could potentially fall off during transport or with additional, newly hatched ones. In addition, an artificial sessile marker (i.e. coloured dots which cannot be mistaken for necrotic areas or disease spots and do not smear) could be used to test perfect detectability, or class 1 oranges with few natural necrotic spots should be employed.

To avoid issues relating to potential background infestations, it would also be best to use clean, disinfested Valencia oranges rather than Navel oranges, which offer convoluted, deep areas that are difficult to inspect before the infestation of individual oranges is organised. These Valencia oranges

should be class 1, and should not be waxed after harvest, but as clean as possible so as to remove as many resident populations of arthropods as possible.

Since inspectors invert the boxes and start examining the bottom layer first, boxes should contain exactly 100 oranges per box and be numbered as described previously.

Because the inspection regime currently operates with examination of 600 units at a time, we should use as many repetitions of 600 as possible, packed in 6 boxes of 100 oranges each.

To ensure that resident population levels are both known and low, a set of small-scale trials should be considered to determine if:

1.   One can use class 1 wax or class 1 un-waxed oranges by infesting them with a known population of arthropods;

2.   Organise a pre-inspection of each orange before artificial infestation to determine both arthropods identity and their levels;

3.   Assess if oranges should be individually disinfested before infestation with the set of selected arthropods if these resident levels are too high (and what too high is);

4.   If scales can be used as a sessile pest and in under what conditions should the infestation be organised (how long do the scales need to settle on the fruit and do they resist cold storage);

5.   If motile pests are to be used again (mealy bugs or mites), additional information on movement needs to be collected: how far do the pests move and under what condition?

6.   For all pests used, information on reproduction rates should also be gathered. Did any of the pests used in this experiment reproduce by the time all inspections were completed?

Following the results out of power/sample size calculation, we propose a set of 3 repetitions at two low prevalence levels, ideally 0.5% and 1%, with a rotation of 3 teams of 3 inspectors (9 inspectors in total) so as to limit the impact of human factors (fatigue, learning etc...).

This would involve the inspection of 324 boxes (or 162 boxes per prevalence level), which represents a total of 32 400 Valencia oranges. The number of artificially infested oranges equals 81 for a 0.5% prevalence level and 162 for a 1% prevalence level. If perfect testing by the inspectors is observed, the lower 95% confidence limit for detection accuracy for 0.5% prevalence would be 96%. An example of the difference in true infestation that could be detected at 80% probability (or power) and a 5% significance level is 99% vs 90%. Alternatively, one could organise 2 repetitions rather than 3, and recruit more teams of inspectors or more inspectors per team.

- **References**

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- **Definitions:**

Prevalence: Prevalence is the percentage of fruit infested or inoculated.

Rate: A quantity or frequency typically measured against some other quantity or frequency.

Appendix 1 Photos
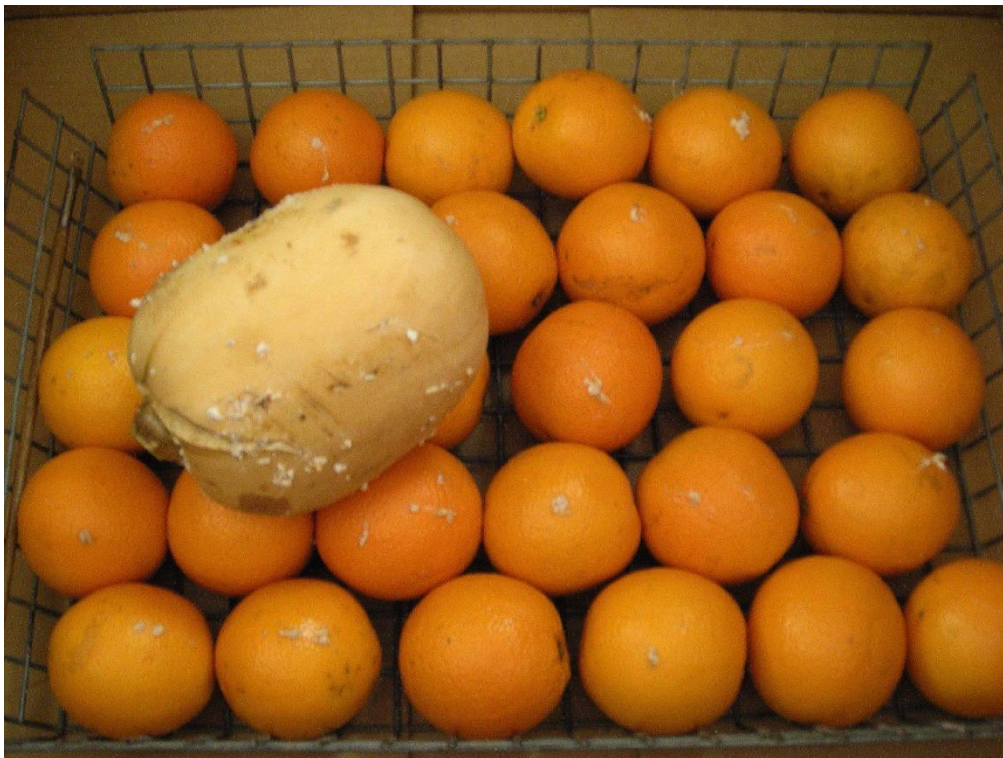
Photo 1: Labelling of individual orange boxes

Photo 2: Labelling of individual oranges

Photo 3: Packing of infested and numbered oranges back in the numbered boxes

Photo 4: Infestation with mealy bugs

Photo 5: Infestation with both species of mites

Photo 6: Sessile markers: Blu-Tack on the calyx

Photo 7: Sessile marker: marker pen dot
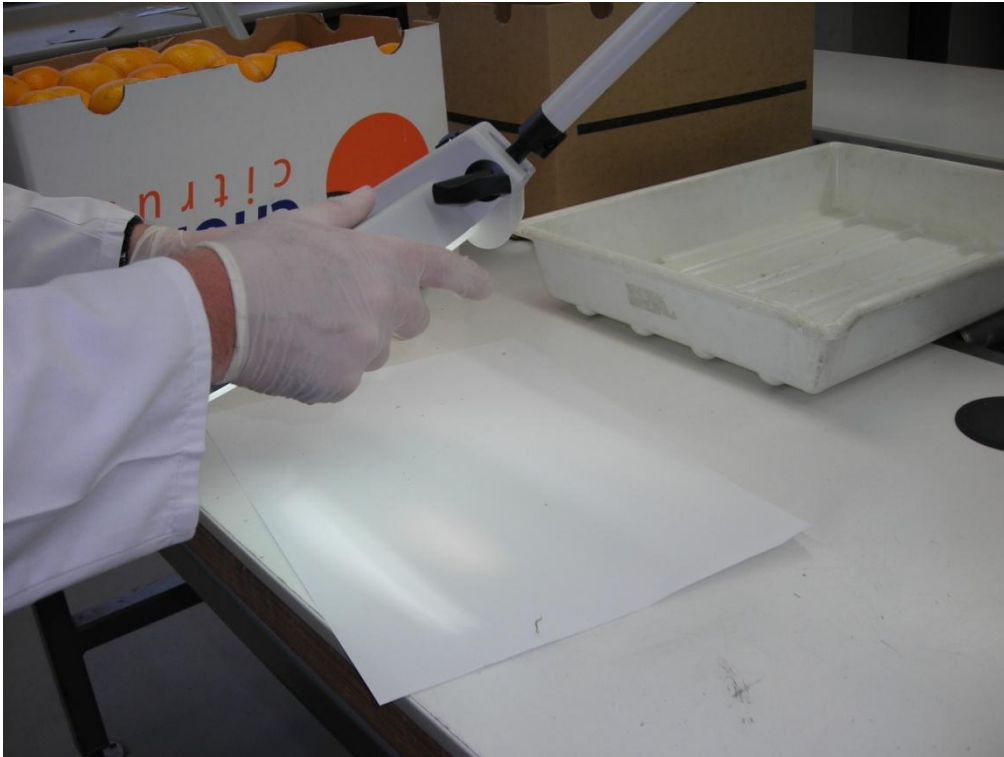

Photo 8: Sessile marker: marker pen dot

Photo 9: Transport to Brisbane and reception by DAFF BA (ex AQIS)

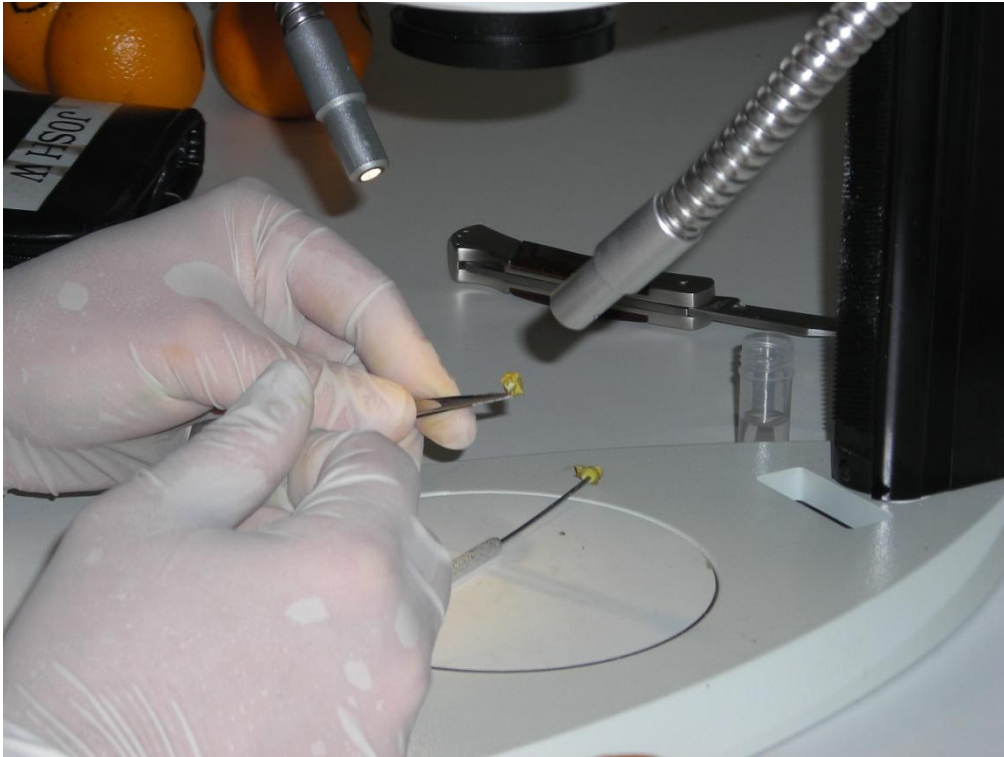**Photo 10 and 11: Inspection of infested boxes by inspectors: inspection of the lids**

Photos 12 and 13: Inspection of calyces

Photos 14 and 15: Inspection of infested citrus

Photos 16: Sampling of suspicious and/or unknown pests for diagnostics

Photos 17: Set of spare infested fruit