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***MBS359 Models for post-border detection
of visibly undetectable exotic plant pests***

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1 *Executive summary*

1.1 *Goal*

This project aimed to assist in defining the specifications for future disease surveys by evaluating a range of sampling strategies through simulation to estimate the probabilities of detection they provide under a range of assumptions regarding incidence and spatial distribution of the unwanted organism, using plum pox potyvirus and brown rot caused by *Monilinia fructigena* as models of diseases where significant aggregation is observed.

1.2 *Context of the project*

The MAF National Plant Pest Reference Laboratory (NPPRL) has been active in carrying out studies to design and estimate the costs of surveillance schemes for high-impact exotic plant pest species (Ganev & Braithewaite 2003, Stephenson et al. 2003). Fundamental research has shown that the pest or disease incidence may be described using different models (e.g. the binomial, hypergeometric, beta-binomial or negative binomial distributions), depending in particular on the degree of aggregation of the affected plants (Madden & Hughes 1999). Plum pox potyvirus and brown rot caused by *Monilinia fructigena* would be devastating to the stone and pome fruit industries, respectively, if they were introduced into New Zealand and became established. These were chosen as trial organisms for testing various sampling schemes for their statistical power to detect incursions when disease incidence, detection efficacy and detection probabilities were varied.

1.3 *Approach*

The possible behaviour of plum pox potyvirus and brown rot (*M. fructigena*) in the event of an incursion in New Zealand was characterised by surveying the published literature. The symptoms, host range, strains, transmission, epidemiology and diagnosis of these organisms were summarised to ensure that disease models to be developed, simulated epidemics, and estimates of detection costs reflected existing knowledge.

Existing equations to describe disease distributions do not consider both aggregation and orchard size, or spatial layout, or spatial distribution of disease other than the extent of aggregation. Therefore, disease epidemics were simulated for a range of parameters including disease incidence (from one tree/orchard to 10%), different pathogen dispersal (using four wind conditions), and different orchard shapes and sizes (square versus long, < 50 trees to 10 ha). Each simulation was run 1000 times. Calculation of index of dispersion and the beta-binomial aggregation statistic (θ) showed that the

extent of disease aggregation in the simulated orchards was appropriate for these two diseases. In addition, six sampling schemes were devised that either randomly sampled a fixed number of trees (50, 60 or 500) or sampled trees in regular patterns, sampling one in four or one in 16 trees, either as individuals or in quadrats. The statistical power of the different sampling schemes to detect an infected orchard was determined by sampling the simulated orchards. The ability of the different sampling schemes to estimate disease incidence was also examined. Resources required to detect and diagnose disease incursions were then estimated using the sampling schemes that were the most powerful for the combination of three disease incidences (one tree/orchard, 0.5% and 5%) and all the various orchard sizes.

1.4 *Outcomes*

For detection of relatively high disease incidence (5% or greater), we showed that the effect of aggregation ($\theta=0.09$) on the power to detect an infected orchard is only slightly reduced (dropping from 90% to 88%) using the sampling scheme described by Richmond et al. (1998) where relatively few trees ($n=50$) are randomly selected. By sampling 60 trees, the confidence of detection would increase to 90%. Therefore, even in the presence of a significant but low level of disease aggregation, the approach of Richmond et al. (1998) will detect a high percentage of infected orchards with 5% or greater disease incidence. The overseas literature on recent plum pox potyvirus outbreaks indicates that orchards with disease incidence as high as 10% occur by the time the disease has been detected (Hughes et al. 2002), therefore this level of sampling ($n=50$) may be appropriate for many diseases (where this degree of aggregation or less is observed) for conducting surveillance to determine whether an incursion has occurred.

However, to delimit the extent of a disease incursion or to ensure disease eradication, reliable detection of infected orchards with much lower disease incidences is required. The effect of disease aggregation is to increase the number of trees that must be selected to maintain a given confidence of detection. For example, if the distribution of trees fits the binomial distribution (i.e. they are randomly distributed), then 511 trees are required to detect an orchard with 0.5% incidence with a 90% confidence of detection and 90% detection efficacy. If modest aggregation is present ($\theta = 0.09$) then 600 trees must be selected to meet the same detection parameters.

To detect infected orchards with aggregated disease at various disease incidences, and at various orchard sizes, the most effective sampling strategies were still those that selected the largest proportion of trees in an orchard. Consequently, for small orchards, strategies that selected a fixed number of trees (50, 60 or 500) were most effective, while for large orchards the sampling strategies that selected the largest proportion of trees were most effective. In the presence of disease aggregation, the sampling scheme that selected one in four trees in quadrats was marginally more effective than a sampling scheme that sampled the same proportion of trees as single trees. In the case where the disease incidence was only one tree/orchard and the orchards were large, the best sampling schemes (selecting one in four trees) would at the best identify 25% of infected orchards.

Using the statistically most powerful sampling schemes for each orchard size, the costs of surveying for plum pox potyvirus by visual observation, ELISA and IC-PCR (immunocapture-PCR) were estimated; and the costs of surveying for brown rot (*M. fructigena*) of pome fruits by visual observation were estimated. For example, sampling one 10 ha orchard to detect a 0.5% disease incidence of plum pox potyvirus with a probability of detection nearing 100% of infected orchards requires 86 hours of surveying, either 5.5 days (ELISA, based on 300 tests/day) or 13.8 days (IC-PCR, based on 120 tests/day) of laboratory diagnostics, and either approximately \$2500 or \$5000 in consumable reagents.

1.5 Summary

This project aimed to evaluate sampling methods through simulation to estimate the probabilities of detecting either plum pox potyvirus or brown rot caused by *M. fructigena* under a range of assumptions regarding incidence and spatial distribution of the unwanted organism in order to assist in defining the specifications for future disease surveys.

Following literature surveys to identify key characteristics of the pathogens and their epidemics, diseased orchards with the relevant spatial distributions were generated by simulation. In particular, the aim was to achieve a mean aggregation of disease consistent with the aggregation that has been observed in overseas studies. Existing equations to describe disease distributions do not consider both aggregation and orchard size, or spatial layout, or spatial distribution of disease other than the extent of aggregation. Therefore, diseased orchards were simulated for a range of parameters including disease incidence (from one tree/orchard to 10%), different pathogen dispersal (using four wind conditions), and different orchard shapes and sizes (square versus long, < 50 trees to 10 ha). The power of different sampling schemes to detect infected orchards was determined by sampling the simulated orchards.

The most appropriate sampling schemes to detect infected orchards with aggregated disease at various disease incidences, and at various orchard sizes, were summarised. The most effective sampling strategies are still those that select the largest proportion of trees in an orchard, therefore different sampling schemes are appropriate for small and large orchards. Using the statistically most powerful sampling schemes for each orchard size, estimates were presented of the costs of surveying for plum pox potyvirus by visual observation, ELISA and IC-PCR (immunocapture-PCR) and of the costs of surveying for brown rot (*M. fructigena*) of pome fruits by visual observation.

2 *Introduction: aims and background*

2.1 *Plum pox virus and brown rot characterisation*

2.1.1 *Plum pox potyvirus*

Plum pox potyvirus (PPV) causes arguably the most significant disease of stone fruit – plum pox disease, also called Sharka disease. The disease was first reported in Eastern Europe and has spread to many stone fruit growing regions of the world including Europe, the Mediterranean and Middle East, India, and recently to Chile. An outbreak of PPV was recently discovered in the eastern USA (Levy et al. 2000b) and adjacent regions of Canada (Thompson et al. 2001). New Zealand, Australia and parts of the USA and Canada are free from plum pox disease.

- *Symptoms.* Symptoms of PPV can appear on fruit, flowers and leaves. Symptoms develop slowly after infection, and may not appear until 2-3 months after aphid inoculation of indicator *Prunus* plants (Quiot et al. 1995). Critically, PPV infection is often characterised by a long latent period (1 or more years) during which no symptoms are apparent. In addition, symptoms show irregular distribution within plants, and may be limited to a single quadrant of a tree, or may appear only on basal or middle sections of growing shoots. The symptoms observed on leaves can include chlorotic rings and bands, vein clearing and general mottling. In some plum cultivars, leaf symptoms can progress to "necrotic shothole symptoms" (Gottwald et al. 1995). Symptoms on leaves are most easily observed in spring, and the leaves showing symptoms are most often associated with rapidly growing shoots (Bodin Ferri et al. 2002). Bodin Ferri et al. (2002) also suggested that the pattern of appearance of symptoms reflects a high virus titre in the growing shoot tissue. The appearance and severity of symptoms, and the effect of infection on plant growth, can differ markedly according to viral strain, cultivar and *Prunus* species (cited in Bodin Ferri et al. 2002). On fruit, symptoms can vary from moderate to severe, and might include the appearance of chlorotic rings with deformations, depressions in the fruit surface, and corky flesh. The result of PPV infection can be severely decreased market quality of fruit and for New Zealand would doubtless threaten access to export markets.
- *Host range.* The host range for PPV is *Prunus* spp, including peach, plum, nectarine, and apricots. Almonds can be infected experimentally by grafting, and cherries can be infected by recently discovered cherry strains of PPV. PPV also infects other genera in plant families including at least *Pisum*, *Melilotus*, *Campanula* and *Lamium* (Levy et al. 2000a). PPV can be propagated in herbaceous hosts such as *Nicotiana* spp, *Pisum sativum* and *Chenopodium foetidum*.
- *Viral strains.* Isolates of PPV can be grouped into 4 strains, PPV-D, PPV-M, PPV-EA, and PPV-C. PPV-D and PPV-M are the predominant strain groupings but have different patterns of distribution in stone fruit orchards. PPV-D is more often found in apricots and plums, while PPV-M is more often found on peaches (Quiot et al. 1995; Dallot et al. 2001).

PPV-M is considered to be faster spreading and more able to establish systemic infections than the D isolate. The EA (El-Amar) isolate was detected on apricot (Wetzel et al. 1991a), and the C isolates are adapted to sweet and sour cherries (Nemchinov & Hadidi 1996). Characterisation of viral strains, including sequence analysis of PPV genomic RNA sequences, has confirmed the existence of recombinant strains with intermediate properties (Revers et al. 1996; Glasa et al. 2002). In the event of a PPV incursion in New Zealand, it will be important to identify the isolate so that its epidemiological behaviour might be predicted. However, Dallot et al. (1998) identified a PPV-D isolate that produced disease epidemics on peach trees.

- *Transmission.* PPV is spread by graft transmission and by aphids. As is the case with other aphid-transmissible members of the potyvirus group, PPV is transmitted non-persistently by aphids. Therefore, aphids are only able to spread the virus for a short time after acquisition, usually less than 1 hour (Levy et al. 2000a). In experiments, more than 20 species of aphids have been shown to transmit PPV, although only about six species are considered to be important vectors of the disease (Levy et al. 2000a). Aphids can acquire virus from infected leaves and fruit at all stages, including from over-ripe fruits that might be found on fruit dumps (Labonne & Quiot 2001). PPV can be mechanically transmitted to some herbaceous hosts (e.g. *Pisum sativum*; Quiot et al. 1995) or from a herbaceous host (*Nicotiana benthamiana*) to *Prunus* (Dallot et al. 2001). Maintenance of the virus in herbaceous hosts may reduce its infectivity (Dallot et al. 2001). Whether PPV is seed transmissible or not is contentious. Early publications report seed transmission of PPV, while more recent publications show that PPV is detectable by ELISA and/or IC-PCR in seed coats and cotyledons, but not in seedlings from infected seeds (Pasquini et al. 2000, and discussion therein).
- *Epidemiology.* The spatial spread of plum pox potyvirus in *Prunus* orchards has been analysed and results are characterised by two papers (Gottwald et al. 1995; Dallot et al. 2003). Gottwald et al. (1995) examined PPV spread in peach and apricot orchards over 4 years. Infected trees were determined using double antibody sandwich-ELISA. The strain (D or M) of plum pox potyvirus present in these orchards was not specified. These authors found that the orientation of the localised systemic infections found in particular scaffold branches was conserved over years, confirming results from other studies on uneven distribution of the virus in infected trees. In the orchards included in this study, disease incidence ranged from 5% to 95%. At lower disease incidences, significant clustering of the disease was not observed (based on the index of dispersion statistic from the beta-binomial distribution). At higher disease incidences, evidence of clustering was observed using this statistic. The spatial pattern of plum pox potyvirus spread found in the orchards suggested to these authors that movement of aphid vectors was preferentially to trees several spaces away rather than to adjacent trees. In a study carried out in peach orchards in southern France, Dallot et al. (2003) examined the spread of the aggressive M strain. In this research, infected trees were determined by observation of visual symptoms by trained teams, with the use of field-based immunoprinting

to confirm scorings. Trees determined to be PPV infected were removed and destroyed each year, as is required in France for the M strain. The disease incidences observed in 18 orchards ranged from 1.9% to 35% of trees. Using two measures of aggregation, the beta-binomial parameter θ and the dispersion index D , these authors also found significant aggregation of disease, particularly at higher disease incidences. Using SADIE (spatial analysis by distance indices, www.rothamsted.bbsrc.ac.uk/pie/sadie), a different measure of aggregation, the authors of this study also found evidence for higher order aggregation, i.e. that non-adjacent clusters of diseased trees could be related. After examining the aggregation patterns in these orchards, Dallot et al. (2003) concluded that aphid transmission of virus to neighbouring trees was not systematic.

- *Diagnosis.* Plum pox virus is the only potyvirus that infects *Prunus* spp., therefore detection methods do not need to distinguish PPV from other related pathogens. Accurate diagnosis of PPV-infected trees is hampered by latency as well as by the uneven distribution and low concentration of the virus in infected trees (Quiot et al. 1995; Bodin et al. 2003). Tests to diagnose PPV infection of host plants include biological indexing, serological testing using ELISA-based methods, and molecular diagnosis using RT-PCR (reverse transcription-PCR)-based methods. The European Plant Protection Organisation (EPPO) has published an approved Standard for PPV diagnosis that incorporates all three methods (EPPO 2004). References to research underlying PPV diagnosis are included therein.

Elsewhere in the literature, data are presented regarding the relative efficiency and sensitivity of ELISA and RT-PCR methods (Adams et al. 1999), or of ELISA methods alone (Hughes et al. 2002). Adams et al. (1999) conclude that immunocapture-PCR (IC-PCR) is about 1000X more sensitive than ELISA. They also show that with ELISA the proportion of positive ELISAs after assaying a single leaf from an infected shoot ranged from 37-97%, but that this increased to 74-100% after taking three samples per shoot (one basal, one middle, one apical). Adams et al. (1999) advised against pooling samples being analysed by ELISA because of difficulties in obtaining significant absorbance values. Adams et al. (1999) also examined detection of PPV in dormant trees by sampling bark or roots. Bark samples gave a positive result (either by ELISA (22/32), or IC-PCR (25/32)) where sampling the leaves on the same stem had given negative results by either method. Root samples were also shown to be valuable for detecting PPV infection in dormant trees. Testing of bark or root samples might be particularly valuable if nursery stock need to be examined for PPV infection.

2.1.2 *Brown rot (Monilinia fructigena)*

Apple brown rot is caused by *Monilinia fructigena* Honey. The host range of *M. fructigena* is fruit trees in the Rosaceae, but it is most characteristically found on apples and pears. Other species of *Monilinia* that cause brown rot of fruit are *M. laxa* and *M. fructicola*. *M. fructigena* is considered to be an "Old World" pathogen, and is prevalent in Europe and Asia. Japanese isolates of *M. fructigena* are considerably different from to European isolates,

and so it has been proposed that Japanese isolates constitute a fourth species, *M. polystroma* van Leeuwen (van Leeuwen et al. 2002). *M. fructigena* does not occur in New Zealand, but *M. fructicola* and *M. laxa* are present here. *M. fructigena* is a quarantine pest in New Zealand, Australia and the USA.

- *Symptoms.* Symptoms of *M. fructigena* infection include blossom blight, twig and branch cankers, and brown rot of fruit. Twig cankers are often small and difficult to detect, but cankers can girdle branches and cause distal portions of branches to collapse. Brown rot of fruit is most often associated with wound sites or with areas where fruit touch. Brown rot begins as a small firm brown lesion and rapidly expands to encompass the entire fruit. *M. fructigena* rots are firm and dry to the touch, and do not squash easily. Conidia appear on the surface of the brown rot lesion.
- *Life cycle and transmission.* New infections in the spring are established from conidia that have over-wintered on mummified fruit and twig and branch cankers. Conidia develop in early spring on blighted blossoms, infected branches and twigs, and on mummified fruit. Spore germination and establishment of infection is stimulated by wet conditions and warm temperatures. Wind and rain disseminate spores, and it is also likely that wounding agents such as insects or birds also transmit spores. The injuries that facilitate development of *M. fructigena* rot may have abiotic causes (hail, wind, rubbing, cracking due to sunburn) or biotic causes (birds, insects, apple scab). Except in the case of fruit touching, *M. fructigena* does not infect uninjured fruit. In addition to being associated with wounding, disease development in orchards is gradual and increases markedly as fruit begins to mature (Xu & Robinson 2000; Xu et al. 2001). *M. fructigena* can also cause rots during storage, but infections will have been established before harvest, and will spread as a result of fruit touching or additional wounding during handling. Transmission between orchards or internationally is likely occur via infected cuttings or nursery stock, or via infected fruit. Transmission on infected fruit in commercial shipments may be less important because wounded fruit or fruit showing rot is removed during grading and packing.
- *Diagnosis.* Identification of *M. fructigena* on rotting pome fruit will first involve biological characterisation by culturing the fungus, followed by application of a diagnostic PCR assay that is capable of distinguishing *M. fructigena* from *M. fructicola* and *M. laxa*. Cultural and morphological characteristics for these three species show quantitative variation, with overlap, so are not completely adequate for species diagnosis. In particular, *M. fructigena* may be misidentified as *M. fructicola* (van Leeuwen & van Kesteren 1998). Several PCR-based diagnosis methods have been developed for identifying *M. fructicola*, and two methods in particular (Hughes et al. 2000; Iloos & Frey 2000) are recommended for distinguishing *M. fructicola* from *M. fructigena* and *M. laxa* (EPPO 2003). These PCR methods produce a positive amplification for *M. fructicola*, but no bands are amplified from *M. fructigena* or *M. laxa*. The European Standard focuses on detecting *M. fructicola* since it is a regulated pest in Europe. More recently, Côté et al. (2004) have published a multiplex PCR method that produces bands of unique length for each of the three species plus *M. polystroma*. PCR amplifications are carried out using a

common reverse primer and three species-specific forward primers. All the PCR-based diagnostic methods stress the importance of using a positive control PCR assay, such as one based on amplification of ITS sequences (Hughes et al. 2000; EPPO 2003), especially to ensure that negative PCR amplification results are not due to template preparations being unsuitable for PCR.

- *Epidemiology.* Only a few studies have examined the epidemiology of *M. fructigena*. Xu & Robinson (2000) studied the factors leading to fruit infection, in particular the effects of wounding, wound age and fruit maturity. Their key findings were that unwounded fruit do not get *M. fructigena* rot, wound age affects the success of infection (recent wounds become infected more readily than old wounds), fruit maturity affects the incidence of rots (mature fruit are more susceptible), and the length of the incubation period after inoculation with conidia depends on wound age and temperature. The spatio-temporal dynamics of *M. fructigena* disease development were examined in two papers (van Leeuwen et al. 2000; Xu et al. 2001). van Leeuwen et al. (2000) found that disease incidence increased relatively constantly throughout the growing season. Clustering of diseased fruits was observed and infected trees tended to contain a greater number of infected fruits than would be expected at random. Infected trees were also clustered. The patterns of between-tree clustering were different in the 2 years of the study. In the first year, positive correlation coefficients for clustering were found for trees that were two, three or four trees down the row. In the second year, however, adjacent trees showed the most significant clustering. Xu et al. (2001) also found clustering of diseased fruit. In particular, aggregation was clearly observed for primary-rotted fruit where the disease incidence was above 0.5%, meaning that significantly more trees had no rotten fruits than would be expected from a binomial distribution. Using the beta-binomial distribution, estimates of θ also indicated aggregation, particularly as disease incidence increased. Xu et al. (2001) concluded that the spatial characteristics of brown rot were probably determined more by the activities of the wounding agents than by dispersal characteristics of conidia. They reasoned that wound is essential for rot to develop. They also contended that dispersal of conidia by rain-splash may be important because infected fruits were observed to be consistently and significantly aggregated within trees compared with between trees.

3 Simulation of disease epidemics

3.1 Calculating the number of trees to sample

3.1.1 MAF sampling scheme

With the MAF sampling scheme described by Richmond et al. (1998), the number of samples to collect (n) is calculated from:

p_3 , the confidence of detecting a pest = 0.9 (90%)

p_4 , the proportion of plants infested = 0.05 (5%)

p_5 , the efficacy of detecting an infected host = 0.9 (90%)

More recently, Ganev & Braithwaite (2003) have calculated n from $p_3 = 0.95$ (95% confidence), $p_4 = 0.05$ (5% incidence) and $p_5 = 0.45$ (45% detection efficiency).

These sets of parameters are used in the following equation, which assumes infected individuals follow a binomial distribution, that is, that they are not aggregated, and the probability of one tree being infected has no bearing on whether an adjacent tree will be infected. In addition, it effectively assumes an infinite (or very large) orchard size, and takes no account of the spatial distribution of the trees.

$$n = \frac{\ln(1-p_3)}{\ln(1-p_4p_5)}$$

With the above values for p_3 , p_4 and p_5 , this gives $n = 50$ and $n = 132$, respectively. These are the numbers of tests or observations to take from an orchard in order to have a 90% chance (Richmond et al. 1998) or a 95% chance (Ganev & Braithwaite 2003) of getting at least one infected tree if there are 5% or more of trees infected in the orchard. If the infection rate (p_4) is lower than 5%, then the chance of getting at least one infected tree in the sample will be below 90% or 95%. This is explored below.

In general, sample size calculations are done with the assumption that the orchard contains an infinite number of trees. For the very largest orchards, this assumption is reasonable, but clearly, this is not so for the smaller orchards. Where there is no aggregation (the assumption for the current scheme) there are alternative calculations that do take account of orchard size (using the hypergeometric distribution, e.g. see Venette et al. (2002)):

$$p_3 = 1 - \frac{\binom{N(1-p_4)}{n}}{\binom{N}{n}}$$

where N is the orchard size, and :

$$\binom{N}{n} = \frac{N!}{n!(N-n)!} \text{ and } N! = N \times (N-1) \times (N-2) \times \dots \times 1 \text{ etc}$$

When p_5 , the efficacy of detecting an infected host, is less than 1, there is no direct modification of this equation (see below). This calculation can be solved iteratively to estimate n for any given values of N , p_3 and p_4 . Ganev & Braithwaite (2003) mention the use of the hypergeometric distribution for small orchards.

The major effect of including total orchard size is that the expected confidence for detecting a pest (p_3) increases as the orchard size decreases, for a given number of samples (n) taken. That is, if 50 (or 132) trees are sampled, the likelihood of the sample including at least one infected tree is greater for the smallest orchards than for larger orchards. In the extreme, if there are 50 or fewer trees in the orchard and there is some infection, the sample is certain to contain the infected tree, whereas in an extremely large orchard, the chances reduce to finding at least one infected tree in only 90% of cases. In contrast, if a constant proportion of trees is sampled (as in schemes Q, I2 and I4 below), then for a given infection level, the chances of getting at least one infected tree in the sample increase as the orchard size increases. If there is a single tree infected, then the chances of the sample containing this tree reduce as the orchard size increases for a fixed number of trees sampled in the orchard, but remain constant for a fixed proportion of trees sampled. These effects are illustrated in Figure 1.

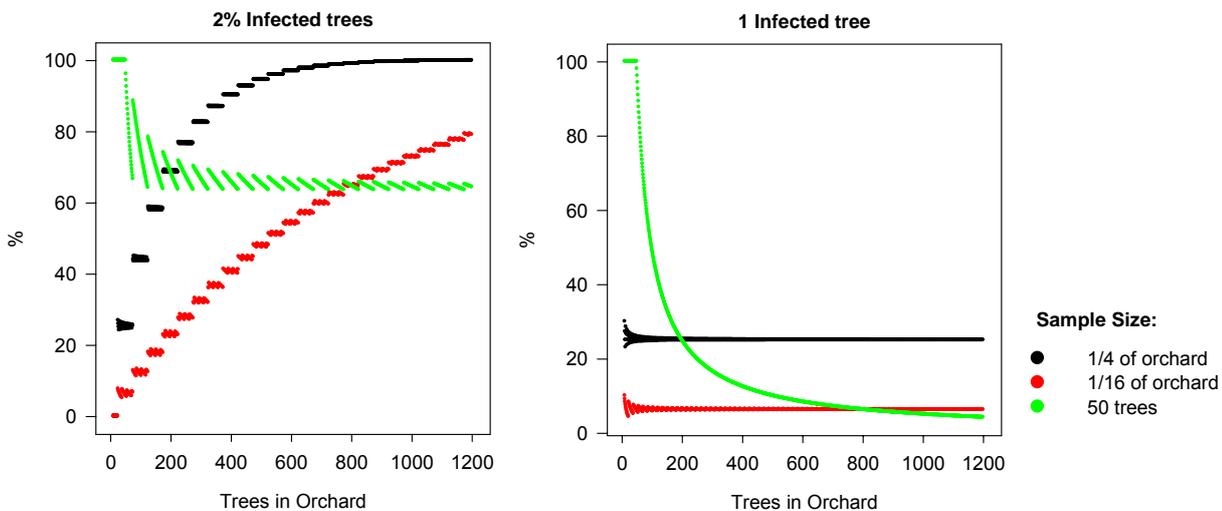


Figure 1: Theoretical % chance of the sample containing at least one infected tree for different for numbers of trees selected, for two infection levels. The groups of points (staggering) are due to rounding to whole numbers of trees. No adjustment has been made for detection efficiency (p_5) less than 1.

3.2 Adjusting for aggregation

For plum pox and brown rot (*M. fructigena*), aggregation has been observed. This means that a tree near an infected tree is more likely to be infected than a tree near an uninfected tree. The beta-binomial distribution can be used to

account for this aggregation (Madden & Hughes 1999). This distribution has an aggregation parameter, θ , which for plum pox was found from sampling orchards in the USA (Hughes et al. 2002) to have an average value of around 0.09. If $\theta = 0$, then there is no aggregation, and the equation reduces to the equation above for binomial sampling. Where there is aggregation, it is more usual to sample groups of trees, rather than single trees, and common practice in the USA is to sample groups of four adjacent trees. An equation to estimate the number of groups (m) of size g where there is aggregation is given by Madden & Hughes (1999):

$$m \approx \frac{-\theta \left[\ln(1-p_3) \right]}{p_4 \left[\ln(1+g\theta) \right]}$$

This equation again takes no account of orchard size. Using the Richmond et al (1998) parameters and assuming that detection efficiency can be accounted for by replacing p_4 in the equation by $p_4 \cdot p_5$ (which may not be appropriate, see below), this equation gives 15 groups of $g = 4$ trees, or 60 trees in total. Generally, where there is aggregation, a larger sample will be needed to reach the same levels of confidence compared with cases in which there is no aggregation, and the sample size will increase as the level of aggregation increases. This second sample size equation can be used to estimate the confidence in detecting a pest (p_3) when there is aggregation and the current scheme of 50 random trees is used, but trees are sampled in groups of four. That is, if $m = 50$, $g = 4$, $\theta = 0.09$, $p_4 = 0.9$, $p_5 = 0.05$, then the equation can be rearranged to give $p_3 = 0.88$. This is a reduction of 2% in power – not that substantial for this level of infection, but it does mean that another 2% of orchards infected at the 5% level are likely to be classified incorrectly as being free from infection. If single trees are sampled rather than groups of trees (as in the current scheme), then aggregation has no effect on the power, since the spatial distribution of the trees has no influence on which particular trees are sampled. However in practice, it is usually very difficult to achieve a truly random sample, particularly in the field: consequently, the chosen sample is usually in some way affected by the spatial layout of the trees within the orchard. Hence, if there is aggregation, the power (p_3) will be affected, since the sample is not independent of the location of the trees.

3.3 *Detecting a level of infection lower than 5%*

From the purposes of eradication rather than national surveying, 5% infection is probably too high a level to tolerate, and a more realistic level might be 0.5% infection or less. If the percentage of plants infected is decreased to 0.5% ($p_4 = 0.005$, again with $p_5 = 0.9$), then with the binomial equation, the number of trees that need to be selected increases to 511 to have a 90% chance of getting at least one infected tree in the sample. With aggregation of $\theta = 0.09$, 150 groups of four trees (= 600 trees) would need to be selected. If the binomial sampling scheme is used (511), but there is aggregation and trees are sampled in groups of four, then the confidence in detecting infection (p_3) when aggregation is present is slightly reduced (to $p_3 = 0.89$), as above. If the aggregation is increased slightly, e.g. to $\theta = 0.15$, then for $p_4 = 0.05$ (5%

infection), the number of groups required would be 16.3, which rounds to 17, and thus 68 trees would need to be selected.

For even lower levels of infection, and increased aggregation, then the number of trees to select increases again, as can be seen in Figure 2.

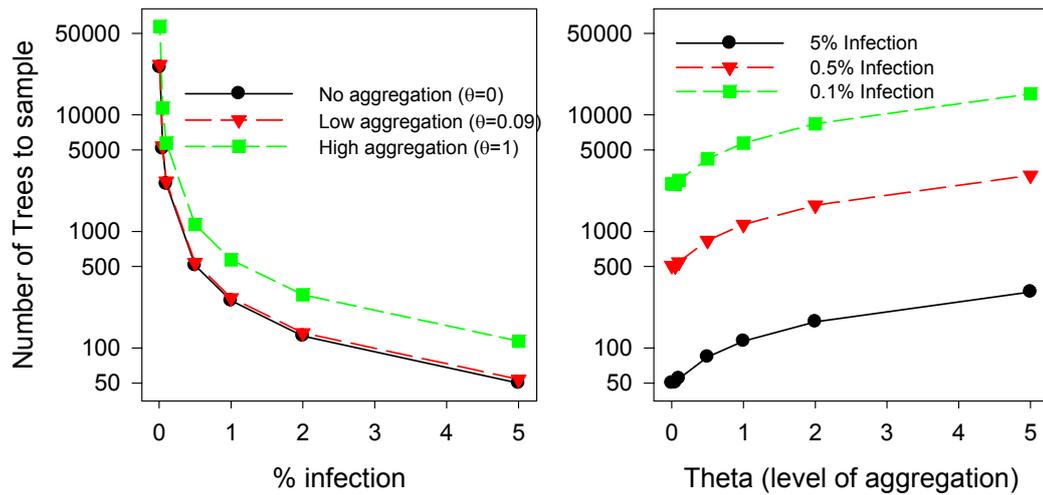


Figure 2: Numbers of trees to select, for 90% chance of there being at least one infected tree in the sample, for various levels of infection and aggregation. This assumes a detection efficiency $p_5 = 0.9$.

3.4 Conclusions

These equations show:

1. To have a reasonable level of confidence ($p_3 = 0.9$) of detecting the pathogen, the number of trees that needs to be selected increases dramatically with a decrease in the infection level p_4 required to be detected.
2. If the infection is aggregated and sampling of groups of trees is done, or the sample is not completely random, then the number of individual trees required also increases as the level of aggregation increases. Even a low level of aggregation ($\theta = 0.09$) increases the number of selected trees that are needed by 20%.
3. However, although more individual trees need to be selected, with a low level of aggregation, the increase in confidence is reasonably low. The reduction in confidence (“power”) through assuming a random distribution when there is aggregation increases as aggregation increases. For the relatively high aggregation of $\theta = 1$ (which is rarely found; Madden & Hughes (1999)), p_3 is below 80%.
4. For a given sampling scheme, the size of the orchard affects the chances of the sample containing at least one infected tree (p_3).

There is no easily available equation for the aggregated case that includes an adjustment for orchard size. Since orchard size affects p_3 where there is no

aggregation, it can be concluded that similar effects would be observed where there is aggregation.

The primary effect of ignoring aggregation in sampling is in reduced efficiency for estimating the level of incidence, rather than in an increase in the chances of getting no infected trees in the sample. The confidence in this estimate is reduced (i.e. the 95% confidence interval is larger).

Given that there are no easily available equations to estimate sample sizes (n) which adjust for both aggregation and orchard size, and given that the equations above also make no adjustment for the spatial layout of the orchard and spatial distribution of infected trees (other than a level of aggregation), equations cannot be used to properly explore various sampling schemes for plum pox or brown rot caused by *M. fructigena*. Instead, these can be studied through simulating orchards, and sampling these with various sampling schemes.

3.5 *Distribution of plum pox and brown rot (M. fructigena) in orchards*

The literature for plum pox indicates that the distribution of infected trees within an orchard is not completely random (Gottwald et al. 1995; Hughes et al. 2002; Dallot et al. 2003), and that there is some aggregation or clumping between infected trees. In addition, the distribution of the virus within an infected tree is far from uniform (Bodin Ferri et al. 2002). The data given by Gottwald et al. (1995) indicate that incidence increases by between two and four-fold between years on average: thus, it is reasonable to assume the trees in one year are infection sources for the disease found in the following year, and these are about $\frac{1}{2}$ to $\frac{1}{4}$ of the infected trees from the second year in number.

The plum pox virus is principally spread by aphids within an orchard, once infected trees have been introduced. Nemecek et al. (1993) found that the distance and direction of aphid flights within a potato crop was strongly correlated with wind direction. They found an approximate mean flight distance of 13 m, with nearly half of the flights below 5 m. Flights were examined for wind speeds below 2.5 m/s, since aphids were not thought to fly in higher wind speeds.

The flights of aphids are quite similar to the distribution of spores by the wind (Dixon 1998), so spread of spores can be used to give a reasonable model for the spread of infection. In a series of papers, Xu and Ridout (1998; 2000; 2001) simulated the spread of infection by spores, including the effect of a prevailing wind. Their model was used with the information from Nemecek et al. (1993) to generate the data in a two-step process, as outlined below.

Literature on the spatial distribution of brown rot (*M. fructigena*) on apple and pear is much less common. Information in Xu et al. (2001) suggests that the spatial characteristics of brown rot disease are sufficiently similar to those for plum pox that the same set of simulations can be used to explore sampling schemes for both diseases. Brown rot is generally spread by wounding agents and spores on the wind, with infection mainly through pre-existing wounds on the tree. Aggregation of infected trees ($0.05 < \theta < 0.321$; Xu et al.

(2001)) appears to be at similar levels to that found in plum pox (e.g. $0.00 < \theta < 0.95$; Dallot et al. (2003)). Therefore, in the absence of more detailed information, the same simulations were used for both diseases.

3.6 Orchard size and shape

Within New Zealand, the number of trees in *Prunus* orchards varies considerably, as does the spacing of trees. From papers within New Zealand journals, spacings of 5×2.5 m, 6×3 m, 5×2 m, and 2×3.2 m were found. Similarly, the size of apple orchards also varies considerably, with several over 10 ha in size, and a median size of around 5 ha. Maps of orchard growing areas show orchards as small as 0.1 ha, and orchards in hobby farms are smaller still. Therefore, the simulations used six orchard sizes, from about 0.05 ha to around 10 ha. Orchard shape also varies considerably. Two basic shapes were used in the simulations for each size, 'square' and 'long', with 'square' orchards being approximately as wide in meters as they were long, and long orchards being about 5 times as long in meters as they were wide. A spacing of 5 m between rows and 3 m between trees within rows was chosen for all simulations. Details of the 12 orchard sizes are given in Table 1.

Table 1: Details of orchards used in simulations.

Size	Shape	Area (ha)	Number of rows	Trees per row	Trees in orchard	Meters	
						Across rows	Row length
Very small	Square	0.07	5	9	45	25	27
	Long	0.07	2	22	44	10	66
Small	Square	0.12	7	11	77	35	33
	Long	0.11	3	25	125	15	75
Medium	Square	1.19	22	36	792	110	108
	Long	1.22	10	81	810	50	243
Large	Square	2.99	35	57	1995	175	171
	Long	2.99	15	133	1995	75	399
Very large	Square	5.06	45	75	3375	225	225
	Long	5.07	20	169	3380	100	507
Huge	Square	9.92	63	105	6615	315	315
	Long	10.00	28	238	6664	140	714

4 *Simulations*

Programs for the simulations, sampling and summary of results were written in GenStat (GenStat Committee 2005).

Up to six incidence levels (one infected tree, 0.5%, 1%, 2%, 5%, and 10% of trees infected) were used for each of the 12 orchards. For the smaller orchards, the lower levels of incidence were either equal to one infected tree, or below one infected tree, so these were not done. Simulations were carried out in several steps: about 1/4 of the required number of infected trees were randomly selected as start trees. For each of these, aphid (or spore) flights were generated randomly, using the method of Xu & Ridout (2001). Random wind events (speeds and angles) were generated and then used to create the aphid (or spore) flights. In each simulation, the randomly generated distances travelled by aphids (spores) were adjusted to have a mean median of around 5 m. The simulation model allowed for the angle of the prevailing wind to be included, so four angles were used: parallel to the rows (0°), at 45 degrees and 90 degrees to the rows, and no prevailing wind. For each start tree, the required number of aphid flights that fell within the orchard were selected, and the trees closest to these chosen to be infected.

4.1 *Detail of steps in the simulation - method*

Choose:

infection level (proportion of trees infected): incidence

number of rows in orchard: nrows

number of columns in orchard: ncolumns

If the infection level was one tree, then one tree from the orchard was randomly selected. Otherwise, these steps were used:

Calculate:

Total number of trees **nt**=nrows x ncolumns

Number of trees require to be infected as **ninf**=round(nt*incidence/100)

Number of start trees as **nstart**=round(ninf/4.2)

Calculate remaining trees that need to be generated as **nrem**=nt-nstart

Calculate number of trees per start tree that need to be generated: rather than an equal number of trees, this was chosen as an arithmetically increasing series;

proportion for each start tree = (1,2...nstart)/sum(1...nstart)

These proportions were then multiplied by **nrem**, and rounded to the nearest whole number.

(In a few cases, this produces 1 less than the required number, so an extra tree was added to one of the start trees.)

Generate:

For each of the 1000 simulations needed for each combination of orchard shape and size by incidence and by wind angle,

1. Randomly select starter trees
2. Generate distance and angles travelled for each start tree.

As in Xu & Ridout (1998; 2000; 2001), wind speed and direction were created as X and Y distances, generated as random samples from a normal distribution. For the three sets with a prevailing wind, the mean and standard deviation (σ^2) used for X and Y were 1,1 and 0,0.6 respectively, to give an elliptical distribution of winds. For the sets with no prevailing wind, the mean and σ^2 used for X and Y were 0,0 and 0.8,0.8 respectively, giving a symmetrical distribution around the starter trees. The generated X and Y were converted into angles and speeds using standard trigonometry. For the three sets with a prevailing wind, angles were adjusted by 0, 45 or 90 degrees to give the required prevailing wind angle. For all sets, the wind speeds were adjusted so that the final aphid travel distance had the required median and mean (with the same adjustment used for all sets and simulations). Wind speeds were converted into distances that aphids travelled using the half-cauchy distribution with two parameters (Xu & Ridout 1998; 2000; 2001); the adjusted speed as a median, and a number randomly generated from a [0,1] uniform distribution.

For each start tree, the number of aphid (spore) flights generated was the number of final infected trees required for that starter tree x 15, except for the very small and small orchards, when it was x 30. These multiples were selected after experimentation, to ensure that most simulations generated sufficient infected trees within the orchard. From the flights generated, a random selection of those falling within the orchard was made, up to the required number for each start tree, and the closest tree to each flight was selected to be the one infected. Sometimes, a simulation did not generate enough trees falling within the orchard, so that simulation was abandoned, and another one done to replace it.

Save:

For each simulation, the position of start trees, the position of infected trees, and the start tree that was associated with each infected tree were saved. The mean and median angle and distance of the aphid flights were calculated and saved.

4.2 *Assessment and summary of simulations*

For each simulated data set, other than those with only one tree, two parameters were estimated to assess whether the simulated data resembled those found in three plum pox survey papers (Gottwald et al. 1995; Hughes et al. 2002; Dallot et al. 2003) and the one brown rot paper (Xu et al. 2001). For each simulated data-set, the orchard was divided into quadrats of 4 x 4 trees, and the number of infected trees within each counted. For most orchard sizes, there were quadrats at the edges of the orchard which contained fewer than four trees, since the number of rows or number of trees per row was not a multiple of four (illustrated in Figure 3 for the very small

square orchard). Thus, the actual number of trees in all quadrats was also recorded. From the numbers infected per quadrat, the θ index of aggregation parameter for the beta-binomial distribution was estimated using the algorithm of Smith (Smith 1983), as implemented in the BBD software (Madden & Hughes 1994). The binomial index of dispersion (D) was also estimated using the same BBD software.

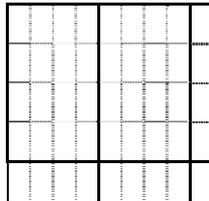


Figure 3: Illustration of incomplete 4 x 4 quadrats, using the example of a Very Small, square orchard. Two complete 4 x 4 quadrats, two 1 x 4 quadrats, one 4 x 1 and one 1 x 1 quadrat are shown.

Figure 4 shows a small selection of simulated orchards, and Figure 5 summarises the estimated binomial index of dispersion (D) and θ parameters. If the binomial index of dispersion exceeds one, this indicates that there is some aggregation in the spatial distribution of infection, and the larger this dispersion is, the greater the aggregation. For 4 x 4 quadrats, Dallot et al. (2003) found values for this parameter which ranged from 0.73 to 7.61, with all but two above 1, and a median value of 1.68. Similarly, if the beta-binomial θ parameter is greater than zero, this indicates aggregation. Values of θ found by Dallot et al. (2003) ranged from 0 to 0.95, with a median of 0.05. For brown rot, Xu et al. (2001) found values of θ ranging from 0.005 to 0.321, with strong evidence that the infection was not randomly distributed, but aggregated. These (and other papers in the literature) strongly suggest aggregation is present in most cases, but that it is usually weak. Where they can be estimated, the ranges of values for these parameters for the simulated data fall mostly within the ranges found (Figure 5), indicating that the simulated data have at least some properties of data from real orchards. (θ could not always be estimated, particularly for the smallest orchards, where there was only a small number of 4 x 4 quadrats of trees).

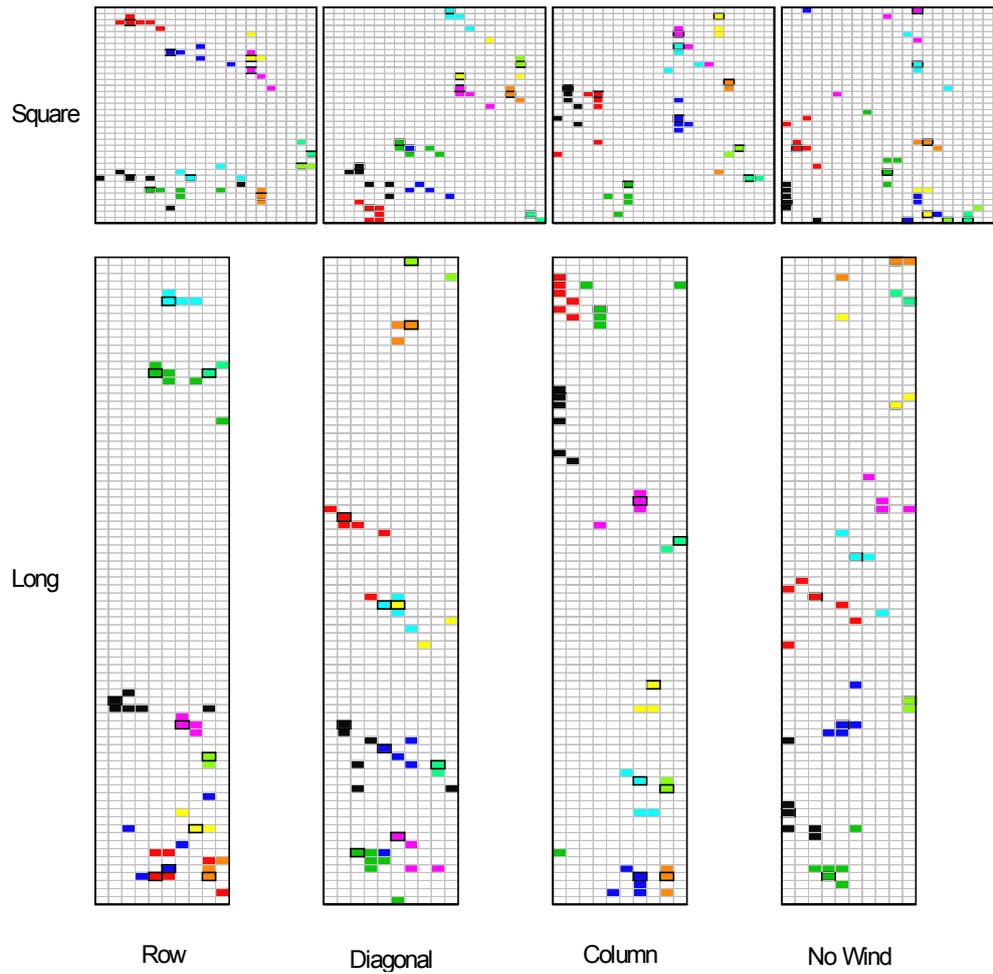


Figure 4: Examples of simulated orchards: Medium size, square and long shapes, with 5% disease incidence, for four wind directions/conditions. Each square is a tree, and coloured squares indicate infected trees. Squares of the same colour indicate trees infected from the same start tree, with the start tree edged in black.

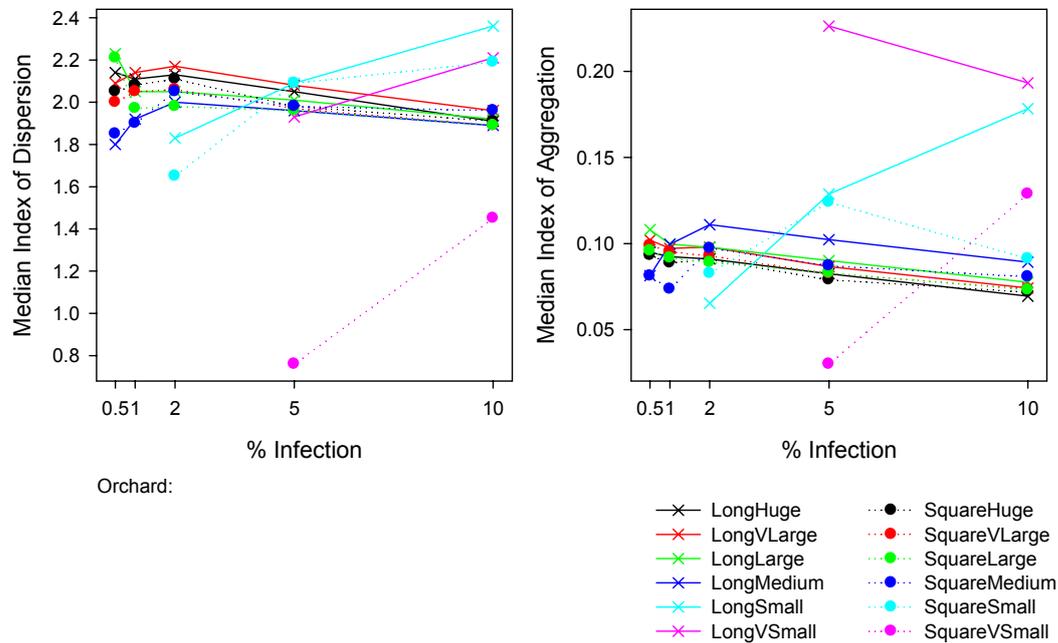


Figure 5: Median estimated binomial index of dispersion D and aggregation parameter θ , for simulated orchards.

5 Sampling schemes

Six sampling schemes were used with each data-set (Figure 6):

1. 50 randomly selected trees per orchard, the Richmond et al. (1998) MAF scheme (**R50**). Where there were 50 or fewer trees in the orchard, all trees were selected.
2. 500 randomly selected trees per orchard (**R500**). Where there were 500 or fewer trees in the orchard, all trees were selected. This is the approximate number suggested to detect 0.5% infection, with 90% confidence and a 90% detection efficiency.
3. One tree randomly selected from the first two trees in the first two rows (four trees), then every second tree in every second row from this tree. One quarter of the trees in an orchard was assessed in this scheme (**I2**).
4. One tree randomly selected from the first four trees in the first four rows (i.e. one tree from sixteen trees), then every fourth tree in every fourth row from this tree. One in every 16 trees (6.25%) was assessed with this scheme (**I4**).

5. Sampling groups of four adjacent trees. The orchard was divided into groups (quadrats) of two rows x two trees within a row. The first group was selected from the first four quadrats (numbered down a row), then every fourth group systematically from then on. All trees within a selected group were sampled. One quarter of the trees in an orchard was assessed in this scheme (**Q**).
6. 15 randomly chosen 2 x 2 quadrats (60 trees in all). The orchard is divided into all possible 2 x 2 quadrats, and 15 of these randomly selected for sampling. If the number of trees to sample is calculated using the same parameters as Richmond et al. (1998) used (to get 50 trees), but allowing for a low level of aggregation ($\theta = 0.09$), then 60 trees in groups of 2 x 2 should be sampled (see above). Therefore, this scheme was included for comparison (**Q15**).

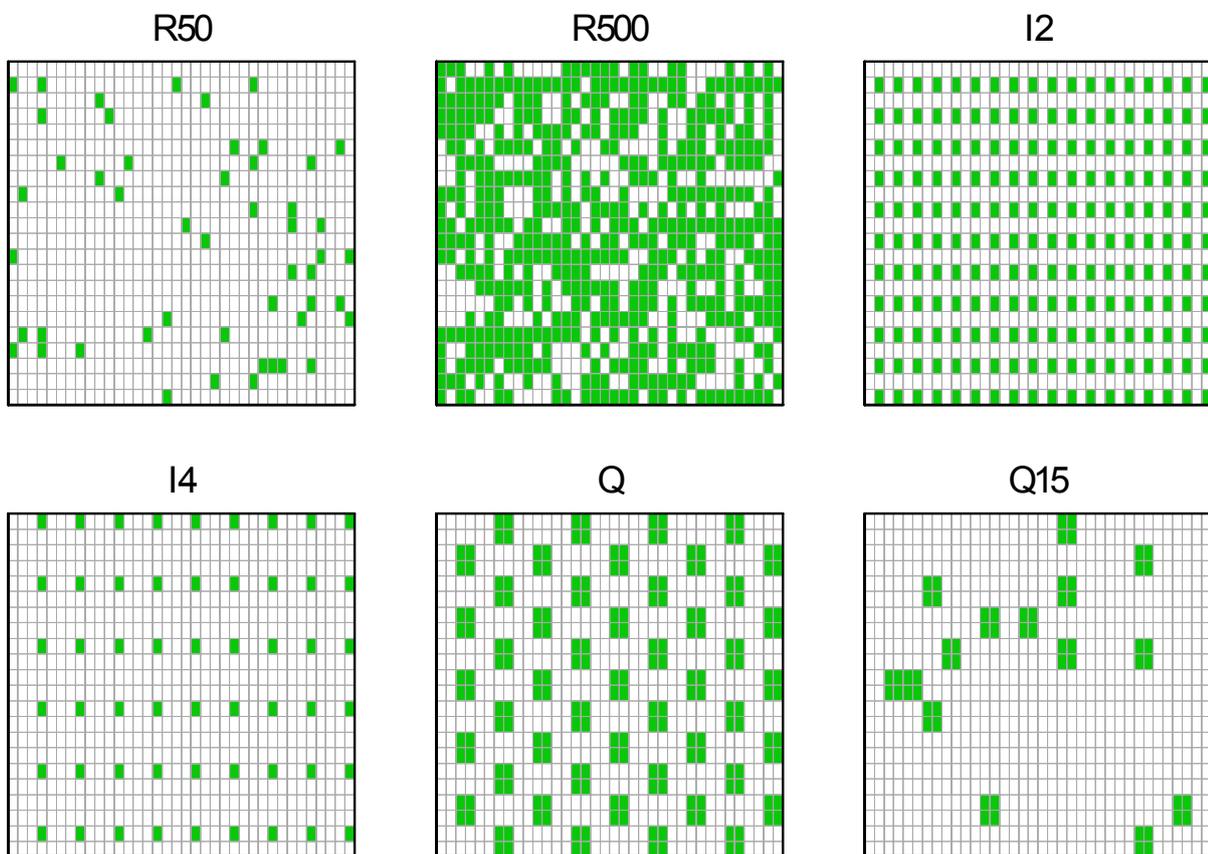


Figure 6: Illustration of the six sampling schemes (for Square, Medium Orchard). See text for details. Squares represent individual trees; green squares are selected trees.

Schemes R50, R500 and Q15 sample a constant number of *trees* regardless of orchard size, whilst schemes I2, I4 and Q sample an approximately constant *proportion* of all the trees in an orchard. The actual number of trees sampled in Q15, and the proportion in Q will vary slightly, since some quadrats at the edges of the orchard may contain fewer than four trees (Figure 3). Similarly, the proportions sampled under schemes I2 and I4 will vary slightly because the numbers of rows / trees in a row will not always be exact multiples of 2 or 4 (Table 1).

Schemes I2, I4 and Q were used by Hughes & Gottwald (1998) to assess sampling schemes for *Citrus* orchards. Scheme Q has since been used to assess *Citrus* and *Prunus* orchards in large-scale surveys (Hughes & Gottwald 1999; Hughes et al. 2002), and the approach is used for sampling by the USDA-APHIS for their plum pox virus surveys (ceris.purdue.edu/napis/pests/ppox/survey/ppv00-survey.html). In these surveys, material from the group of four trees is pooled for assessment of infection by ELISA, reducing the number of tests required for a given number of trees sampled.

In the results we present below, estimated numbers of infected trees are not adjusted for testing efficacy, that is, not adjusted to allow for p_5 less than 1. Consequently, if testing were not fully efficient, then the results from the simulations would need to be adjusted to account for this. The effect of reduced detection efficacy (p_5) on the proportion of samples detected as containing at least one infected tree is also presented below.

5.1 Results

Figure 4 shows some examples of orchards generated with square or long shapes and with the four different simulated wind conditions. Where a prevailing wind was included, infected trees were generally downwind of the start tree. For no wind, trees were approximately evenly spaced in all directions from start trees.

The theoretical chance of a sample containing an infected tree was explored in Figure 1, in which the infected trees were assumed to be randomly placed, and the orchard shape was ignored. The samples taken from the simulations can be used to show how well the theoretical calculation matches a more realistic situation, with some aggregation, two shapes of orchard, and differing sampling schemes. The results from the simulations (Figure 7) are in line with the theoretical calculations. There was little difference between the long and square orchards, and little difference between wind directions (details not shown). This suggests that the low level of aggregation found in the plum pox and brown rot surveys and used in the simulations is not large enough to have a major deleterious effect on sampling schemes assuming no aggregation. For all but the smallest orchards, schemes Q and I2 are most effective at detecting the lowest levels of infection.

The proportion of samples containing at least one infected tree increases rapidly with increasing disease incidence (Figure 8). Schemes R500, Q and I2 are almost 100% efficient at detecting infection where incidence is above 2% for all but the two smallest orchard sizes. For the smallest orchards, schemes R50, R500 and Q15 are most efficient, principally because a greater proportion of the orchard is sampled. Scheme I4 falls somewhere between these groups, except for small and very small orchards, where it is very inefficient. For the lowest levels of infection (one tree, 0.5%) schemes Q and I2 are most effective for the larger orchards, but scheme R500 is more effective for the smallest orchards. This suggests that if very low levels are important to detect, then the sampling scheme should vary, depending on the orchard size. However, even schemes Q and I2 have less than a 30% chance of detecting a single infected tree.

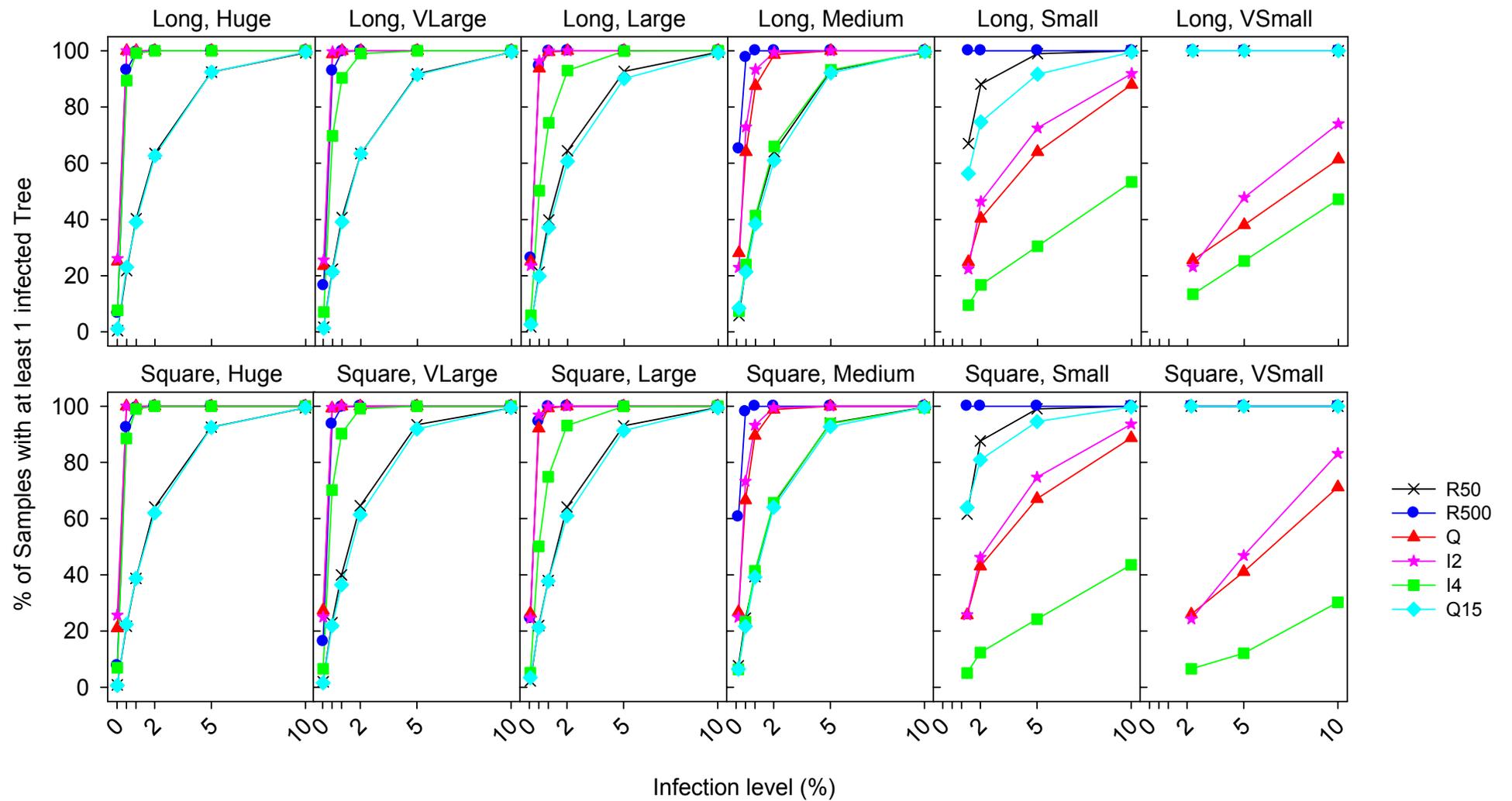


Figure 7: Percentage of samples containing at least one infected tree, for each infection level, orchard size and sampling scheme.

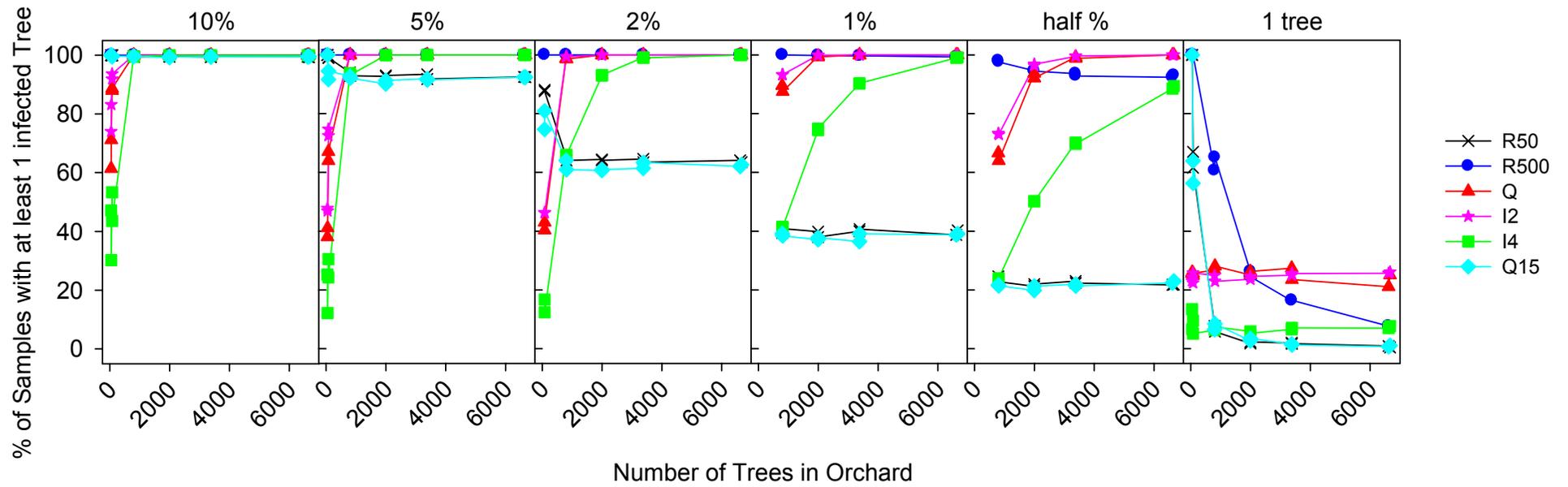


Figure 8: Percentage of samples containing at least one infected tree, vs number of trees in an orchard, for each sampling scheme

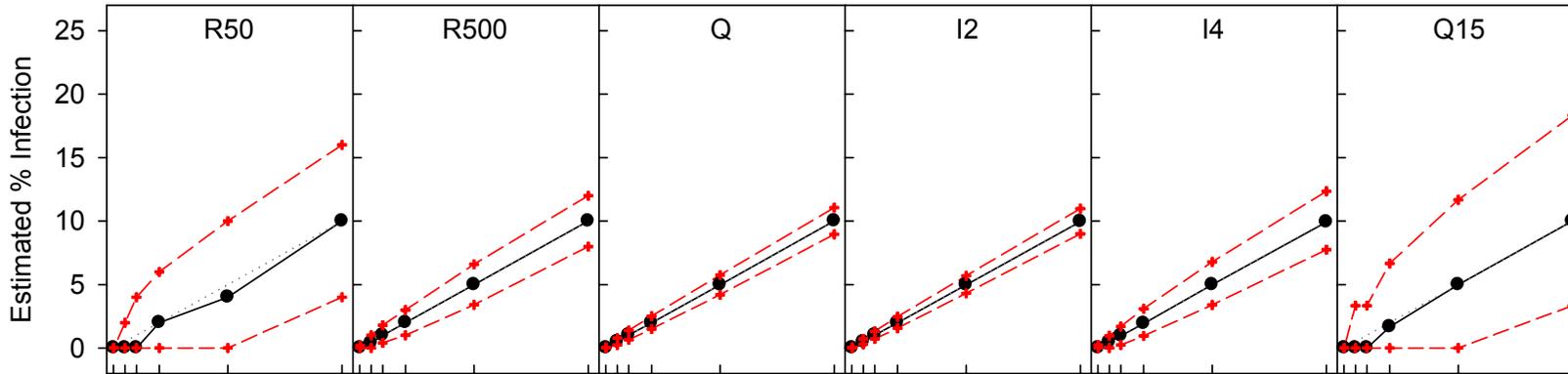
6 *Estimating disease incidence*

The proportion of samples containing at least one infected tree is the *power* of the sampling scheme (p_3). As well as detecting that infection is present, it is usually desirable to obtain an accurate estimate of the level of infection (or disease incidence) present, p_4 . The proportion of infected trees in a sample is an estimate of the level of infection in the sampled orchard, and if this is summarised across all of the simulated orchards, it is possible to see how well the underlying level of infection is estimated. Figure 9 shows the median level of infection, and the 5% and 95% quantiles: that is, if the simulations are sorted in order of estimated infection level, 50% have infection below the median, 5% have estimated infection below the 5% quantile, and 5% have estimated infection above the 95% quantile. If the 5% and 95% quantiles are close to each other, this indicates that most samples have similar estimated infection levels, indicating a reliable (or precise) sampling scheme. If the median lies close to the underlying infection level, then the sampling scheme gives an accurate estimate of infection.

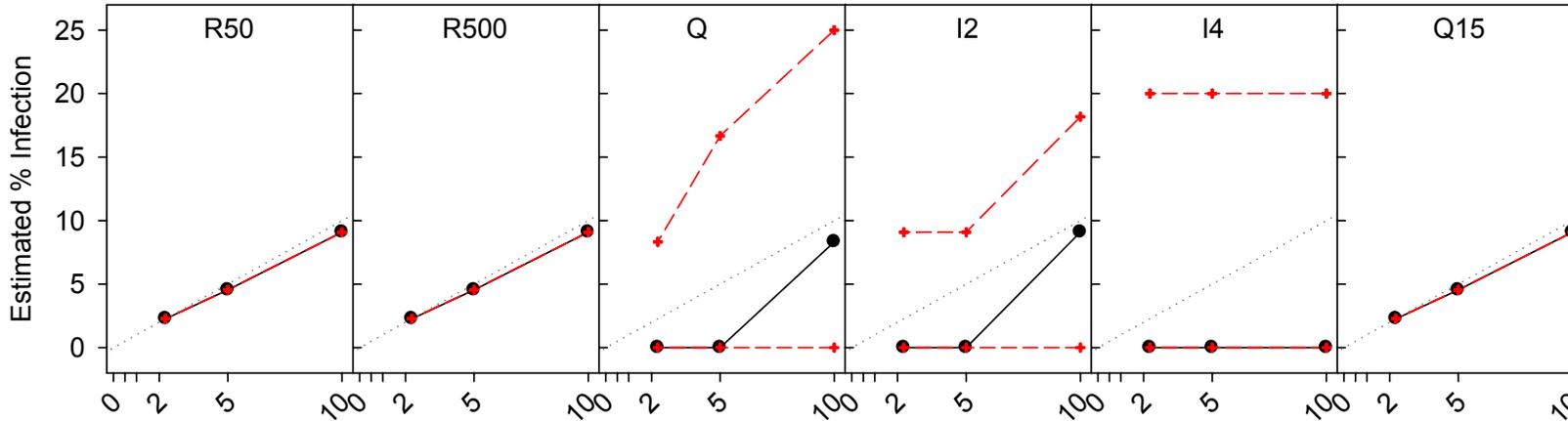
For the smallest orchard (Long, Very Small), infection was generally underestimated by all of the schemes. In part, this is because the actual number of infected trees for a given infection level is lower than the defined percentage because of rounding. For example, for 10% and 5% nominal infection rates, the actual infection rates were 9.1% and 4.5%. Thus, for R50, R500 and Q15, the estimated equals the 'true' actual for all samples, since all the trees in the orchard (44) were sampled under these schemes. However, for schemes I2 and Q (a quarter of the orchard sampled, or around 11 trees) the two lower levels of infection (one tree, 5%) were generally underestimated as no infection. I2 was a little more precise than Q, since the 95% quantile is closer to the actual infection than for Q, indicating that with this scheme, estimated infection would be closer to the true value for more samples than with the Q scheme. Using scheme I4 (1/16 of the orchard, or about five trees sampled), infection was not detected, even at 10%.

Effect of wind: The varying wind directions had little or no effect on either the precision and accuracy of estimated infection levels, or on the percentages of samples that contained no infected trees. With higher levels of aggregation, or stronger prevailing winds, this might not be the case, but this has not been explored here.

Long, Huge Orchard



Long, VSmall Orchard



Actual % Infection ● Median -+ 5% Quantile
 Estimated = Actual -+ 95% Quantile

Figure 9: Estimated infection v. actual infection for six sampling schemes: Median over all samples, 5% and 95% quantiles (see text), with Estimated=Actual marked. Top: Long, Huge Orchard, Bottom: Long, Very Small Orchard.

7 *Adjusting for detection probability, $p_5 < 1$*

The probability of there being at least one infected tree in a sample (power, p_3) is usually calculated by working out the probability of there being no infected trees in the sample, and subtracting this from one:

$$p_3 = 1 - \text{prob}(\text{sample contains no infected trees})$$

Therefore, to estimate the power when detection probability (p_5) is less than one, a similar approach is required. For a sample of size n from a field containing N trees, with x infected trees in the sample, then the probability that none of the x infected trees will be detected as being infected is:

$$\text{prob}(\text{all tests negative/ } x \text{ infected trees in sample}) = (1-p_5)^x \quad (1)$$

using standard probability calculation rules, including assuming the result of the test for one tree is independent of the test for another tree.

If the number of infected trees in the sample follows a hyper-geometric distribution (with a proportion p_4 infected), then:

$$\text{prob}(x \text{ infected trees in sample of size } n) = \frac{\binom{N \cdot p_4}{x} \binom{N(1-p_4)}{n-x}}{\binom{N}{n}}$$

Thus, using standard rules for combining probabilities,

$$\begin{aligned} &\text{prob}(\text{no infected trees detected in sample of size } n) \\ &= \sum_{x=0}^{\min(n, N \cdot p_4)} (1-p_5)^x \cdot \text{prob}(x \text{ infected trees in sample of size } n) \\ &= \sum_{x=0}^{\min(n, N \cdot p_4)} (1-p_5)^x \cdot \frac{\binom{N \cdot p_4}{x} \binom{N(1-p_4)}{n-x}}{\binom{N}{n}} \end{aligned}$$

Thus, the power to detect at least one tree in the sample as being infected is:

$$\begin{aligned} &\text{prob}(\text{at least 1 infected tree detected in sample of size } n) \\ &= 1 - \sum_{x=0}^{\min(n, N \cdot p_4)} (1-p_5)^x \cdot \frac{\binom{N \cdot p_4}{x} \binom{N(1-p_4)}{n-x}}{\binom{N}{n}} \end{aligned}$$

The binomial or beta-binomial distributions could be included in place of the hyper-geometric distribution in a similar manner.

The calculations used to estimate sample size (as used by MAF; Richmond et al. 1998, detailed above) include an adjustment for detection efficiency, by multiplying the orchard infection rate p_4 by p_5 . This is appropriate, providing the probability of detecting an infection in material from an infected tree is independent of the infection rate in the orchard, and that the infection has a binomial distribution within the orchard, and also that detection of infected trees in a sample also follows a binomial distribution. That is, if the binomial distribution is used instead of the hyper-geometric distribution:

prob (t trees detected as being infected in sample of size n)

$$= \sum_{x=0}^n \binom{n}{x} p_3^x (1-p_3)^{n-x} \cdot \binom{x}{t} p_5^t (1-p_5)^{x-t}$$

This simplifies to a binomial distribution with probability $p_4 p_5$:

prob (t trees detected as being infected in sample of size n)

$$= \binom{n}{t} (p_4 p_5)^t (1-p_4 p_5)^{n-t}$$

Rearranging this gives the first equation above for sample size calculations (Richmond et al. 1998).

In the general case:

prob (t trees detected as being infected in sample of size n)

$$= \sum_{x=0}^n \text{prob}(x \text{ in } n) \times \text{prob}(t \text{ detected})$$

where:

prob(x in n) = prob(x infected trees in sample of size n)

prob(t detected) = prob(t of the x infected trees detected as being infected)

Prob(x infected trees in a sample of size n) does not always have a binomial distribution, and prob(t of the x infected trees infected as being infected) may not have a binomial distribution. Therefore, in general prob(t trees detected as being infected in a sample of size n) will not have the same distribution as prob(x infected trees in a sample of size n), but with parameter $p_5 p_4$ instead of p_4 (thus, p_5 should probably not be included in the beta-binomial as was done in the section 'Calculating the number of trees to sample' above).

In the current study, because of the above, it is not appropriate to estimate p_3 (power) by adjusting infection level in the simulated orchard to be p_5 times the infection rate p_4 . It is more appropriate to examine how a detection rate of $p_5 < 1$ affects the power p_3 using the simulated orchards and then adjust for p_5 . This also allows the effect of p_5 on p_3 to be examined for the lowest levels of infection (half%, one tree), when $p_5 \times p_4$ would result in no infected trees to include in the simulation! (A new set of simulations would also be very time-consuming, since each set of simulations requires about 10 hours of computer running time, plus another 2.5 hours to summarise the results).

As an alternate approach, using the existing simulations, prob(no infected trees detected in a sample of size n) can be calculated in an analogous

manner to that used for the hyper-geometric distribution, using equation 1 with each individual simulation, summing over simulations, and dividing by the number of simulations.

prob (at least 1 infected tree detected in sample of size n)

$$= 1 - \left(\sum_{i=1}^{\text{no.simulations}} (1 - p_5)^{x_i} \right) / \text{no.simulations}$$

where x_i is the number of infected trees in the sample taken from simulation i . This can be calculated very quickly for a range of values for p_5 . The results of such an analysis are presented below and in Table 2.

Table 2: Sampling schemes with the greatest power to detect disease in infected orchards varying for orchard size and three levels of disease incidence (p_4), assuming detection probabilities (p_5) 1 (i.e. no false negatives from either surveying or laboratory analysis), 0.75 and 0.5. Details of orchard sizes are presented in Table 1. The Power (p_3) is presented as calculated from the simulations

Detection probability (p_5) of 0.75						
Orchard size	5% incidence		0.5% incidence		1 tree infected	
	Scheme	Power	Scheme	Power	Scheme	Power
Huge	R500, Q, I2, I4	>99.99	Q, I2	99.9	Q	17.3
					I2	19.4
Very large	R500, Q, I2, I4	>99.99	Q	96.7	Q	19.1
		>99.9	I2	97.8	I2	19.0
Large	R500, Q, I2, I4	>99.99	R500	87.5	R500	19.1
		99.3	I2	90.0	I2	18.1
					Q	19.3
Medium	R500	>99.99	R500	91.7	R500	47.1
	Q	>99.9				
Small	R50	93.8	n/a	n/a	R500	75.0
	R500	99.6	n/a	n/a		
Very small	R50, R500, Q15	93.8	n/a	n/a	R50, R500, Q15	75.0

Detection probability (p_5) of 0.5						
Orchard size	5% incidence		0.5% incidence		1 tree infected	
	Scheme	Power	Scheme	Power	Scheme	Power
Huge	R500, I4, Q, I2	>99.99	Q,	98.6	Q	11.6
		>99.99	I2	98.9	I2	13.0
Very large	R500, Q, I2	>99.99	Q	89.2	Q	12.7
	I4	99.6	I2	90.8	I2	12.7
Large	R500, Q, I2, I4	>99.99	R500	73.6	R500	12.7
		96.0	I2	76.0	I2	12.1
					Q	12.9
Medium	R500	>99.99	R500	77.3	R500	31.4
	Q	99.5				
	I2	99.7				
Small	R50	80.1	n/a		R500	50
	R500	93.8				
Very small	R50, R500, Q15	75.0	n/a		R50, R500, Q15	50

Detection probability (p_5) of 1						
Orchard size	5% incidence		0.5% incidence		1 tree infected	
	Scheme	Power	Scheme	Power	Scheme	Power
Huge	R500, Q, I2, I4	>99.9	Q, I2	>99.9	Q	23.1
					I2	25.9
Very large	R500, Q, I2, I4	>99.9	Q	99.0	Q	25.5
			I2	99.6	I2	25.3
Large	R500, Q, I2	>99.9	R500	94.5	R500	25.4
	I4	99.9	I2	96.6	I2	25.7
					Q	24.1
Medium	R500, Q, I2	>99.9	R500	97.8	R500	62.8
Small	R50	99.0	n/a		R500	100
	R500	100				
Very small	R50, R500, Q15	100	n/a		R50, R500, Q15	100

8 *Summary and comparison of sampling schemes*

8.1 *Detection methods*

8.1.1 *Detection of PPV*

Methods for detecting PPV-infected trees include visually observing symptoms on leaves or fruit, biological indexing by chip grafting, conducting ELISA-based methods, and conducting RT-PCR-based methods (EPPO 2004). Difficulties in reliably detecting PPV infection are due to various factors including latency of infection, low virus titres and uneven distribution within plants.

- *Symptoms.* As discussed above, the appearance of symptoms is variable with time of year, growth of the plant, severity of the infecting PPV strain and plant cultivar. Latent infections are common and appearance of symptoms can occur months or years after infection (Quiot et al. 1995; Bodin et al. 2003). Dormant trees cannot be scored for the presence of symptoms on leaves and fruit. If symptoms do appear, they are best observed on leaves in spring, but can also appear as flower petal discolouration, or symptoms on fruit.
- *Sampling of trees.* Since PPV is unevenly distributed in infected trees and can be present in low concentrations, selection of samples from individual trees is critical for ELISA or RT-PCR-based detection. If symptoms are present, then these tissues (leaves, flowers or fruit) should be sampled since they will contain the highest virus titres. Otherwise, samples should be taken in quadrants (or greater) around the tree, with leaves collected from the middle of branches, and within the tree canopy.

The timing of leaf sampling is important – and is best carried out in spring or early summer before temperatures get too high (EPPO 2004). Adams et al (1999) showed that bark and root samples can be used very successfully to detect PPV in dormant trees, but this approach was not incorporated in the EPPO Standard.

- *ELISA*. Kits for DAS-ELISA (Double Antibody Sandwich-ELISA) or for DASI-ELISA (Double Antibody Sandwich Indirect ELISA) are available from REAL, Durviz (www.durviz.com), and have been validated in European ring tests. Methods for DAS- and DASI-ELISA are detailed in the EPPO Standard (EPPO 2004). ELISA is less sensitive than IC-PCR and therefore has limited tolerance for pooling of samples. For example, Hughes et al. (2002) found that ELISA reliably detected two infected leaves in a pooled sample of 16 total leaves using the standard antibody dilution (1:1000).
- *IC-PCR*. Protocols for IC-PCR were developed by Wetzel et al. (1991b; 1992) and Olmos et al. (1997). IC-PCR has advantages over ELISA methods, in particular its greater sensitivity of 1000-5000X; (Adams et al. 1999; López-Moya et al. 2000). Plant extracts prepared for ELISA detection can be used for IC-PCR, rather than specific RNA extractions that are required for conventional RT-PCR (therefore the time required for sample preparation is no greater for IC-PCR than for ELISA). In addition, IC-PCR effectively concentrates virions thereby increasing the sensitivity of the test. Coating of PCR tubes or plates with antibodies (polyclonal or monoclonal 5B-IVIA) is carried out as for ELISA, then the immunocapture phase is carried out by adding plant extract to the coated plate/tube, incubating, then washing. It is important to remove washes by pipetting using fresh pipet tips rather than by decanting to avoid cross-contamination of wells because of the increased sensitivity of IC-PCR. RT-PCR products can be detected by agarose gel electrophoresis or colorimetrically using a digoxigenin-labeled probe (EPPO 2004).

8.1.2 *Detection of M. fructigena*

Brown rot (*M. fructigena*) infected apple or pear trees are identified by observing blighted blossoms, rots associated with wounds or regions of contact on fruit, and/or cankers on twigs and branches, by culturing the fungus and examining its morphology, and by PCR diagnostics. EPPO (2003) has published a Standard for diagnosis of the related pathogen, *M. fructicola*.

- *Symptoms*. *M. fructigena* symptoms on blossoms, branches and stems, and fruit have been described and illustrated by EPPO (2003). Symptoms are most easily observed on fruit that is approaching maturity, where firm rots associated with wound sites are seen. Characteristically *M. fructigena* is seen on apples and pears, but other fruit trees in the Rosaceae also serve as host plants.
- *Biological characterisation*. Preliminary identification of the fungus as *M. fructigena* can be carried out by culturing infected material on potato dextrose agar (PDA), then by observing the morphology of hyphae and conidia in particular (EPPO 2003). The morphologies of *M. fructigena*,

M. fructicola and *M. laxa* overlap, therefore cannot be used alone for definitive identification.

- **PCR.** A multiplex PCR method that distinguishes *M. fructigena* from *M. fructicola*, *M. laxa* and *M. polystroma* (Côté et al. 2004) amplifies different sized bands from each of these three pathogens. Fungal DNA can be extracted either from cultures or from hyphae protruding from infected fruit and dissected away using a sharp needle. It is important that positive control amplifications, for example based on ITS sequences (Hughes et al. 2000; EPPO 2003), are done to ensure that extractions are suitable for directing PCR.

8.2 *Estimating resources required for detection*

Resources required to detect PPV or *M. fructigena* by surveying and diagnostic tests (for plum pox virus) were estimated by first considering the sampling schemes that provide the greatest power for detecting at least one infected tree in orchards of varying sizes (from very small to huge) at three disease incidences: 5%, 0.5% and one tree. In addition, the effects of three levels of detection probability (p_5 ; 1, 0.75 and 0.5) on the power of the various sampling schemes are also presented. The results (Table 2) provide a basis for selecting sampling schemes that are most efficient for determining whether infection within an orchard can be detected.

Data from simulations on the power of the R50 sampling scheme for various orchard sizes, with three disease incidence levels (5%, 0.5% and one tree), and assuming a detection probability (p_5) of 1, are summarised in Table 3. The R50 sampling scheme was the basis for the pioneering work on surveillance methods by Richmond et al. (1998). This summary shows that the power to detect at least one tree in a sample is more than 90% when the disease incidence is 5% (as expected), but that the detection power drops off considerably at lower disease incidences, also as expected, except for the smallest orchard sizes.

Table 3: Power to detect infected orchards of various sizes and at three incidence levels using the R50 sampling scheme (for detection probability $p_5 = 1$).

Orchard size	Power (%) to detect infected orchards		
	5% incidence	0.5% incidence	1 tree/orchard
Huge	92.5	21.8	0.7
Very large	92.6	22.7	1.9
Large	92.8	21.6	2.1
Medium	93.5	23.7	6.8
Small	99.0	n/a	64.3
Very small	100	n/a	100

Estimates of the resources required for detection and subsequent diagnosis are presented in Table 4. The estimates are presented for examining single orchards, ranging in size from huge to very small. The times required to survey orchards for *M. fructigena* (3.6 min/tree) and PPV (3.1 min/tree) were taken from Ganey and Braithewaite (2003) and do not include travelling time. The times required to carry out ELISA or IC-PCR to detect or diagnose PPV were taken from Lopez-Moya et al. (2000) and Levy et al. (2000a). These authors estimated that one person could process 300 ELISA samples/day and 120 IC-PCR samples/day. Some automation will be required to meet a throughput of 300 ELISA samples/day, particularly of sap extraction, plate washing and plate reading. López-Moya et al (2000) also estimated the consumable costs/individual test of the ELISA and IC-PCR diagnostic tests as 0.7 and 1.4 Euros, respectively. For the purposes of Table 4, these values have been doubled to reflect the value of the New Zealand dollar. The times and consumable costs required to identify and diagnose *M. fructigena* brown rots by culturing and PCR have not been estimated since these methods are identification tools that would be applied once suspected *M. fructigena* rots have been observed and are not routine screening tools.

Table 4: Estimation of resources required to sample orchards of various sizes using the sampling schemes that give the best power to detect infection incidences of 0.5% or 1 tree/orchard.

Orchard size (mean number of trees) ^a	Scheme	Number of trees sampled ^b	BR survey time (hr) ^b	PPV survey time (hr) ^c	PPV ELISA time (days) ^d	PPV IC-PCR time (days) ^d	ELISA costs (\$NZ)	IC-PCR costs (\$NZ)
Huge (6640)	R500	500	30.0	25.8	1.67	4.17	700	1400
	Q	1660	99.6	85.8	5.53	13.83	2324	4648
	I2	1660	99.6	85.8	5.53	13.83	2324	4648
	I4	415	24.9	21.4	1.38	3.46	581	1162
Very large (3378)	R500	500	30.0	25.8	1.67	4.17	700	1400
	Q	845	50.7	43.7	2.82	7.04	1183	2366
	I2	845	50.7	43.7	2.82	7.04	1183	2366
	I4	211	12.7	10.9	0.70	1.76	295.4	591
Large (1995)	R500	500	30.0	25.8	1.67	4.17	700	1400
	Q	499	29.9	25.8	1.66	4.16	698.6	1397
	I2	499	29.9	25.8	1.66	4.16	698.6	1397
	I4	125	7.5	6.5	0.42	1.04	175	350
Medium (801)	R500	500	30.0	25.8	1.67	4.17	700	1400
	Q	200	12.0	10.3	0.67	1.67	280	560
	I2	200	12.0	10.3	0.67	1.67	280	560
Small (76)	R50	50	3.0	2.6	0.17	0.42	70	140
	R500	76	4.6	3.9	0.25	0.63	106.4	213
Very small (44.5)	R50	44.5	2.7	2.3	0.15	0.37	62.3	125

a number of trees averaged for long and square orchard dimensions.

b number of trees sampled averaged over the simulations for long and square orchard dimensions.

c times for surveying taken from Ganev & Braithwaite (2003).

d times and costs for laboratory analysis taken from Levy et al. (2000) and Lopez-Moya et al. (2000).

9 *Generalisations that can be drawn from this work*

Our conclusions regarding the power of various sampling schemes for detecting the presence of a plant pest in a horticultural or agricultural sample would most likely be similar for diseases or pests that show a similar or lesser amount of aggregation than the diseases examined in this report. Orchard (or sample) size and the requirement to detect low pest or disease incidences are the major factors affecting the choice of sampling scheme. In addition, the results of the approach taken would hold when there is a number of discrete units such as trees, grains or fruits; and would apply to a “structured” population regardless of shape. We draw this conclusion because the spatial layout (square versus long orchards in this case) did not have a significant effect on the power of the various sampling schemes.

The simulation approach is valuable if it is necessary to take into consideration various aspects of detection with one sampling scheme (for example, both field and laboratory detection methods), because the combining of equations can be algebraically very complex and it may not be possible to reach a theoretical solution.

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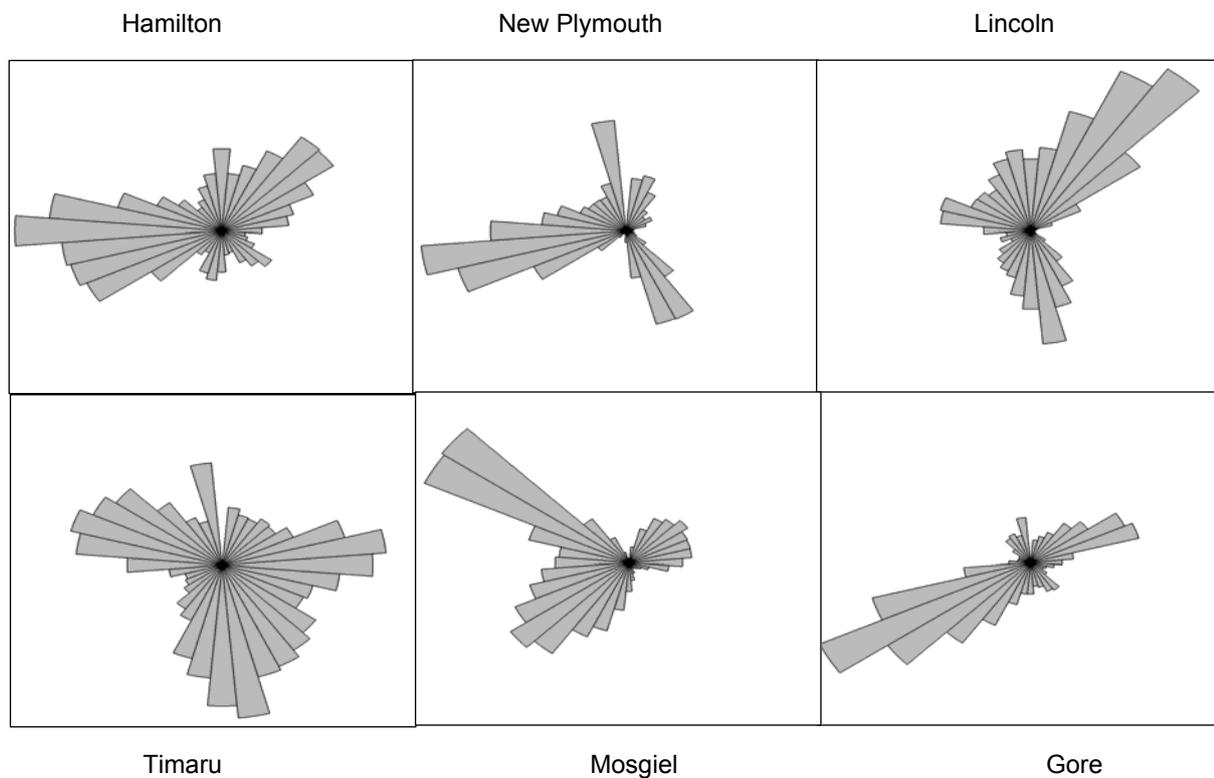
Appendices

Appendix I Review of report: Models for post-border detection of visibly undetectable exotic plant pests

David Baird, Biometrician, AgResearch, Lincoln

Overview

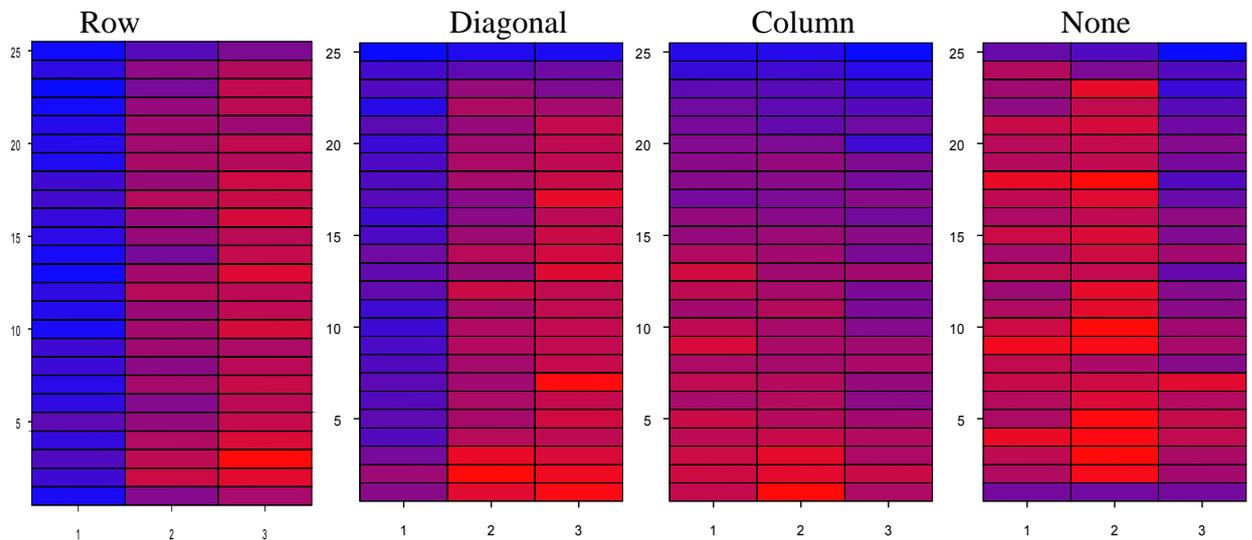
1. This report is a comprehensive analysis of sampling methodologies of a wind-dispersed pathogen in orchards. The sampling and simulation techniques used are appropriate for this analysis. I have checked statistical methods and have found them to be sound. The key result in this paper is the assessment of different sampling methods for aggregated infections. The results critically depend on the appropriateness of the dispersal model to generate the type of aggregation that will be found in the field. After examining the literature review and references, I am satisfied that the Gaussian plume model simulated represents a reasonable model for the two pathogens considered. This model is commonly used in wind dispersal situations and has been validated under a range of conditions (Erbrink, 1995; de Jong et al, 2002). The choice of parameters of the aphid spread being extrapolated from a potato crop to trees is perhaps suspect, as the higher take off points for aphids on trees would be expected to increase their range. However, provided that the level of aggregation matches that observed in actual studies, this is unlikely to affect the results. I have checked the simulation programs and cannot find any mistakes in these, and the results examined are consistent with what I would expect.
2. The examination of the model under very strong prevailing winds (row, column and diagonal) will generate more dispersed patterns than found in practice in New Zealand conditions. The following figure shows the wind roses for six sites around New Zealand. It can be seen that some sites do have some strong prevailing directions, but most do not have a single prevailing direction, as simulated in the data.



Wind roses (down-wind run) for six New Zealand sites

3. The sampling schemes also tend to confound the sampling method and the number of trees sampled. It would have been useful to keep these separate and look at a factorial combination of sample type (random or systematic), plot size (single or four tree plots) and sample size. In addition, there seemed to be another obvious sample type (stratified random) which falls between the extremes of random and systematic that could have been examined. It would be expected that a systematic sampling scheme would be better than a random scheme, with the gain increasing as the level of aggregation increases. This was what was found, although the gains were modest with the levels of aggregation simulated from the dispersal model used.
4. The analysis of the sampling methods by orchard shape and size is useful in validating the techniques over a range of conditions. However, I would have been surprised if these had produced differing results, beyond the effects due to small sample sizes and the number of samples collected. With small orchards, the simulation technique used will be influenced by edge effects, with rejected samples tending to have samples more often near the edges. This is a typical problem in spatial analysis and will have influenced the departures from the expected median index of dispersion, as seen in Figure 5 for very small and small orchards. The edge effects can be seen in the following plots of the mean number of infections observed

over all 1000 simulations for the long small orchard at the 10% infection level.



Edge effects in Long Small Orchard at 10% infection rate by wind direction

5. The section on adjusting for aggregation I believe only applies when groups of plots are being sampled. For example, a simple random sample of single trees, the level of aggregation cannot change the probability of detection. These calculations are of use when multiple samples are bulked together (as in the quad plots). This result indicates that under aggregation, single tree plots are more efficient, as seen in the simulation study. However, cost-effectiveness of multiple tree plots may ameliorate this.

Although under the hypergeometric and beta-binomial models the effect of the efficacy of detecting an infected host, p_5 , is not included in the simulations, in any reasonable-sized sample this would be a second order effect, and would be unlikely to shift the results by much.

6. The differential costs of the different sampling schemes should have been considered in Table 4, where a common cost per tree sample is used. However, a random study, due to the time taken to identify the trees in the sample, is going to take more time than a systematic sample in which only the first tree has to be located, with subsequent trees able to be found very quickly. As previously mentioned, quad tree plots will also be more time-efficient than single trees.
7. The option of grouped testing (where samples from multiple trees are bulked) could have been examined in more detail. This technique is referenced in the discussion of the USDA plum pox surveys. A cost-benefit basis could be carried out, including in the calculations a component which allows for differing probabilities of detection based on the number of infected trees in the bulked sample. It seems that

perhaps provided two leaves are positive in a sample of 16, the plum pox virus can be detected “reliably” (Hughes et al. 2002), but are the probabilities of detection constant for one leaf in 16 and three or more leaves in 16? With two in 16 being detected reliably, does this mean that eight trees can be bulked with two leaves per tree, or four trees with four leaves per tree, two trees with eight leaves per tree, etc? Table 4 seems to assume that trees are not bulked.

8. The work on simulating the single tree case was not necessary, as in this case, there can be no aggregation effect, and all sampling schemes with any form of randomization (i.e. even the systematic sample has a random starting point) must logically give the common result of the probability of detection being $p_5 \times f$, where f is the sampling fraction.

The most important conclusion of this paper is that systematic sampling is the best approach for aggregated spatial distributions. The sampling fraction can then be set for a required level of precision, based on simulation (further simulations may be required to give a wider range of sampling fractions/numbers).

Details

- a) The sentence on the goal could be usefully split into several simpler sentences (although it is a feat to get it all into one sentence).
- b) The sentence “Fundamental research has shown that the spatio-temporal distribution of plant disease epidemics may be best described using different models (e.g. the binomial, beta-binomial or hypergeometric distributions), depending in particular on the degree of aggregation of the affected plants (Madden & Hughes 1999).” is not correct as these models apply to the counted number of infected plants in a sample and not their spatial dispersion.
- c) In the section, “Detail of steps in the simulation - method” the Number of start trees is given as $\text{round}(n_{inf}/3.5)$, but the simulation program uses $\text{round}(n_{inf}/4.2)$.
- d) The distribution of the number of trees generated per start tree seems arbitrary, and there is no justification to the method used. Given that most infected orchards are likely to start from a single case, a non-uniform size distribution is more likely, with one large patch and with several smaller patches due to escapes from the initial infection. However, the patterns in the orchards examined by Gottwald et al. (1995) show a few large patches, and then many singletons. Thus, the pattern of patch sizes used is a compromise between these two cases. Again, the key outcome is that the level of aggregation in the simulated samples matches that likely to be found in the field, so this is probably of minor importance, given that this has been demonstrated.
- e) Figure 7 may be better plotted on a logit scale to better separate the curves as they approach 100%. In Figure 9, for Long Very Small orchard, plot 14 is missing its 95th percentile which should be at 20% by my calculations, and so is off the scale of the plot.

References

de Jong, M.D.; Bourdot, G.W.; Hurrell, G.A.; Saville, D.J.; Erbrink, H., Zadoks, J.C. 2002: Risk analysis for biological control - simulating dispersal of *Sclerotinia sclerotiorum* (Lib.) de Bary ascospores from a pasture after biological control of *Cirsium arvense* (L.) Scop. *Aerobiologia*, 18: 211-222.

Erbrink, J.J. 1995: Use of boundary-layer meteorological parameters in the Gaussian model 'STACKS'. *Boundary-Layer Meteorology*, 9431: 1-25.

Appendix II Authors' responses

Paragraphs 1 and 2: Prevailing wind.

It would be interesting to explore the effect of the higher take-off points of aphids and how this affects the flight distance of the aphids, particularly under New Zealand conditions. There was little easily available information in the literature on this, and we did not find information on flight distances of aphids within New Zealand orchards.

Paragraph 3: Confounding of sampling schemes and number of trees.

Given the resources available, we chose to limit the number of sampling schemes to the current MaffOpps scheme and those used by other researchers in this area. However, for a future project, the suggestion to explore combinations with a range of schemes (random, systematic, systematic with groups of trees, and stratified) with a range of number of trees sampled is excellent and one we would wish to pursue.

Paragraph 4: Edge effects.

Again, this is a topic worth pursuing, which we had some awareness of, but insufficient resources to pursue thoroughly.

Paragraph 5: Adjusting for aggregation.

We have adjusted the text to address this point. Our understanding of the literature and the equations is that aggregation in the field can lead to a reduction in power (p_3), and biased estimates of the level of infection, if the sample size is determined assuming no aggregation, and the sample is not chosen in a truly random way. Logic suggests that if there is aggregation and a simple random sample is taken (i.e., individual trees, completely randomly chosen), then the power of a sample of a given size to detect a given level of infection should not be affected by aggregation, since each tree is equally likely to be included in the sample. However, as now stated in the text, in practice, it is very difficult to take random sample in the field that is chosen completely independently of the spatial location of trees. Thus, in practice, the power of the sample will inevitably also be affected by any aggregation of disease within the orchard. The equation for sample sizes derived for the beta-binomial is an approximation: thus, estimates for reduction in power (p_3) are also approximate.

Paragraph 6: Cost of sampling trees.

We agree that the sampling time for a random sample would be greater than that for a systematic sample. However, we have no data to estimate how much less time would be needed.

Paragraph 7: Grouped testing.

We were aware that this is an area that requires more work. Each testing laboratory will need to determine the extent of pooling of leaf samples that will allow reliable detection of PPV infection. Since PPV in particular can occur "systemically" in only one quadrat or scaffold branch of an infected tree, appropriate sampling of a tree will involve taking at least eight leaves –

so at the most (following the work by Hughes et al. 2002) then two trees could be pooled. Use of IC-PCR may actually be a more efficient method for testing for infected trees than ELISA since it is likely to allow a greater amount of pooling because of its increased sensitivity.

Paragraph 8: Single trees.

This point is true, and was recognised before the simulation was done. However, we included single trees to allow direct comparison of this extreme case with greater levels of infection, using the same methods to summarise the single tree case and all other level of infection.

Details

b) This change has been made.

c) This was an error and has been corrected.

d) The distribution of numbers of trees generated per start tree is arbitrary, and was chosen to be fairly simple, but reasonably consistent with the data was had available, in the absence of more detailed information.

e) The 95% percentile was indeed outside the axis range: the axis ranges were chosen so as to allow the major part of the data to be easily visible. We have extended the y-axes in this figure to address this point.