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Everett KR, Xu G

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Executive summary

BS20149 Pollen pathway risk review

Everett KR, Xu G
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September 2020

From the evidence considered for the preparation of this review, it is concluded that pollen is a substrate that has been shown to transmit bacteria, fungi, viroids, phytoplasmas and viruses from diseased to healthy hosts. There is no evidence for oomycetes being transferred on pollen. Isolations of bacteria have been made from commercial pollen, which shows that these microorganisms can survive the pollen milling process. It is therefore likely that other microorganisms could also survive the pollen milling process, which is conducted at temperatures that are not likely to kill plant pathogens. Because milled pollen also contains floral debris, there is a risk that propagules of pathogens that infect flowers will contaminate pollen. However, contamination does not necessarily equate to transmission.

The evidence for pollen transmission is strong for bacteria, some viruses (including viroids), and some fungi. Some fungi and viruses (including viroids) infect pollen and are found inside pollen grains, but no bacteria have been found inside pollen grains. American foulbrood is a bacterial disease of bees caused by *Paenibacillus larvae* which has been detected on pollen. It is well recognised that contaminated pollen is an important means of spread of American foulbrood. There are also examples of pollen transmission for several plant bacteria including Psa, walnut blight, fire blight, a biocontrol *Pseudomonas*, and bacterial blast. Two kiwifruit fungal pathogens have been shown in other crops to be able to infect pollen (botrytis and sclerotinia). Pollen transmission has been shown for sclerotinia but not for botrytis. A fungal pathogen of bees, chalkbrook (*Ascosphaera apis*), has been reported to be spread from contaminated pollen to infect healthy bees. Recently a new pollen transmitted kiwifruit virus has been discovered, *Actinidia seed-borne latent virus* (ASbLV). Several kiwifruit adapted viruses have only recently been discovered, and in many cases the means of transmission of these viruses is not well understood. Other of these viruses may yet be found to be pollen transmitted. Only two viruses cause economically damaging losses for kiwifruit, *Cherry leaf roll virus*; CLRV and *Pelargonium zonate ringspot virus*; PZRV. Both are pollen transmitted, and PZRV is not present in New Zealand.

If pollen is imported from overseas, there is a high risk of pollen transfer of PZRV and new strains of Psa with effectors that could overcome the tolerance of Gold3 into New Zealand. Within New Zealand, there is a high risk that CLRV could be spread on pollen. Sclerotinia control may be able to be improved by using clean pollen. Application of Psa contaminated pollen to already infected orchards is unlikely to worsen Psa epidemics. However, it is likely that application of Psa contaminated pollen to uninfected orchards will introduce Psa to those orchards. Botrytis might be spread in pollen, but is likely to be a minor means of transmission of this ubiquitous pathogen.

Current methods used for removing pathogens from pollen either kill or damage the pollen, are not very effective, or are not commercialised. Using several mitigation methods in tandem could be investigated, and are likely to be more effective than each method alone.

A number of novel methods used to mitigate pathogens for fresh food products, plant propagation material and human diseases were examined. The most promising for use on kiwifruit pollen to eliminate fungal and bacterial contamination are ozone, nitric oxide, modified atmospheres and thermotherapy. Fungicides and antibiotics could also be used to suppress fungal and bacterial populations. For production of virus-free pollen, it is best to source pollen from high health plants that are regularly tested. Usually high health plants are within insect exclusion enclosures to prevent insect vectored diseases infecting these plants, and may be necessary to prevent introduction of Psa, sclerotinia and botrytis on bee borne pollen. Because the recently discovered kiwifruit adapted viruses are not well researched, it is possible that some of these could also be insect vectored.

The greatest risk of economic damage from spread of pollen borne diseases within New Zealand is for *Cherry leaf roll virus*, and therefore the greatest need is for high health indexed plants to be established for virus-free pollen production.

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1 Introduction

Biosecurity incursions typically have a lag phase from when an organism arrives, to when symptoms are expressed and detection occurs. This lag phase can be months or even years. Eradication success typically depends on early detection and the ability to prevent spread of unknown organisms during this lag phase. For the kiwifruit industry, the movement of plant material is considered our highest risk pathway and Kiwifruit Vine Health (KVH) has been implementing risk management practices such as the Kiwifruit Plant Certification Scheme (for rootstock) to better manage this risk, Such Standards are designed to manage a broader range of threats than only kiwifruit canker (*Pseudomonas syringae* pv. *actinidiae*; Psa), and reflect focus of KVH to shift from focusing on a single organism (Psa), to better preparing the industry for future biosecurity incursions. This approach is proposed to be formalized under a National Pathway Plan for the kiwifruit industry, a tool under the Biosecurity Act.

KVH will be implementing controls for the movement of rootstock, budwood and mature plants, however the risk associated with pollen is largely unknown. To determine if pollen should also be included in this risk management approach we need to understand what organisms could potentially be spread on this pathway, which would inform the controls necessary to manage this risk. This is a knowledge gap.

In order to address this knowledge gap KVH have requested a review of available literature on pollen and associated floral debris as a means of pathogen spread to inform the need to address pollen in the Pathway Management Plan (PMP). Although there is nothing published about floral debris contaminating pollen, unpublished micrographs clearly show the presence of associated plant material (Figure 1) which are probably floral remnants such as filaments, anthers, or petals. Pieces of other male floral parts are probably also present.

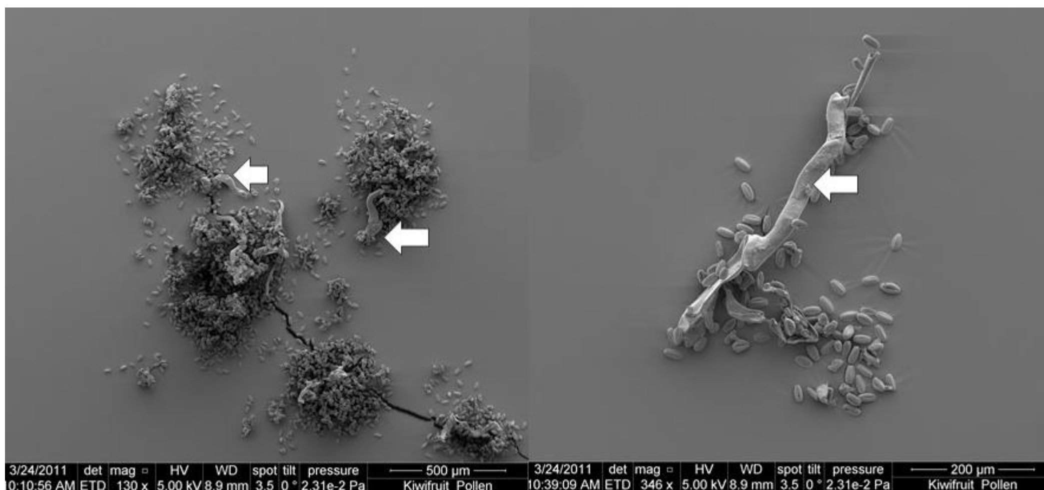

Figure 1. Scanning electron micrograph of commercially milled pollen showing associated plant debris (arrows). Photo courtesy of Paul Sutherland, Plant & Food Research.

The evidence for pollen as a vector for plant diseases in the New Zealand kiwifruit industry was not well understood prior to 2010 (Card et al. 2007) due to the paucity of evidence for pollen transmission of bacterial pathogens in the literature. However, commercially processed pollen also contains remnants of other floral parts (Figure 1). Therefore any discussion of pollen transmission of pathogens needs to consider the risks of transmission by associated floral debris.

This review aims to extensively search the published literature, and critically assess the evidence for the role of pollen and associated floral tissue in transmission of bacterial plant disease. In addition, the risk of pollen and associated floral tissue for transmitting other plant pathogens, bee pathogens and human pathogens is reviewed, including any mitigation measures that are used. Likely reasons for lack of information on certain pathogens or pathways are provided.

2 Methods

A literature search was conducted using Centre for Agriculture and Bioscience International (CABI) and Web of Science (WOS) abstracts, International System for Agricultural Science and Technology (AGRIS), Scopus and other databases for relevant information on pollen and flowers (all pollen, not just kiwifruit). Databases of leading plant protection organisations, including the Animal and Plant Health Inspection Service for the United States Department of Agriculture (APHIS), European Plant Protection Organisation (EPPO), and the Australian Department of Agriculture and Water Resources (ADAWR), amongst others, were also consulted, including relevant information held by the European Food Safety Authority (EFSA).

3 Review

3.1 Pollen production by kiwifruit

3.1.1 Flower initiation and differentiation

Kiwifruit (*Actinidia* spp.) are dioecious, requiring pollen produced by anthers on male plants to be transported to receptive styles on female plants (Figure 2), most commonly by bees (Matheson 1991), but also by other insects (Howlett et al. 2017). The amount of pollen transferred to styles influences the size of the resultant fruit; more pollen grains equals larger fruit (Alspach et al. 1991). Therefore, reliable delivery of quantifiable numbers of pollen grains to styles greatly enhances grower returns, and has led to the development of artificial pollination as a standard practice (Stevenson 1990).

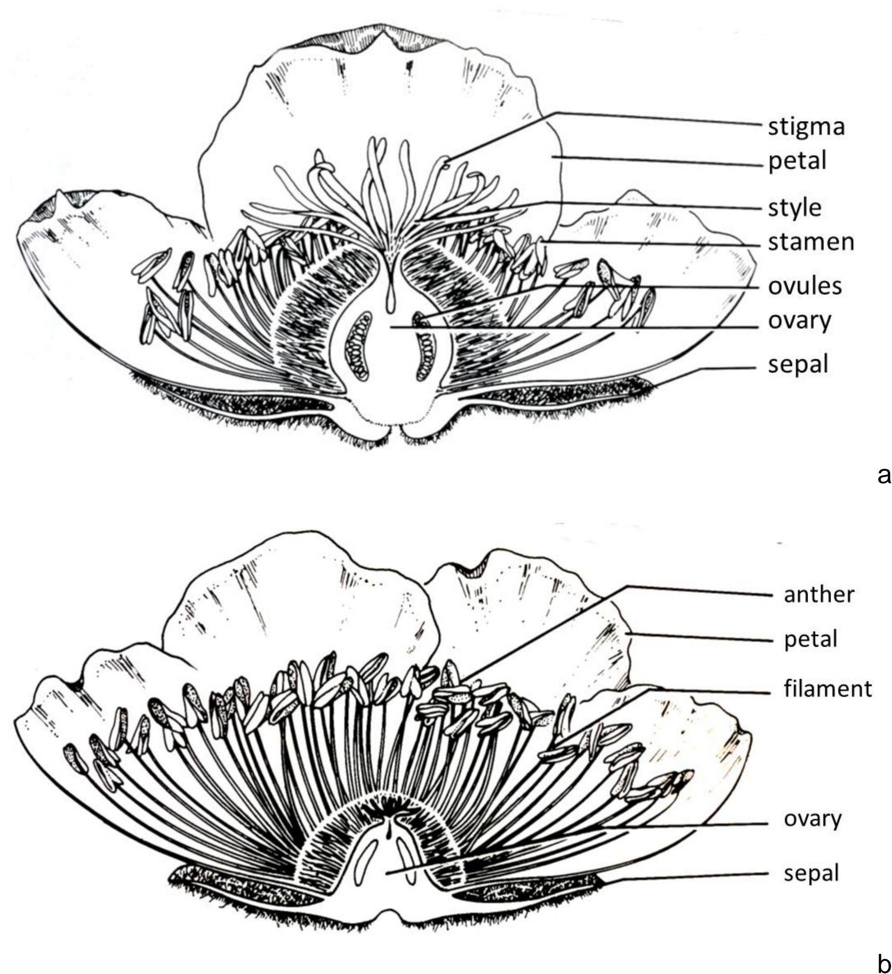

Figure 2. Female (a) and male (b) *Actinidia chinensis* var. *deliciosa* 'Hayward' floral structures. (Slightly redrawn from Hopping (1990).

Kiwifruit is a deciduous plant and growth is dormant during winter. Flower production starts in axillary buds on a flowering shoot growing from 1-year-old canes. These buds change into a reproductive state (are evoked) starting in February for *Actinidia chinensis* var. *deliciosa* 'Hayward' kiwifruit (Hopping 1990). The first of these buds to be evoked is found on new canes immediately after fruit from flowers produced the previous season (Figure 3). The remaining buds are evoked in order until late summer (early May), when the terminal bud is evoked (Figure 3) (Hopping 1990). At the developmental state when they are evoked, these buds have about 22 meristematic primordia in the axis of the bud scales, of which nodes 5–12 are potential reproductive meristems. The first three nodes are bud scales, node 4 is an aberrant reproductive meristem, and nodes 13–22 form leaves (Hopping 1990).

Flower buds were initiated just before shoot growth resumes in the spring (mid-late September) (Brundell 1975) which is approximately 2 months before full bloom (Polito and Grant 1984). Development of both male and female flowers was similar until approximately 15 days after initiation, when the female flower parts (i.e. ovary, ovules, styles and stigma) started to appear (Brundell 1975).

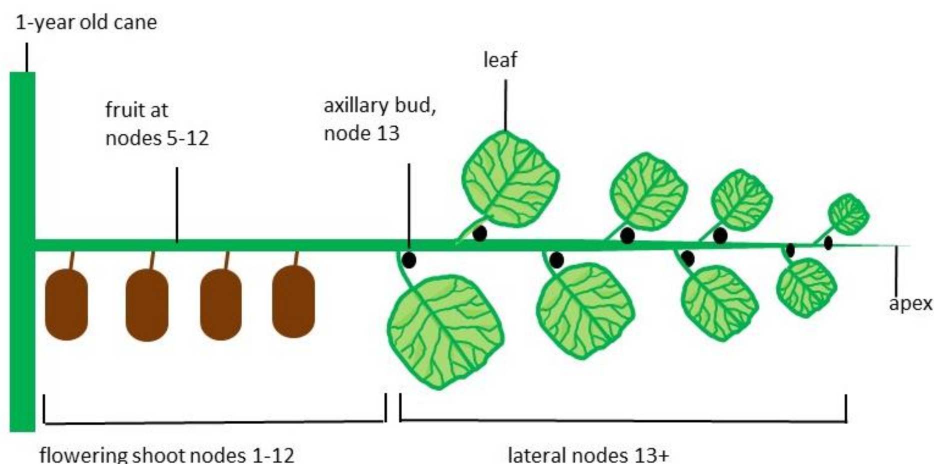

Figure 3. Diagrammatic representation of a kiwifruit flowering shoot in February showing axillary buds at node 13 + for which flowers are evoked at this time. Diagram after Hopping (1990).

The first change indicating flower initiation was swelling of reproductive meristems approximately 10 days before bud burst, followed by initiation of bracts and sepals 6 days before, then bud burst and initiation of petals. About five days after bud burst, stamens were initiated, and after this male flower development differed from female flower development. In males, stigmas were initiated about 9 days after bud burst, and in females about 11 days after. Gynoecial (sexual parts) development stopped approximately 16 days after bud burst in male flowers. Anther and filament initiation took place about 27 days after bud burst in flowers of *A. chinensis* 'Alpha' (male) and after 34 days in 'Hayward' (female) kiwifruit vines (Brundell 1975). Pollen grain formation occurred in male flowers about 38 days after bud burst (20 days before flower opening), followed by calyx splitting after about 40 days, and the flower opened after approximately 53 days. Flowers on these male 'Alpha' plants opened about 7 days before opening of flowers on the female 'Hayward' plants (Hopping 1990) which opened approximately 60 days after bud burst. Stamens on female flowers are fully functional but only produce empty (sterile) pollen grains (Figure 2a) (McNeilage 1991). Stamens on flowers from male plants produce functional pollen grains which contain cytoplasma (McNeilage 1991). Flowers from

male plants do not contain ovules and are generally small with vestigial styles (Figure 2b) (McNeilage 1991).

The flowers form clusters of a primary and two secondary flowers in an inverted ‘T’ shape. Axillary flower buds are formed in the leaf axils of flowering shoots initiated from one-year-old canes (Figure 3). Each flower bud has the potential to initiate eight floral clusters, but often clusters between nodes 5 and 12 lack flowers due to bud abortion. Buds at node 5 cease development before petals are initiated (Brundell 1975). Most male vines produce at most 60% of their flower clusters, although some do produce eight flower clusters. Flowers can develop normally but abort at a late stage.

‘Hayward’ flowers remain receptive to pollen for 8 days after opening (Hopping 1990) and pollen tubes take a maximum of 72 h to fertilise ovules.

The causes of flower abortion are thought to be lack of winter chilling, sharp temperature fluctuations during flower development after bud burst and competition for nutrients within the axillary bud and flowering shoot (Hopping 1990). Expanding leaves towards the apex likely compete with flower buds, causing their abortion.

Aberrant flowers can also be produced in the leaf axils of nodes 4 and 5, by fusion of terminal and lateral flowers. This is caused by a growth check during development. These aberrant flowers set and develop into fan shaped fruit (Hopping 1990).

3.1.2 Pollen production

Kiwifruit pollen is produced on anthers that comprise two chambers, one on either side of a vascular strand. Pollen dehiscence is through longitudinal slits in each chamber (Hopping 1990). Dehiscence takes place early in the morning (about 0920 h) and reaches a peak between 1000 h and 1100 h, then drops to a constant low after 1300 h (Goodwin 1995), but the drop off in the afternoon is due to bees collecting pollen. Pollen supply continued to increase throughout the day in the absence of bees (Goodwin 1995). Anthers released pollen each morning for up to 3 days (Goodwin 1986; Hopping 1990). Male kiwifruit vines can produce 3700–8000 flowers (Hopping 1990), and each flower produces 2–3 million pollen grains weighing c. 9.5 mg. Each plant produces about 14 kg of flower buds per season from which approximately 100 g of pollen can be extracted under ideal conditions (Hopping 1990). Pollen viability ranges from 40 to 80% depending on the clone (Hopping 1990; Wang et al. 2017). Cultivars examined in Wuhan, China, yielded 1160–1520 grains per anther, with as low as 900 grains (Wang et al. 2017). Poor vine management, including inadequate nutrition and too much shade, can significantly impact on pollen viability, resulting in deformed pollen tubes (Hopping 1990).

Flower thrips consume pollen, and it was shown that *Thrips imaginis* and *T. obscuratus* consumed 29 to 843 grains per day, equivalent to 0.2–0.7% of the average total pollen production of a flower per thrips per day. These results suggest that thrips feeding may be negatively affecting crop yield (Kirk 1987).

Irradiation with weak-intensity extremely high frequency (EHF) microwaves improved germination and pollen tube growth (Calzoni et al. 2003).

Pollen is milled by harvesting male flowers in the ‘popcorn’ stage, just before opening. Flowers are then lightly chopped up by a machine, and then placed in a spinning drum consisting of a metal grill through which the anthers fall onto a conveyer belt leaving the larger flower parts to

be discarded on a separate conveyer (Figure 4). The anthers are transferred on trays to a drying chamber in which air at 25°C is passed over the anthers for 12-16 hours. The anthers are then placed in a cyclone separator, in which spinning air separates the pollen from the anthers. Any pieces of flower tissue that are the same mass as the pollen will also be collected with the pollen at this stage (Hopping 1990).

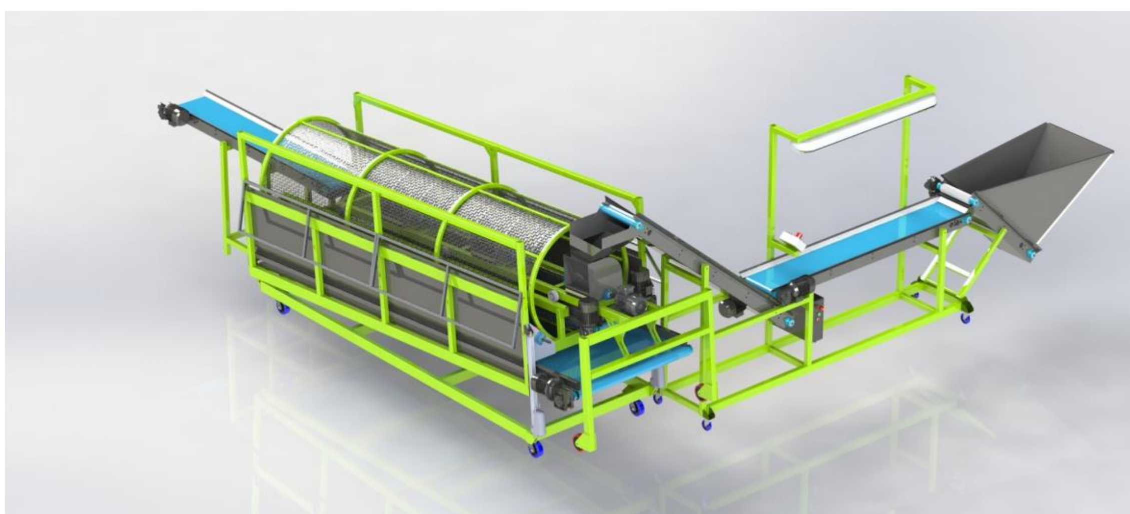

Figure 4. Spinning drum that separates kiwifruit flower parts from anthers. https://frasergear.co.nz/products/pollen-extraction/tri-mill/

3.2 Microbial populations on pollen

3.2.1 Bacteria

Kiwifruit

Very few studies are reported in the literature examining what microorganisms are associated with kiwifruit pollen. In the one study found, bacterial populations were studied by extracting DNA and testing using metabarcoding, which is a next generation sequencing method for examining microbial populations (Kim et al. 2018). Although extraction of DNA can be from dead as well as live microorganisms, it has been shown elsewhere that Psa can be isolated from pollen, and can survive the pollen milling process (Gallelli et al. 2011b; Stefani and Giovanardi 2011; Vanneste et al. 2011; Everett et al. 2012b; Loreti et al. 2014; Tontou et al. 2014; Miller et al. 2015; Balestra et al. 2018a) (Table 1). If Psa can survive the dessication and raised temperatures common during pollen milling (Hopping 1990), there is a risk that other non-spore forming bacteria will also survive, as well as spore-forming bacteria which are more able to survive adverse conditions (Agrios 2005).

Three bacterial phyla were present (Proteobacteria, Actinobacteria and Firmicutes), and *Pseudomonas* spp. was the most abundant genera on pollen from New Zealand and China (Kim et al. 2018). For pollen sourced from South Korea, the most common genera and species were *Pantoea* spp., *Enterobacter cloacae*, *Acinetobacter calcoaceticus*, *Stenotrophomonas* spp., *Pseudoxanthomonas* sp., *Ketogulonogenium vlugarum*, *Methylobacterium jeotgali*, *Exiguobacterium acetylicum*, *Weissella cibaria*, and several uncultured bacteria in the Alcaligenacaea, Sinobacteraceae, Propionibacteriaceae, Micrococcaceae and

Staphylococcaceae. *Pseudomonas* spp., *Duganella zoogloeoides*, *Erwinia aphidicola*, *Rosenbergiella australaborealis*, *Leudonostoc* spp., *Empedobacter* sp., and uncultured bacteria in the Moraxellacaea, Aerococcaceae, and Enterobacteriaceae were most common in imported pollen from China and New Zealand.

None of these bacteria are serious plant pathogens. The only bacterium reported as a plant pathogen was *Erwinia aphidicola*, first isolated from aphids. Recently this bacterium has been reported to cause a fruit spot on pepper in China (Luo et al. 2018).

There are many reports of the virulent bacterial kiwifruit canker pathogen, *Pseudomonas syringae* pv. *actinidiae* (Psa) (known first as Psa3 (Chapman et al. 2012), then later as PsaV (McCann et al. 2013) and biovar 3 (Vanneste et al. 2013)) contaminating pollen. Psa3 was detected from naturally infected pollen by isolations and polymerase chain reaction (PCR) using a number of different primer sets (Table 1) (Gallelli et al. 2011b; Stefani and Giovanardi 2011; Vanneste et al. 2011; Everett et al. 2012b; Loreti et al. 2014; Tontou et al. 2014; Miller et al. 2015; Balestra et al. 2018a). Pollen could also be artificially contaminated with Psa3 (Gallelli et al. 2011a; Everett et al. 2012a; Biondi et al. 2013; Everett et al. 2016; Balestra et al. 2018b).

Table 1. Results of isolations and polymerase chain reaction (PCR) tests for field sourced pollen. Positive indicates detection of *Pseudomonas syringae* pv. *actinidiae*.

Results of isolations	PCR method	Primers	Isolation	Pollen source	Reference
+	conventional	RG[1], GAL[2]	+	Italy	Gallelli et al. (2011a)
+	conventional	KN[3.], RG	+	Italy	Vanneste et al. (2011)
+	conventional	KN, RG	+	Italy	Stefani and Giovanardi (2011)
+	quantitative	RG, RA[4.]	+	New Zealand	Everett et al. (2012b)
+	conventional	RG, GAL, LO[5.]	+?[6.]	Italy	Loreti et al. (2014)
+	conventional	RG, KN,	+	Italy	Tontou et al. (2014)
+	quantitative	RG, RA	+	New Zealand	Miller et al. (2015)
-	conventional	KN2[7], L[8.]	+	New Zealand and China	Kim et al. (2016)
+	conventional	RG, GAL	+	Argentina	Balestra et al. (2018a)

[1.] Rees-George et al. (2010)[2]. Gallelli et al. (2011a) [3].Koh and Nou (2002)[4.]Andersen et al. (2018)[5.] Louws et al. (1994) [6.] Results of pollen isolations were not individually reported, but it was stated that it was difficult to isolate Psa from pollen. [7.] Koh et al. (2014) [8.] Lee et al. (2016)

Other plant pollen

Many studies have been conducted of the microbial populations that contaminate pollen other than kiwifruit. There is no doubt that many different species and genera of bacteria are present, including bee pathogens such as American foulbrood caused by *Paenibacillus larvae* (Bakonyi et al. 2003; Nofouzi and Razmaraii 2015; Teixeira et al. 2018; Moreno Andrade et al. 2019; Pereira et al. 2019), human pathogens including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis* (subspecies *hominis*), *Staphylococcus sciuri* (subspecies *sciuri*), members of the Enterobacteriaceae (e.g. *Escherichia coli*, *Enterococcus faecalis*), *Salmonella* spp. and *Listeria* spp. (Belhadj et al. 2014; Beev et al. 2018; Dinkov 2018) and plant pathogens including fire blight, (*E. amylovora*) (Wilson et al. 1989b), bacterial blast (*P. syringae* pv. *syringae*) (Wilson et al. 1989) and walnut blight (*X. juglandis*) (Olsen et al. 1976). Examination of the microbial communities by next generation sequencing has identified genera of which several species are plant pathogens, such as *Xanthomonas*, *Pseudomonas* and *Erwinia* (Anderson et al. 2013; Shevtsova et al. 2016b; Syromyatnikov et al. 2019).

Also present on bee collected pollen are Firmicutes, Actinobacteria Thermotogae and Proteobacteria. Those bacterial orders that were most common were Thodobacterales, Rhodospirillales, Rickettsiales, Lactobacillales, Petrotogales and Pseudomonadales (Lozo et al. 2015; Moreno Andrade et al. 2018). The most common bacterium was *Pantoea agglomerans* (62%), followed by *Bacillus licheniformis* (9.5%), *Curtobacterium floccumfaciens* (9.5%), *Leuconostoc mesenteroides* (9.5%), *Bacillus megaterium* (4.8%) and *Bacillus subtilis* (4.8%) (Lozo et al. 2015). In a study of bee collected pollen in Russia, the most abundant bacteria were *Acinetobacter* spp. (26%), *Tatumella citrea* (5%), *Lactobacillus* (7%) and *Lactococcus* (13%) (Syromyatnikov et al. 2019). None of these organisms are plant pathogens.

Pollen from silver birch contained members of the Enterobacteriaceae, and mesophilic aerobic bacteria (Shevtsova et al. 2014). Culturing from *Betula* and *Pinus* spp. pollen showed the presence of mesophilic aerobic and anaerobic bacteria, lactobacilli and total coliforms (Shevtsova et al. 2015). *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Arthrobacter*, *Pantoea*, and *Klebsiella* were identified on pollen from *Pinus* spp. using MALDI-TOF mass spectrometry (Shevtsova et al. 2016a). Using the same method, aerobic and anaerobic mesophilic bacteria, lactobacilli, coliform bacteria and *Pantoea agglomerans* were found associated with pollen of *Betula verrucosa* (Shevtsova et al. 2016b). *Pseudomonas* species can be plant pathogens.

Commercially milled bee pollen was examined for bacterial contaminants, and although aerobic mesophilic bacteria were isolated, none were human pathogens (Altunatmaz & Aksu 2016).

Pollen is stored in the hive as beebread which is compacted pollen mixed with exudates from the bee crop (honey stomach) including enzymes that discourage biofilm formation and protective bacteria. Next generation sequencing of 16S ribosomal DNA yielded bacteria from the Xanthomonadales, Bacteriodetes, Enterobacteriaceae, Betaproteobacteria, Halomonadaceae and the Firmicute *Clostridia* (Anderson et al. 2013). No plant pathogens were described.

Isolations showed that the amount of bacterial contamination varied depending on the batch of pollen and on the type of bacteria, but generally ranged from 10^1 to 10^5 cfu/g (Beev et al. 2018; Nikolaieva et al. 2019; Simunovic et al. 2019; Fernandez et al. 2020).

Examination of the source of endotoxins, which are air borne allergens, showed that the source bacteria were not independently present in the air, but instead were associated with *Artemisia vulgaris* (mugwort) pollen. The endotoxin was related to the presence of

Pseudomonas spp. and *Pantoea* spp. on pollen (Oteros et al. 2019). The *Pseudomonas* spp. bacteria were not identified to species level, and therefore it is not possible to determine whether these bacteria were plant pathogens.

3.2.2 Viruses

Kiwifruit

The first report of a virus on kiwifruit was from cuttings imported from China into New Zealand (Clover et al. 2003).

This virus (*Apple stem grooving virus*) was found on kiwifruit imported from Shaanxi Province in China, and caused chlorotic (yellow) symptoms on leaves in mosaic and ringspot patterns, as well as interveinal mottling. The effect on kiwifruit growth was not known. Although predominantly latent, on sensitive varieties of citrus and apple it can cause graft union necrosis (Yanase 1983, Broadbent 1994). Transmission in kiwifruit was by grafting and mechanical transmission, but may also have been by other means because isolates of *Apple stem grooving virus* from *Lilium longiflorum* and *Malus platycarpa* are seed-transmissible (van der Meer 1976; Inouye et al. 1979).

Since then, another 18 viruses have been described (Biccheri et al. 2012; Blouin et al. 2013; Chavan et al. 2009; Pearson et al. 2011; Veerakone et al. 2018; Wang et al. 2016, 2020; Zhao et al. 2019, 2020b; Zheng et al. 2017;). These are classified into three virus groups; non-specialist, kiwifruit adapted and disease inducers.

The non-specialist viruses include two tobamoviruses (*Ribgrass mosaic virus* and *Turnip vein clearing* virus), an alfamovirus (*Alfalfa mosaic virus*; AMV), a tombusvirus (*Cucumber necrosis virus*), a potex virus (*Potato virus X*), a cucumovirus (*Cucumber mosaic virus*; CMV) and a capillovirus (*Apple stem grooving virus*) (Chavan et al. 2009; Clover et al. 2003; Pearson et al. 2011; Zhao et al. 2019). These viruses do not have debilitating effects on kiwifruit and infect many hosts, including weeds that may be under the canopy in orchards acting as reservoirs. Most of these viruses are either symptomless, or cause yellow spots and/or patterns on leaves.

The kiwifruit adapted viruses include eight novel viruses; three vitiviruses (*Actinidia virus A*, *Actinidia virus B* and *Actinidia virus C*; AcVC), a citrivirus (*Actinidia citrivirus*) closely related to *Citrus leaf blotch virus*, two emaraviruses (*Actinidia chlorotic ringspot-associated virus*; AcCRaV and *Actinidia emaravirus 2*; AcEV-2), a potex virus (*Actinidia virus X*), a prunevirus (*Actinidia seed-borne latent virus*), a tospovirus (*Tomato necrotic spot-associated virus*; TNSaV) and a closterovirus (*Actinidia virus 1*; AcV-1) (Blouin et al. 2013; Pearson et al. 2011; Veerakone et al. 2018; Wang et al. 2016; Zhao et al. 2020b; Zheng et al. 2017). These viruses can cause chlorotic leaf symptoms of ringspots, mottles, vein and leaf clearing or interveinal chlorosis, but can also be latent.

The viruses that cause disease include a nepovirus (*Cherry leaf roll virus*; CLRV) (Woo et al. 2002) and an Anulavirus (*Pelargonium zonate spot virus*; PZSV) (Biccheri et al. 2012). CLRV causes leaf spots, fruit deformity, yield reduction, bark cracking and cane wilting (Blouin et al. 2013), and PZSV causes concentric chlorotic and necrotic ring and line patterns on leaves, and forms depressions in fruit resulting in deformity (Biccheri et al. 2012).

Viruses infect plants through the vasculature (Garcia-Ruiz 2019). Distribution of viruses throughout the plant, including the flowers, is likely, but varies from virus to virus. Floral debris in milled pollen could also contain virus particles.

Only recently have extensive virus surveys been conducted in China (Zhao et al. 2019), but these surveys only covered the Shaanxi province. It is possible that there are more viruses in the kiwifruit germplasm found throughout the natural range of kiwifruit in Asia. Most species are found in southern China between 25° and 30° North (Liang 1983; Ferguson and Huang 2007). The center of biological diversity for kiwifruit species spans the provinces of Yunnan, Guangxi, Guizhou, and Hunan, and numerous kiwifruit species are found throughout the rest of the southern part of China. They are less abundant in Tibet, northern India, Japan, Korea, Siberia, and south from Vietnam to Sumatra and Java. For this reason it is possible that there may be more pollen transmitted viruses found in kiwifruit that were previously not known. The discovery rate for new viruses of kiwifruit has accelerated since the first were found in kiwifruit in New Zealand now that kiwifruit in China is being investigated (Figure 5).

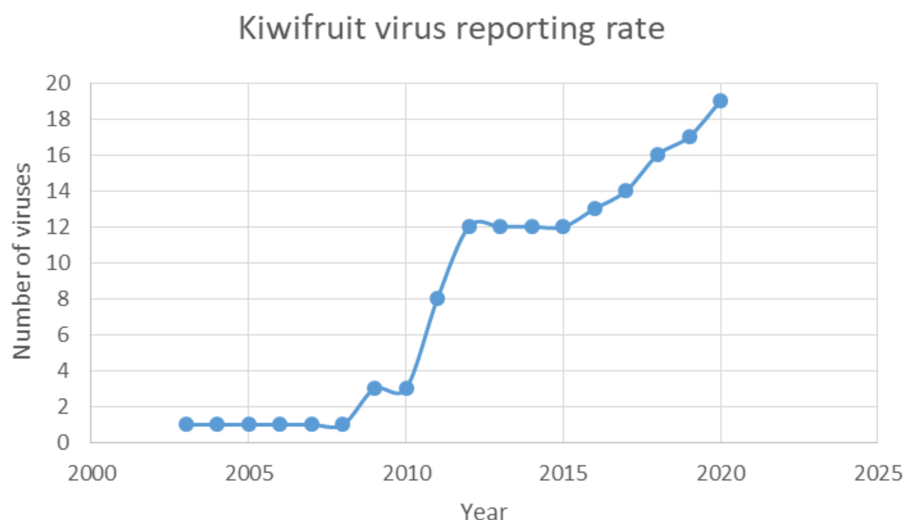

Figure 5. Cumulative number of viruses reported from kiwifruit by year.

Viroids and phytoplasmas

No viroids or phytoplasmas have been reported infecting kiwifruit.

Other plant pollen

Many plant viruses can be pollen transmitted, but of over 1000 known viruses only 44 are pollen transmitted. Transmission is either horizontal, where the plant is infected through the fertilized flower, or vertical, where the seed that results from pollination is infected. A recent review listed 39 viruses from 16 genera that are pollen transmitted (Card et al. 2007), of which all are vertically transmitted and of these 17 are also horizontally transmitted. The hosts range from annual crops (e.g. beans, spinach, cucumbers, peas, potatoes, tomatoes, tobacco, soybean, maize), berries (blueberries, strawberries, raspberries) through to ornamentals such as petunias, chrysanthemums, roses, flowering cherries and beech to productive trees such as apples, walnuts and cherries.

Since the review, pollen transmission has been demonstrated for a further five viruses (*Tobacco mosaic virus* (TMV) (Ellis et al. 2020), *Zucchini yellow mosaic virus* (Harth et al. 2017), *Gentian ovary ring-spot virus* (Isogai et al. 2017), *Apple latent spherical virus* (Nakamura et al. 2011) and *Potato virus Y* (Otulak et al. 2017)) bringing the total to 44.

A reverse transcription-polymerase chain reaction (RT-PCR) assay has been developed that can detect virus contamination of one pollen grain with *Tobacco ringspot virus* (TRSV). This test could be used for testing pollen at the border (Shiller et al. 2010). However, PCR testing depends on knowing what viruses are likely to be present. For kiwifruit, a more general test that detects for e.g. all potyviruses (Wei et al. (2009) would be more useful. Other general virus tests are reviewed in Pallás et al. (2018). In other words, because new viruses are still being discovered in kiwifruit, PCR tests that could detect all viruses in any group would be useful. However, such PCR tests are not currently available. Next generation sequencing techniques have the capability of detecting any known virus, and as costs decrease and ease of use increases could be developed for testing pollen in the future, but again, this test cannot detect unknown viruses. Rubbing samples into herbaceous indicators can detect undescribed new sap-transmissible viruses in the absence of a universal DNA based test, and its use should be continued.

Viroids

Ten viroids have been detected on pollen: *Avocado sunblotch, Coconut cadang-cadang, Hop stunt, Chrysanthemum stunt*, *Potato spindle tuber*, *Peach latent mosaic*, *Columnea latent*, *Tomato planta macho*, *Tomato chlorotic dwarf* and *Pepper chat fruit* (Barba et al. 2007; Bhuvitarkorn and Reanwarakorn 2019; Card et al. 2007; Gramazio et al. 2019; Matsushita et al. 2018; Yanagisawa and Matsushita 2018).

Phytoplasmas

"*Candidatus liberibacter asiaticus*" (CLas), associated with citrus huanglongbing (citrus greening), was detected from pollen collected from infected trees (Lou et al. 2012). Anthers were also found infected by CLas (Stover & McCollum 2011).

3.2.3 Fungi

Kiwifruit

No fungi have been reported from kiwifruit pollen.

Other plant pollen

Fungi from several saprotrophic genera contaminate pollen and do not seem to affect it in any other way. This includes *Acremonium*, *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma* associated with chestnut (*Castanea sativa*) pollen (Kacaniova et al. 2010). Isolations from pollen from dogwood (*Cornus mas*), *Secale strictum*, *Cucurbita pepo* var. *styriaca* and grapes (*Vitis vinifera*) found most frequently *Alternaria alternata*, *Mucor racemosus*, *Mucor mucedo* and *Cladosporium cladosporoides* (Kacaniova et al. 2014). Marigold pollen was contaminated with fungi including *Aspergillus niger*, *A. flavus*, *A. nidulans*, *Chaetomium globosum* and *Phoma* sp. (Sinha 2011). Pollen from *Corylus avellana* contained *Cladosporium*, *Penicillium*, *Alternaria*, *Rhizopus*, *Paecylomyces*, *Aspergillus*, *Mucor* and mycelia sterilia. Isolations were not consistent across 14 samples collected from geographically diverse regions, but numbers ranged from cf. 10^1 to 10^4 cfu/g pollen (Nikolaieva et al. 2019). Several of these genera contain species that are plant pathogens.

Bee harvested pollen from unspecified plants in Slovakia was contaminated most commonly with *Alternaria*, *Cladosporium* and *Penicillium* (Kacaniova et al. 2009), and similar pollen from

Turkey with *Aspergillus*, *Penicillium, Rhizopus, Alternaria, Mucor, Trichothecium, Cladosporium, Monascus* and *Geotrichum* species (Altunatmaz & Aksu 2016).

Pollen from *Pinus* spp. was contaminated with *Penicillium*, *Aspergillus*, *Cladosporium* and *Debaryomyces* identified by MALDI-TOF mass spectrometry (Shevtsova et al. 2016a).

Slovenian bee pollen was shown to contain yeasts and moulds, ranging from zero detection of moulds in three of 14 geographically diverse samples, to a maximum of 10^6 cfu/g for moulds and 10^8 cfu/g for yeasts (Simunovic et al. 2019). Bee pollen from Argentina contained c. 10^4 cfu/g filamentous fungi and c. 10^5 cfu/g yeasts (Fernandez et al. 2020).

Pollen that have blown into water reservoirs are decomposed by 89 species of the Chromista (fungal-like pathogens that produce motile zoospores of which *Phytophthora* is the most well-known), from 75 angiosperm plants (including both dicotyledons and monocotyledons) (Czeczuga & Muszynska 2004), and most commonly in the Peronosporales and Chytridiales from pollen grains of 36 gymnosperm taxa (Czeczuga & Muszynska 2001). Chromista were also responsible for pollen decomposition in tropical soils (Phuphumirat et al. 2011). Several plant pathogens are in the Chromista group.

Other plant pathogenic fungi parasitize pollen, including the ascomycete, *Sydowia japonica*, which infects pollen of Japanese cedar (Hirooka et al. 2013) and has been considered as a control for limiting pollen release by this tree. Cotton pollen was parasitized by *Verticillium dahliae* which penetrated pollen cell walls and destroyed the pollen grains (Ma et al. 2000). *Colletotrichum acutatum* was shown to penetrate pollen and sporulate from infected stigmas of citrus (Marques et al. 2013). *Fusarium proliferatum*, causing rice spikelet rot disease, was shown to damage pollen grains (Sun et al. 2019). The fungus *Mycoceros antennatissimus* gen. et sp. nov. was shown to capture Pinaceae pollen grains and then utilise them as a food source (Magyar et al. 2018)

Alfalfa (*Medicago sativa*) pollen was colonised and parasitized by two mycoparasitic fungi, *Coniothyrium minitans* and *Gliocladium catenulatum,* by directly penetrating pollen cell walls, often through germ pores, resulting in destruction of pollen grains (Huang et al. 2003).

3.2.4 Evidence for the importance of floral parts in pathogen life cycles

Kiwifruit

Blossom blight of kiwifruit is caused by a bacterium that does not yet have a proper name (*Pseudomonas* sp., formerly known as *Pseudomonas viridiflava*). This bacterium rots anthers of flowers, which become a chocolate brown colour visible when the flower opens. Severe infections can rot the entire bud before opening, which then drop from the vine. Milder symptoms can result in misshapen fruit with a ‘dropped shoulder’ (Young et al. 1988), or fruit that develop normally. Quantification and identification of bacteria isolated from macerated buds, flowers and developing fruitlets showed that the population of *Pseudomonas* sp. increased to a maximum at the peak of flowering, then declined as flowers senesced (Everett & Henshall 1994). The overwintering mechanism for this bacterium is not known, therefore the source of inoculum for infections of closed buds in spring is unclear. Because this bacterium infects flowers, it is likely that it also contaminates pollen (Phatak 1980). Blossom blight symptoms can be mild, and therefore it is possible that infected flowers could be harvested for pollen production. Psa3 survives the pollen milling process, which suggests that the closely related blossom blight bacterium would also survive and be present on commercial

pollen. Whether application of contaminated pollen leads to new infections is not known, but is certainly possible. Close monitoring of a blossom blight epidemic in a Kumeu orchard showed that it was polycyclic, which means that there was spread from the first flowers showing symptoms to healthy flowers (Everett & Henshall 1994) which may have been facilitated by bees during pollination with contaminated pollen. However, there is no direct evidence for the means of secondary spread of blossom blight during epidemics.

Psa3 is reported to infect flowers (Everett et al. 2011). Flower buds and petals were proposed to become infected in spring by exudates produced from cankers on twigs, leaders and the trunk (Ferrante et al. 2012). However, infection of young shoots by systemic migration and of twigs by rainsplash was most commonly from leaf spots (Ferrante et al. 2012). Infected flowers were observed to become necrotic and fall to the ground, presumably providing another source of rain-splashed inoculum for infections during spring. These authors proposed that the disease cycle was driven by inoculum produced from leaf spots rather than infected flowers. However, inoculum is on and in necrotic flower tissue, and it is likely that some infections would result from rain-splash to healthy kiwifruit tissue.

Flower infections by the fungal fruit rot pathogen *Botrytis cinerea* are considered to be an important part of the life cycle for blossom end rots of kiwifruit that develop during ripening for kiwifruit grown in California (Michailides and Elmer 2000). Inoculations of flowers were shown to result in a higher incidence of blossom end rots. Michailides and Elmer (2000) suggest that botrytis infections of kiwifruit in California originate from infected receptacles and sepals. Other Californian researchers assert that flower parts do not have a major impact on the development of botrytis rot (Sommer and Suadi 1985). In New Zealand, flower or sepal infections were not related to botrytis rot incidence, but necrotic leaf discs incubated to express botrytis were related (Manning et al. 2010). It is possible that the importance of flower infections in California is due to the climatic differences from New Zealand. Similar to the results described from California, botrytis stem end rots of kiwifruit in Chile were correlated to botrytis infections of styles, sepals and receptacles, but there was considerable seasonal variability (Riquelme-Toledo et al. 2020). However, botrytis can infect kiwifruit flowers, and therefore there is a possibility that it could contaminate pollen, either directly or in floral debris.

Neofabraea actinidiae, another fungal pathogen causing postharvest fruit rots (Tyson et al. 2019), was shown to infect mainly during flowering (Rheinlander et al. 2005; Fullerton et al. 2007) following inoculations with Nit mutants. Presumably developing fruit are infected, because *N. actinidiae* infects lenticels. The disease cycle of *N. actinidiae* is largely unknown, and the role of floral parts remains undetermined (Tyson et al. 2019).

Sclerotinia sclerotiorum causes scarring on developing fruitlets, and badly affected fruit drop off the vine. Ascospores of *S. sclerotiorum* do not directly affect fruit, but first invade flower petals or stamens which, if in contact with fruit, will invade in wet humid conditions (Hoyte et al. 2001) to cause a lesion. The following spring ascospores are produced from apothecia produced from sclerotia in rotting fruit that have fallen to the ground (Agrios 2005). Because this fungus infects flowers, it is possible that contaminated floral debris containing propagules of sclerotinia could be in milled pollen. There is no evidence that contaminated pollen can cause disease when applied to healthy flowers, but it is possible given the right environmental conditions.

A review of the scientific literature did not find any reports of viruses infecting kiwifruit flower parts. However, because viruses are spread through the vasculature of infected plants (Garcia-Ruiz 2019), it is likely that virus particles would be found in flowers on infected vines. Once again, there is no evidence that viruses can be spread to healthy plants to cause new infections on contaminated pollen, but it is possible.

In conclusion, several kiwifruit pathogens infect flowers, that is: Psa, blossom blight, botrytis, *N. actinidiae* and *S. sclerotiorum*. Inoculum produced from infected flowers could be contributing to new infections, and thus flowers are part of the disease cycle for these pathogens.

Other plants

Fungi

A large number of fungi infect flowers, comprising 105 fungal species from 48 genera (Ngugi and Scherm 2006) (Table 2). These can be categorised into three broad groups, those that are opportunistic and unspecialised (52 species), and specialised fungi that infect through either the female flower parts (22 species) or through the growing tip (30 species). Flowers are excellent microbial habitats, excreting various nutrient rich food sources comprising nectar, stigmatic exudate and pollen exudate. Some floral tissues, such as petals and the stigmatic surface, are very thin-walled, allowing easy entry by fungal pathogens. The stigma-style pathway allows entry into the plant tissue similar to an open wound. Flowers are typically positioned to enable easy dispersal of pollen by wind or pollinators, which also advantages dispersal of flower infecting fungi, regardless of whether they are vectored, wind or water dispersed. Infection of flowers allows access to seeds, with obvious advantages to fungal dispersal and nutrient supply. High rates of vertical transmission (infection of seeds) of as much as 100% can ensue (Schardl 1996; Clay and Schardl 2002). Endophytic colonisation of flowers can result in stem-end rots of the resultant fruit, as in the case of infection of mangoes by *Nattrassia mangiferae* (Johnson et al. 1992).

Group 1 fungi are typified by general necrotrophs such as *Botrytis* and *Monilinia*. These pathogens infect other parts of the plant as well as flowers, and typically do not produce specialised survival propagules in flower parts. Infection results in partial sterilisation as some flowers are destroyed.

Group 2 fungi invade the ovary of the host through the female flower parts. Ergot pathogens *Claviceps purpurea* and *C. africana* and the corn smut fungus *Ustilago maydis* are examples. These all produce survival structures in inflorescences e.g. sclerotia, pseudosclerotia or teliospores. Infection results in partial sterilisation as some flowers are destroyed.

Group 3 fungi infect flowers systemically through the growing tips and include many seed-borne smut fungi (e.g. *Ustilago nuda* f.sp. *hordei*). Also in this group are pathogens that infect host seedlings from soilborne teliospores (e.g. *Tilletia controversa,* wheat dwarf bunt) or can infect through vegetative tissue (e.g. *Microbotryum violaceum*, which causes anther smut). Infection of flowers can completely replace host tissue with resting spores, and they can grow endophytically within the host (Ngugi & Scherm 2006).

Pollen can stimulate fungal spore germination, and Fusarium head blight of wheat caused by *Gibberella zeae* was more severe when inoculated to intact flowers rather than those from which anthers were removed (Ngugi & Scherm 2006). Thus nutrients exuded from pollen can aid infection by fungal pathogens.

Buds are commonly a means of overwintering for bacterial and fungal plant pathogens of deciduous trees (Miller and Bollen 1946; Agrios 2005). Floral structures, including bud scales, fall to the ground after fertilisation of the ovule, and can be a source of inoculum for later infections by *Colletotrichum acutatum* during late summer in New Zealand (Everett et al. 2018).

Some plant pathogens “have evolved sophisticated strategies to change the physiology and development of their hosts in a manner that favours pathogen reproduction and spread” (Haueisen & Stukenbrock 2016). For instance, the smut fungus *Microbotryum lychnidis-dioicae* produces teliospores only inside anthers of its host, to contaminate pollinators that thereby spread the fungus to healthy plants. To overcome limitations to its dispersal due to its host being dioecious, the fungus stimulates anther production in female plants (Haueisen and Stukenbrock 2016).

Bacteria

Several bacterial pathogens infect flowers or catkins (the structure that produces wind-dispersed pollen) including fire blight (*E. amylovora*) (Agrios 2005), *Erwinia stewartii* (maize bacterial wilt) (Phatak 1980), *P. syringae* pv. *syringae* (bacterial blast) (Mansvelt and Hattingh 1987) and walnut blight (*X. juglandis*) (Ark 1944 a,b). The life cycle of *Pseudomonas syringae* pv. *tabaci* also includes flower capsules on which this bacterium overwinters (Agrios 2005).

Viruses

If a virus infects a plant it often spreads in the vasculature throughout the plant (Garcia-Ruiz 2019), and it is thus common for flowers to be infected. Agrios (2005) states “systemic distribution of some viruses is quite thorough and may involve all living cells of a plant”. Other viruses are restricted to local lesions, and the distribution of viruses in plants does depend on the individual virus. An example of viruses that infect flowers are *Strawberry latent ringspot virus*, *Prunus necrotic ringspot virus*, *Apple mosaic virus*, *Prune dwarf virus* and two uncharacterised viruses that were easily transmitted from flowering cherry flowers to herbaceous indicators (Everett et al. 1993).

Viruses are not motile, and must rely on internal plant transport systems (phloem and xylem) and cell to cell transmission to spread within the plant.

For plant to plant spread, viruses can be vectored (by insects) or they can be mechanically transmitted by animals brushing infected plants to pick up virus particles and then depositing these on healthy plants. Usually viruses will enter through damaged tissue caused by the passing of these animals. Viruses can also pass from an infected leaf by wind rub to an adjacent healthy leaf (Agrios 2005).

Viruses can also be seed transmitted, graft transmitted or transmitted by pollen. However, of the over 1000 known plant viruses, only 44 are reported to be transmitted by pollen (Card et al. 2007). The role of flowers in the pollen transmitted virus life cycle is by attracting pollinating insects to pick up infected pollen. The role of catkins is to release infected pollen that is wind disseminated. Otherwise flowers can transmit viruses by rubbing on adjacent healthy plant parts, or by animals brushing past and carrying virus particles to healthy plants (Agrios 2005).

The role of flowers in the life cycle of viroids and phytoplasmas, which are also non-motile, is similar to viruses. They are similarly transmitted by vectors, infected pollen, seed, grafting, and mechanical transmission, and can also be distributed throughout the plant in the vasculature, dependent on the individual organism (Agrios 2005).

Table 2. Fungal genera and corresponding number of species that infect flowers, and those genera reported on kiwifruit according to Pennycook (1989).

Genus	No. of species	Genera reported on kiwifruit
Albugo	1	-
Alternaria	5	+
Antbracoidea	3	-
Ascochyta	1	-
Atkinsonella	1	-
Balansia	1	-
Botryotinia	1	-
Botrytis	4	+
Brachysporium	1	-
Calonectria	2	+
Cercosporella	1	-
Ciborinia	1	-
Cladosporium	1	-
Claviceps	13	-
Cochliobolus	2	-
Colletotrichum	5	+
Diaporthe	1	+
Didymella	2	-
Dothiorella	1	+ [1.]
Epichloe	4	-
Fusarium	1	+
Gibberella	2	-
Glomosporium	1	-
Lewia	1	-
Microbotryum	2	-
Microsphaera	1	-
Monilinia	7	+
Mycosphaerella	2	-
Nattrassia	1	-
Oidium	1	-
Ovulinia	1	-
Peronophythora	1	-
Phaeosphaeria	1	-
Phytophthora	3	+
Pycnostysanus	1	-
Rhizopus	1	-
Sclerophthora	1	-
Sclerotinia	1	+
Sorosporium	1	-
Sphacelotheca	3	-
Sporisorium	2	-
Thecaphora	1	-
Tilletia	4	-
Tolyposporium	1	-
Ustilago	11	-
Venturia	1	-

[1.] *synonym for Botryosphaeria*

Chromista: Oomycetes

The Oomycetes are fungus-like micro-organisms in the kingdom Chromista. Species of two of the genera in this kingdom, *Phytophthora* and *Pythium*, inhabit the soil and generally cause root rots, damping off (a collapse of young seedlings) and trunk diseases. Some also infect fruit from spores splashing onto low hanging fruit or fruit that have fallen onto the ground, but reports of flower disease were not found. If a plant in flower is killed by these fungi, it is possible that the dead tissue would be invaded and could produce spores to infect healthy plants. It is also possible that fungal spores in the soil could be rain-splashed to contaminate flowers, although there is no evidence to support this hypothesis.

Ngugi and Scherm (2006) include three reports of *Phytophthora* infecting flowers (Table 2). Further literature searching found a report of *Phytophthora ramorum* infecting flower buds of *Camellia* (Tjosvold et al. 2006), *Phytophthora* spp. attacking young buds and flowers of mature orchids (Ann 1995) and *Phytophthora arecae* attacking the flower stalks of areca nuts (Stamps 1985).

Late blight, caused by *Phytophthora infestans*, causes primarily aerial symptoms of leaf spots, blight and total collapse of potato and tomato plants. There are no descriptions of flower disease (Agrios, 2005), although if a plant in flower is blighted the flowers will also be infected and killed. Dead infected tissue produces more spores to infect healthy plants.

Downy mildew is also caused by genera in the Chromista, in the family Peronosporaceae. *Peronospora*, *Plasmopara*, and *Pseudoperonospora* species cause a foliar disease known as downy mildew. After infection large, angular or blocky yellow patches appear on the tops of leaves which eventually turn brown. Associated water-soaked lesions form on the underside of the leaves which can coalesce to destroy leaf tissue without affecting stems or petioles. Young flower clusters of grapes can be covered with downy growth and die. Dead infected tissue will produce more spores to infect healthy plants (Agrios, 2005).

Relevance to kiwifruit

There is no record of downy mildew infecting kiwifruit, and the *Phytophthora* species that infect kiwifruit cause root rots and trunk diseases (Pennycook 1989). Infection of the lower parts of the plant by these fungi disrupt the flow of water and nutrients to the upper part of the plant leading to wilting and death. Colonisation of flowers is likely only after the entire plant is killed. It is unlikely that flower debris infected by these members of the Chromista would contaminate milled kiwifruit pollen, as decayed flowers would not yield anthers containing viable pollen. However, it is possible that rain splashed spores could contaminate flowers and floral debris containing these spores be a component of milled pollen.

3.2.5 A critical review of the evidence for pollen transmission

There can be no doubt that pollen is contaminated with viruses, viroids, a phytoplasma, bacteria, yeasts and fungi, as described above. This is not surprising as pollen consists not just of pollen grains but also contaminating floral tissue which may harbour these organisms. Even if pollen is not itself colonised or invaded by plant pathogens, it may be externally contaminated by microorganisms that colonise flower parts, either directly or in pieces of floral tissue.

There is evidence of contamination of kiwifruit pollen with several bacteria and one virus. Even though there are no reports of fungi contaminating kiwifruit pollen, there are three pathogens that infect floral parts (botrytis, sclerotinia and *N. actinidiae*) that could be contaminating milled

pollen. This is supported by evidence from examination of pollen from other plants, fungi have been directly detected on pollen.

No viroids or phytoplasmas have been detected in kiwifruit, but it is possible that these are present in yet to be tested remote kiwifruit populations in the native habitats of South-East Asia. Some viroids and a phytoplasma (citrus greening) contaminate pollen from other plants, therefore there is a possibility (if these are ever found on kiwifruit) of pollen contamination with these microorganisms.

However, contamination does not necessarily mean that pathogens are transmitted on pollen. Therefore the evidence for pollen transmission also needs to be available to accurately assess the risk.

Evidence for pathogen transmission on pollen is sometimes indirect, by pollinators e.g. bees. However, if pollen is contaminated by a pathogen, then it can also spread the pathogen to bees directly or on pollen that the bees could transfer onto healthy plants.

Transmission of pathogens by the pollinating insect is probably facilitated by damage to the plant during pollination, therefore a lower rate of transmission would be expected if pollen was applied in the absence of bees. Some pollen is wind spread, and during commercial production kiwifruit pollen is applied artificially by spraying onto flowers, also in the absence of pollinating insects. There is evidence that wind spread pollen can transmit a bacterial disease, which is discussed further below, and thus it is possible that artificially applied kiwifruit pollen could also spread disease.

Bacteria

Phatak (1980) conducted a literature review of the role of seeds and pollen in the spread of bacteria, suggesting that *Erwinia stewartii* (maize bacterial wilt) and *X. juglandis* (walnut bacterial blight) may contaminate pollen and pollen transmission may occur. He indicates that pollen transmission of pathogens is relatively little understood and conclude[s] by stating “there is real danger of host pollen contamination of practically any bacterial pathogen [which] may have infected the host before or at the time of pollen formation/maturation”.

There is evidence for pollen transmission of bacterial walnut blight, *Xanthomonas juglandis,* (Ark 1944a), which was found to overwinter in diseased leaves and catkin buds. Catkins are the structures that produce pollen. The bacterium was present on the pollen of diseased catkins. Walnut pollen is spread by wind, therefore catkins are not visited by pollinating insects. Frequently catkins were partially infected, and pollen was readily contaminated and broadcast for a considerable distance causing infection whenever environmental conditions were favourable. Leaves experimentally dusted with contaminated pollen developed blight lesions. Ark (1944b) presents further evidence of pollen dissemination of walnut blight when he detected recently pollinated nuts that were completely blighted on trees that had no foliar blight or other lesions. Wash water from young healthy leaves and nuts yielded virulent cultures of blight and he concluded that the disease was induced by abundant contaminated wind borne pollen on leaves and young nuts.

In a different climate (Oregon) Miller & Bollen (1946) could not demonstrate the presence of *X. juglandis* on pollen from walnut blighted catkins. This is possibly because of the difference in climate, or some other unknown factor. In support of the earlier work of Ark (1944a,b) *X. arboricola* pv. *juglandis*.was later isolated from walnut pollen in France (Giraud et al. 2010).

Overall, there is both evidence of *X. juglandis* contaminating pollen, and evidence of infection following application of contaminated pollen to healthy plants.

Fire blight (*Erwinia amylovora*) infects blossoms of pear or apple, which remain on the tree whilst blighted. The bacteria have been observed multiplying on the stigmatic surfaces of the flower, and was shown to heavily contaminate pollen grains (Wilson et al. 1989b). Inoculation of stamens of freshly opened flowers resulted in *E. amylovora* colonising anthers, invading anther locules via the ruptured dehiscence zone, and rapid multiplication within the locule which resulted in heavy contamination of pollen grains.

The bacterium can be transferred to uninfected blossoms by bees or other pollinating insects (Johnson et al. 1993; Sabatini et al. 2006; Wilson et al. 1989b). Sabatini et al. (2006) set pollen collection monitoring stations at the entrance to honey bee hives in orchard environments known to be contaminated with fire blight (*E. amylovora*). They detected *E. amylovora* in at least one sample of collected pollen.

Although a direct link between contaminated pollen and spread of fire blight has not been shown, and bees could also become contaminated by *E. amylovora* in nectar and as droplets on flowers, it is likely that contaminated pollen is also a source of spread of fire blight to healthy trees.

In summary, there is evidence that pollen is contaminated by *E. amylovora*, and that bees can spread fire blight to healthy plants. It is therefore likely that *E. amylovora* can be spread to healthy plants by contaminated pollen.

Supporting the possible direct spread of fire blight on contaminated pollen, pollen artificially contaminated with two species of *Pseudomonas* used for biological control of fire blight successfully spread these bacteria to apple flowers by bees (Thomson et al. 1992; Johnson et al 1993a,b; Vanneste 1996; Johnson and Stockwell 1998; Pusey 2002).

Pattemore et al. (2014) showed that bees doused with pollen contaminated with a strain of *Pseudomonas syringae* pv. *syringae* (Pss) spread the bacterium throughout the hive within 24 h. Pss could still be detected 14 days after introduction of doused bees into the hive. Although not tested, it is likely that Pss was also spread by bees from the contaminated hive to visited blossoms.

American foulbrood is a bacterial disease of bees caused by *Paenibacillus larvae* which has been detected on pollen by several authors (Bakonyi et al. 2003; Teixeira et al. 2018; Moreno Andrade et al. 2019; Pereira et al. 2019). It is well recognised that contaminated pollen is an important means of spread of American foulbrood (Moreno Andrade et al. 2019).

The evidence for this is based on feeding pollen contaminated with *Paenibacillus larvae* to healthy honey bees leading to infection of the hive with foulbrood (Gochnauer & Corner 1974). Moreno Andrade et al. (2019) and Melathopoulos et al. (2004) state pollen transmission is an important means of spread for foulbrood. As a consequence pollen that is fed to honey bees is irradiated to prevent spread (Anonymous 2013). There is no doubt that foulbrood is transmitted by pollen to healthy bees.

Kiwifruit

There is evidence for only one bacterium transmitted to healthy vines by kiwifruit pollen, and that is Psa. This has been controversial, with conflicting reports in the literature and criticisms of the methodology used in various studies which are catalogued and discussed in the Judgement

for the Strathboss versus Attorney-General court case conducted in 2017 (also known as the Kiwifruit Claim) (Mallon 2018).

The judgement by Judge Mallon was that the incursion of Psa into New Zealand was most probably from a consignment of what is termed in the industry as ‘rough pollen’ (Mallon 2018). This consists of anthers, as well as pollen, and clearly in this case transmission was from either the pollen, the associated plant material, or from both. A detailed examination of the evidence from which Judge Mallon reached this judgement during the Kiwifruit Claim trial is in Appendix 1.

Spread of Psa3 during 2014 and 2015 in South Korea was attributed to Psa3-contaminated pollen imported from New Zealand and China (Kim et al. 2016). The evidence was based on PCR testing of imported pollen, which was positive for Psa3, and inoculation experiments with infected pollen applied during flowering and inspected for symptoms 3 weeks later. Bacteria were isolated from resulting symptomatic material and identified as Psa3, proving Koch’s postulates (Kim et al. 2016).

Examination of naturally contaminated pollen showed that 4–5% of pollen harvested from closed flowers and 10–80% of pollen from open flowers was contaminated with Psa. The authors suggested that bumble bees used to pollinate kiwifruit orchards were contaminating pollen in open flowers, as well as natural spread from inoculum sources in the orchard facilitated by rain and wind (Balestra et al. 2018b). Microscopical examination showed that infection of anthers by inoculating flowers with bacterial suspensions resulted in pollen contaminated on the outside with Psa (Balestra et al. 2018b). Psa was never found inside pollen grains.

There is also experimental evidence of transmission of Psa by milled pollen (Table 3). As is shown in Figure 1, milled pollen is also contaminated with other plant tissue, so where milled pollen is used, remnants of floral tissue are also part of the pollen sample.

Stefani and Giovanardi (2011) conducted pollination experiments in the glasshouse and in the field. Koch’s postulates were proven when plants in the glasshouse were pollinated with an aqueous suspension artificially contaminated by Psa3. The trial was not set up in a randomised block design. Application of pollen in the field was by dusting, but symptoms were not caused nor was Psa3 re-isolated, and thus Koch’s postulates were not proven.

Pollen transmission in the field was proven by Tontou et al. (2014) following planting a study orchard 100 km away from commercial kiwifruit production in Italy. Pollen was applied using both wet and dry application methods, and leaf spot symptoms were observed in leaves during the second season after application. Psa3 was re-isolated from these leaf symptoms, thus proving Koch’s postulates. In the second season Psa3 was also isolated from a few control plants. These infections could have been caused by secondary spread from inoculum produced on plants pollinated with contaminated pollen. Cankers were observed in autumn of the second season.

In New Zealand application of PCR positive pollen in an aqueous suspension to wounded and unwounded leaves of kiwifruit seedlings in the glasshouse did not result in detection of Psa3 when leaves were harvested and tested by PCR and isolated 3 weeks later (Miller et al. 2015),.

However, a similar study in New Zealand (Everett et al. 2012b) using different methodology did induce leaf spots following inoculation of naturally contaminated pollen to kiwifruit seedlings. The stored kiwifruit pollen was PCR positive, and in an experiment in which pollen was added to

bacterial growth media it was shown that the amount of Psa increased over time, proving that the Psa was not only PCR positive, but was alive. Psa was re-isolated from the leaf spots, proving Koch's postulates.

A fluorescent labelled strain of Psa3 was used to contaminate pollen that was then used to inoculate flowers in a glasshouse study in Italy (Donati et al. 2018). Bacterial cells were observed to spread from styles to the flower stalk 10 days after inoculation, and were observed to invade the vasculature. In some flowers visible bacterial ooze was produced with the nectar (Donati et al. 2018). Leaf spots were observed after 2 months, and 4 months after inoculation the bacteria was distributed throughout the plant, including roots.

Exposure of female kiwifruit plants 'Hort16A' to naturally infected male vines resulted in 100% contaminated flowers after 10 days, with 48% leading to systemic infection, and in 'Hayward' 44% of flowers became infected leading to 25% systemic infections (Balestra et al. 2018b).

Table 3. Inoculation studies with pollen contaminated by *Pseudomonas syringae* pv. *actinidiae* (Psa) biovar 3.

Koch's postulates proven	Method	Artificially contaminated	Symptoms	Glasshouse or field	Reference
-	1. dusting during flowering (naturally contaminated and control pollen)	-	No symptoms	GH	Stefani and Giovanardi (2011)
+	2. spray aqueous suspension (artificially contaminated pollen)	+	Leaf spots		
-	1. dusting during flowering (naturally contaminated and control pollen)	-	No symptoms	F	Stefani and Giovanardi (2011)
+	1. dusting and spray aqueous suspension	-	Leaf spots, canker	F	Tontou et al. (2014)
-	1. spray aqueous suspension on leaves - half of which were wounded	-	No symptoms	GH	Miller et al. (2015)
+	1. incubate pollen for 4 hours in nutrient broth followed by rub inoculation with carborundum	-	Leaf spots	GH	Everett et al. (2012b)
+	Natural spread	-	Systemic infection	GH	Balestra et al. (2018b)
+	Spraying flowers with fluorescent labelled Psa3 contaminated pollen	+	Leaf spots oozing	GH	Donati et al. (2018)

In conclusion, Psa3 has been conclusively proven to contaminate kiwifruit pollen by a number of different authors, either by PCR, by isolations or by microscopical examination (Gallelli et al. 2011a, Vanneste et al. 2011, Stefani and Giovanardi 2011, Everett et al. 2012b, Loreti et al. 2014, Tontou et al. 2014, Miller et al. 2015, Balestra et al. 2018a). Secondly, application of contaminated pollen to kiwifruit plants was shown to cause symptoms of Psa, thus proving

Koch's postulates (Table 3) (Tontou et al. (2014), Donati et al. (2018), Stefani and Gionvanardi 2011, Everett et al. 2012b, Balestra et al. 2018).

Viruses

Pollen transmission of viruses has long been considered, with the first observations made in 1918 (Reddick & Stewart 1918) and first evidence in the 1960s and 70s. In those experiments, reviewed in Mink (1993), glasshouse grown squash flowers were tied to prevent pollination, then selected flowers pollinated with *Prune dwarf virus* (PDV) contaminated pollen and others with healthy pollen, resulting in 10% infection of treated plants. A further experiment was conducted in the field, where sour cherry trees, some healthy and others infected with PDV and *Prunus necrotic ringspot virus* (PNRSV), were grown in screened compartments. Arthropods were introduced into selected compartments during bloom, and only the introduction of honey bees resulted in previously healthy trees becoming infected. Thrips were not implicated in disease transmission. In a third set of experiments, sour cherry trees were debloomed and spread of PDV + PNRSV compared with trees that flowered. No spread was observed to debloomed trees. Virus contaminated pollen was used to pollinate various *Prunus* species by hand or by bees, resulting in some virus transmission, albeit very low (Mink 1992). When both thrips and contaminated pollen were introduced to herbaceous plants, transmission rates were a lot higher (Mink 1992). It was concluded that pollen transmission requires contaminated pollen, an insect that moves pollen from infected to healthy flowers, and mechanical damage of flower parts by an insect such as thrips or honey bees for successful transmission of ilarviruses and nepoviruses (Mink 1993). This was later confirmed, and applied to the sobemoviruses and carmoviruses as well (Hull 2014).

Two mechanisms for pollen transmission were shown, direct infection of the mother plant (horizontal transmission) and infection of the gamete only (vertical transmission). Numerous techniques such as electron microscopy, dot blot, RT-PCR, in situ hybridization and immuno-gold labelling were used to show that virus particles of *Barley stripe mosaic virus* (BSMV), TRSV, PNRSV, AMV were inside pollen grains (Hull 2014). Although this suggests that infection occurs via infected sperm, PNRSV particles were found in the vegetative but not the generative cell, precluding infection of the embryo by the sperm cell. However, infection via sperm cell probably occurs for the other viruses. Therefore it is suggested that some viruses are picked up by the germinating pollen tube and carried into the ovule. The ability to transmit is related to their ability to invade meristematic regions at the appropriate time. Presence of virus on pollen does not necessarily equate to transmission. For example, TMV occurs in high concentrations on the exine of pollen grains, but is not pollen transmitted.

The importance of pollen transmission for spread of viruses that have this mechanism has not always been explored. However, horizontal transmission resulting in infection of the mother plant was demonstrated for PDV and by *Raspberry bushy dwarf virus* (RBDV) in raspberry (Hull 2014). Neither of these viruses can be transmitted except by pollen. Most viral infections only result in infected seed (vertical transmission) and 19 out of 44 can be horizontally transmitted as well (Hull 2014) (Table 4).

In conclusion, the evidence for pollen transmission for plant viruses is strong and is derived from pollination with virus infected pollen, and subsequent testing of the mother plant and seeds. Although the transmission rate can be low, when facilitated by insects that damage the flowers, the rate is higher.

Card et al. (2007) reported pollen transmission for five viroids (*Avocado sunblotch, Coconut cadang-cadang, Hop stunt, Chrysanthemum stunt* and *Potato spindle tuber*). Since the

review, pollen transmission has been demonstrated for a further five viroids: *Peach latent mosaic* (Barba et al. 2007), *Columnea latent* (Bhuvitarkorn and Reanwarakorn 2019), *Tomato planta macho* (Matsushita et al. 2018; Yanagisawa and Matsushita 2018), *Tomato chlorotic dwarf* (Gramazio et al. 2019) and *Pepper chat fruit* (Yanagisawa and Matsushita 2017), bringing the total to 10 (Table 4).

Pollen transmission for citrus greening, "*Candidatus liberibacter asiaticus*" (CLas), was considered a real risk in Florida (Stover & McCollum 2011).

Table 4. Viruses and viroids that are pollen transmitted, and whether transmission is horizontal (infects mother plant) (H) or vertical (infects seeds) (V).

Genus	Species	Name	Transmission
Viruses			
Alphacryptovirus	ACV1	*Alfalfa cryptic virus 1*	V
	BCV1	*Beet cryptic virus 1*	V
	BCV2	*Beet cryptic virus 2*	V
	RYEV	*Radish yellow edge virus*	V
	RGCV	*Ryegrass cryptic virus*	V
	VCV	*Vicia cryptic virus*	V
Alfamovirus	AMV	*Alfalfa mosaic virus*	H,V
Anulavirus	PZSV	*Pelargonium zonate spot virus*	V
Badnavirus	KTSV	*Kalanchoe top-spotting virus*	V
Comovirus	CPSMV	*Cowpea severe mosaic virus*	V
	ALSV	*Apple latent spherical virus*	V
Cucumovirus	CMV	*Cucumber mosaic virus*	V
Goravirus	GORSV	*Gentian ovary ringspot virus*	H,V
Hordeivirus	BSMV	*Barley stripe mosaic virus*	H,V
Idaeovirus	RBDV	*Raspberry bushy dwarf virus*	H,V
Ilarvirus	AV2	*Asparagus virus 2*	H,V
	BlShV	*Blueberry shock virus*	H,V
	FCILV	*Fragaria chiloensis latent virus*	H,V
	PDV	*Prune dwarf virus*	H,V
	PNRSV	*Prunus necrotic ringspot virus*	H,V
	SPLV	*Spinach latent virus*	H,V
	TSV	*Tobacco streak virus*	H,V
Nepovirus	AVB	*Arracacha virus B*	V
	AYRSV	*Artichoke yellow ringspot virus*	H,V
	BLMOV	*Blueberry leaf mottle virus*	H,V
	CLRV	*Cherry leaf roll virus*	H,V
	LALV	*Lucerne Australian latent virus*	V
	RpRSV	*Raspberry ringspot virus*	V
	TRSV	*Tobacco ringspot virus*	V
	TBRV	*Tomato black ring virus*	H,V
	TORSV	*Tomato ringspot virus*	H,V
Nucleorhabdovirus	EMDV	*Eggplant mottled dwarf virus*	V
Potyvirus	BCMV	*Bean common mosaic virus*	V

Genus	Species	Name	Transmission
	LMV	*Lettuce mosaic virus*	V
	PSbMV	*Pea Seed-borne Mosaic virus*	V
	PVY	*Potato virus Y*	V
	SMV	*Soybean mosaic virus*	H,V
	SCMV	*Sugarcane mosaic virus*	H,V
	ZYMV	*Zucchini yellow mosaic virus*	V
Sobemovirus	SoMV	*Sowbane mosaic virus*	H,V
Tepovirus	PVT	*Potato virus T*	V
Tobamovirus	TMV	*Tobacco mosaic virus*	V
Tobravirus	TRV	*Tobacco rattle virus*	V
Tymovirus	TYMV	*Turnip yellow mosaic virus*	V
Viroids			
Avsunviroid	ASBVd	*Avocado sunblotch viroid*	V
	PLMVd	*Peach latent mosaic viroid*	H
Hostuviroid	HSVd	*Hop stunt*	V
Pospiviroid	CSVd	*Chrysanthemum stunt viroid*	H,V
	CCCVd	*Coconut cadang cadang viroid*	V
	CLVd	*Columnea latent viroid*	V
	PCFVd	*Pepper chat fruit viroid*	H,V
	PSTVd	*Potato spindle tuber viroid*	H,V
	TCDVd	*Tomato chlorotic dwarf viroid*	V
	TPMVd	*Tomato planta macho viroid*	H,V

Data from (Card et al. 2007; Hull 2014; Matsushita et al. 2018). Only viruses in the EPPO Global database are reported.

Kiwifruit

Blouin et al. (2013) comments that pollen transmission of CLRV and PZSV still needs to be proven for kiwifruit, even though PZSV has been shown to be pollen transmitted in tomato (Lapidot et al. 2010). CLRV shows host-specific strain differentiation, probably due to its exclusive mode of transmission through seed and pollen (Rebenstorf et al. 2006), so it seems likely that it is also pollen transmitted in kiwifruit (Table 5).

Although there is no evidence for pollen transmission of CMV and AMV in kiwifruit, these two viruses have been reported to be easily pollen transmitted elsewhere, e.g. in spinach, *Medicago* spp., *Arabidopsis* for CMV and lettuce, clover, *Solanum* spp. and *Medicago* spp. for AMV (Valkonen et al. 1992; Pathipanawat et al. 1995; Yang et al. 1997; Card et al. 2007; Cobos et al. 2019) (Table 5).

Recently an additional seed-borne virus has been found, *Actinidia seed-borne latent virus* (ASbLV) (Veerakone et al. 2018). Research has shown that ASbLV is pollen borne (Amponsah et al. in preparation). In this research pollination with ASbLV infected pollen to healthy female flowers resulted in 81-86% transmission as determined by PCR testing of seedlings.

The kiwifruit adapted viruses have only recently been discovered, and in many cases the means of transmission of these viruses is not well known. These viruses may yet be found to be pollen transmitted (Table 5).

In summary, the only virus proven to be pollen transmitted in kiwifruit is *Actinidia seed-borne latent virus* (Amponsah et al., in preparation) which is already present in New Zealand. There are a number of non-specialist viruses that are reported to be pollen transmitted in other hosts (AMV, CLRV, CMV and PZSV), but it is not known if they are pollen transmitted in kiwifruit. All these viruses, except for PZSV, are present in New Zealand. The kiwifruit adapted viruses have only recently been discovered and it is not known if they are pollen transmitted. Five of these viruses (AcCRaV, AcEV-2, AcV-1, AcVC and TNSaV) are not present in New Zealand. Of the two viruses that cause disease in kiwifruit (CLRV, PZSV), both are pollen transmitted in other plants, and PZSV is not present in New Zealand.

Table 5. Viruses reported from *Actinidia* spp. indicating those that can be pollen transmitted in kiwifruit and in other plants.

Virus	Acronym	Genus	Family	Virus category*	Present in NZ		Pollen transmitted		References**
					Kiwifruit	Other plants	Kiwifruit	Other plants	
Actinidia citrivirus	AcCV	Citrivirus	Betaflexiviridae	KA	+	-	?***	?	Pearson et al. (2011)
Actinidia chlorotic ringspot-associated virus	AcCRaV	Emaravirus	Fimoviridae	KA	-	-	?	?	Zheng et al. (2017)
Actinidia emaravirus 2	AcEV-2	Emaravirus	Fimoviridae	KA	-	-	?	?	Wang et al. (2020)
Actinidia seed-borne latent virus	ASbLV	Prunevirus	Betaflexiviridae	KA	+	-	+	?	Veerakone et al. (2018)
Actinidia virus 1	AcV-1	Closterovirus	Closteroviridae	KA	-	-	?	?	Blouin et al. (2018)
Actinidia virus A	AcVA	Vitivirus	Betaflexiviridae	KA	+	-	?	?	Blouin et al. (2012)
Actinidia virus B	AcVB	Vitivirus	Betaflexiviridae	KA	+	-	?	?	Blouin et al. (2012)
Actinidia virus C	AcVC	Vitivirus	Betaflexiviridae	KA	-	-	?	?	Zhao et al. (2020b)
Actinidia virus X	AVX	Potexvirus	Alphaflexiviridae	KA	+	-	?	?	Pearson et al. (2011)
Apple stem grooving virus	ASGV	Capillovirus	Betaflexiviridae	NS	-	+	?	-	Clover et al. (2003)
Alfalfa mosaic virus	AMV	Alfamovirus	Bromoviridae	NS	+	+	?	+	Pearson et al. (2011)
Cherry leaf roll virus	CLRV	Nepovirus	Secoviridae	KDV	+	+	?	+	Woo et al. (2012)
Cucumber mosaic virus	CMV	Cucumovirus	Bromoviridae	NS	+	+	?	+	Pearson et al. (2011)
Cucumber necrosis virus	CNV	Tombusvirus	Tombusviridae	NS	+	-	-	-	Pearson et al. (2011)
Pelargonium zonate spot virus	PZSV	Anulavirus	Bromoviridae	KDV	-	-	?	+	Biccheri et al. (2012)
Potato virus X	PVX	Potexvirus	Alphaflexiviridae	NS	+	+	-	-	Zhao et al. (2019)
Ribgrass mosaic virus	RMV	Tobamovirus	Virgaviridae	NS	+	-	-	-	Chavan et al. (2009)
Tomato necrotic spot-associated virus	TNSaV	Tospovirus	Bunyaviridae	KA	-	-	?	?	Wang et al. (2016)
Turnip vein clearing virus	TVCV	Tobamovirus	Virgaviridae	NS	+	-	-	-	Chavan et al. (2009)

*Non-specialist viruses (NS), Kiwifruit adapted viruses (KA), Kiwifruit disease causing viruses (KDC). **Data also from: Blouin et al. 2013, Brunt et al. 1996, Manaaki Whenua-Landcare Research: New Zealand Fungi Database. http:nzfungi2.landcareresearch.co.nz. ***?= These newly discovered viruses have not been well researched, and it is not known if they are pollen-transmitted.

Viroids

Evidence for pollen transmission by viroids is the same as for viruses, application of infected pollen followed by detection of the viroid in the resultant seeds, or in the mother plant (Card et al. 2007), and is convincing.

Fungi

Direct infection of pollen grains has been shown for *B. cinerea* infecting alfalfa, penetrating through pollen germ tubes. *S. sclerotium* and *Verticillium albo-atrum* can also infect alfalfa pollen. Other examples include *Peronoslerospora sacchari* infecting maize pollen, and *Coniothyrium minitans* and *Gliocladium catenulatum* infecting alfalfa pollen (Ngugi and Scherm 2006). Whether these fungi are commonly transmitted with pollen by pollinating insects, similar to transmission of some viruses and viroids, "remains an intriguing but unanswered question", according to Ngugi & Scherm (2006).

There are definitive examples of fungal spores being transported flower-to-flower on infected pollen, such as ascospores of *S. sclerotiorum* (Stelfox et al. 1978). Other fungi have been shown to replace pollen with their own spores of an equivalent shape and size and thus be disseminated by pollinating insects, such as *Fusarium semitectum*, which attacks flowering heads of *Rudbeckia auriculata* and becomes transmitted by bees (Diamond et al. 2006). Smut fungi may replace pollen of infected flowers with their own spores and be spread by pollinators, such as *Thecaphora capensis* infecting *Oxalis* (Curran et al. 2009). *Colletotrichum acutatum* was shown to produce conidia on the surface of stigma of *Citrus sinensis* suggesting that pollen may play a role in the spread of this pathogen (Marques et al. 2013).

Although not a plant pathogen, a fungal pathogen of bees has been shown to be pollen transmitted. Pereira et al. (2019) showed that feeding bumble bees with pollen naturally contaminated with *Ascosphaera apis*, the fungus that causes the bee disease chalkbrood, resulted in symptoms and/or dead larvae in all four replicate colonies, but the control bumblebee colony fed with irradiated pollen was disease free. *A. apis* was identified by DNA sequencing to be the same strain on both affected larvae and on contaminated pollen. Because isolations of live fungi were not made, this does not fulfil Koch's postulates. Honey bee collected pollen used to feed managed colonies of bumble bees was found contaminated with another fungal bee pathogen, *Microsporidium* sp. (Pereira et al. 2019). Chalkbrood has been reported to be spread from contaminated pollen to infect healthy bees (Flores et al. 2005).

In summary, there is evidence of fungi infecting and contaminating pollen, and evidence of spread of fungi on pollen. In view of the above examples, it is likely that fungi found on pollen are able to be spread by pollen.

3.3 Mitigation measures

3.3.1 Pollen

Irradiation is routinely used to clean pollen that is fed to bee colonies because of contamination by fungal and bacterial bee pathogens (Hornitzky 1994; Liu and McRory 1994; Melathopoulos et al. 2004). A dose above 4 kGy removed 99.9% of contaminating microorganisms (Snizhko et al. 2015). Similar results were obtained after irradiating rape pollen at 5 kGy or more (Kim et al. 2013). However, irradiation of kiwifruit pollen using 700 and 900 Gy significantly reduced seed

set (Musial and Przywara 1998) as did 200–1500 Gy (Chalak and Legave 1997). Clearly this decontamination method is not suitable if the pollen is subsequently used for pollinating kiwifruit.

Acetone treatment has also been used for decontaminating pollen in Korea. Although acetone treatment significantly reduced contaminating bacterial numbers, it also reduced pollen germination. After treatment, 13% of apple pollen, 22% of peach pollen, 19% of pear pollen and 3% of kiwifruit pollen germinated (Lee et al. 2016) (Figure 6). The reductions of bacterial numbers for kiwifruit pollen was from c. 10^8 cfu/g to 10^4 cfu/g, or a 4 log-fold reduction (Figure 6).

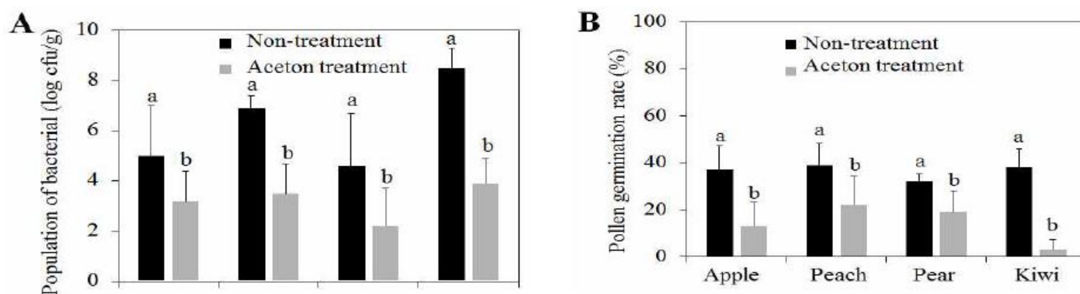

Figure 6. Changes in bacterial population and pollen germination after acetone treatment. (Lee et al. 2016). Cfu = colony forming units.

High velocity electron beams at a radiation level of 3 Mrad HVE per exposure to control foul brood when a 3 cm layer of pollen was exposed, but not a 12 cm layer (Shimanuki et al. 1984). There was no determination of the effect on pollen viability.

Kiwifruit pollen treated with chlorine dioxide (ClO_2), benziothiazolinone or copper oxychloride (kocide) retained over 50% germinability and bacterial populations were reduced by 99.7, 99.6 and 99.4% (Fan et al. 2018). However, if the bacterial load is 10^8 cfu/g, a 99.9% reduction equates to 10^5 cfu/g, or a 3 log-fold decrease. As kiwifruit pollen is usually contaminated with 10^4 cfu/g Psa bacteria (Miller et al. 2015; Vanneste et al. 2011), these measures will not remove all cells.

Heat treatments have also been used to kill Psa on artificially contaminated pollen. It was shown that the combination of 35°C and 30% relative humidity (RH) for 20 h resulted in a 2–3 log-fold reduction of Psa but pollen survived (Everett et al. 2012a). Exploration of this in more detail showed that 70 h at 35°C and 11.25% RH in anoxic conditions (achieved by applying pure nitrogen gas; N_2) resulted in a 7.9 log-fold reduction, whilst retaining 44% pollen germinability (Figure 7). In air there was a 4.9 log-fold reduction (Everett et al. 2016), and 34% of treated pollen germinated (Figure 7). When a flow-through drying method similar to commercial drying was used in the laboratory, the pollen did not survive (data not shown). Ozone was also shown to reduce Psa on artificially contaminated pollen without affecting pollen viability (unpublished data).

When several mitigation procedures are used in sequence it is called hurdle technology. Hurdle technologies have not been used to de-contaminate pollen. Several of the treatments described above could be used in combination, and some separately, to achieve the 4-log-fold reduction required to remove Psa3 from kiwifruit pollen.

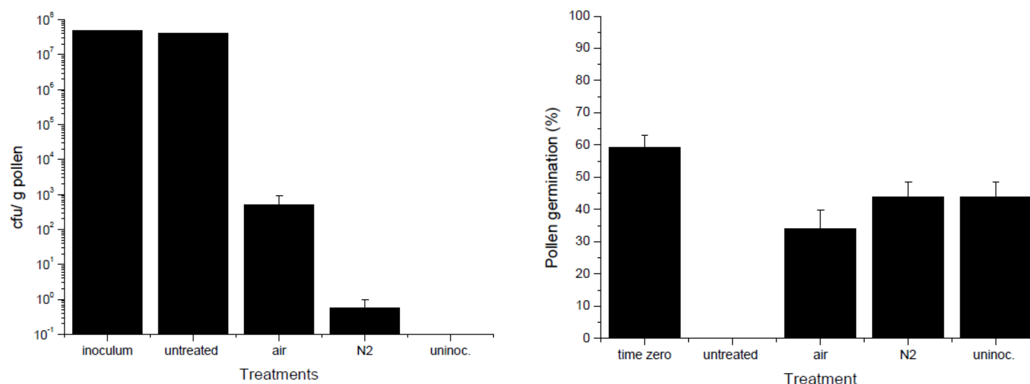

Figure 7. Pollen treated for 70 h at 35°C and 11.25% relative humidity (RH) in air or nitrogen (N_2) showing the effect on survivability of *Pseudomonas syringae* pv. *actinidiae* bacterial cells applied to the pollen and pollen germinability after treatment. The experiment was repeated three times (Everett et al. 2016). Cfu = colony forming units.

3.3.2 Fresh food products

Fresh food products need to remain pathogen free until they are purchased and consumed. Food pathogens include fungi, viruses and bacteria, and can either decay the food products or contaminate the products and induce disease in the consumer (food poisoning). Some mitigation methods that are used to eliminate or reduce food microbes could be used to reduce microbial contamination of pollen. However, most methods used for food do not completely eliminate these pathogens, but simply depress the populations to slow down decay, or reduce the risk of infection by human pathogens. Their use on pollen will depend on what is required. Treatments would also need to be tested for their effect on pollen viability.

If pollen treatments are used to reduce the amount of inoculum introduced to orchards as part of a systems approach in combination with a number of other measures, microbial populations may not need to be completely eliminated. However, if pollen is being exported to another country, or to another region in New Zealand that does not have a pathogen, and such pathogens either infect flowers or are known to contaminate pollen, then complete elimination will be the best outcome. If complete elimination is not achieved, technical data may need to be provided to establish the number of pathogen propagules required to infect a host under optimum conditions of humidity and temperature.

The most common method for preserving fresh food products is chilling combined with chlorine sanitisers. Modified atmospheres are also common (Boonyakarn & Mahakarnjanakul 2002; Lee et al. 2009; Sun et al. 2012; Efimochkina et al. 2016; Mir et al. 2018; Praeger et al. 2018; Kaczmarek et al. 2019; De Corato 2020). More innovative solutions such as lactic acid and hydrogen peroxide (Dyakova 2007; Martinez-Tellez et al. 2009; Augspole et al. 2017), ozone, calcium-based solutions, antioxidants and antimicrobials, electrolyzed water, heating, irradiation, ultraviolet light, high-power ultrasound, pulsed light, cold gas plasma and high hydrostatic pressure have more recently been used, as reviewed by De Corato (2020) (see Appendix: Table A2). Additionally, innovative packaging systems such as moderate-vacuum packaging, active and intelligent packaging, nanocomposite packaging, edible protective films, food coatings and anti-microbial films and the use of combined technologies (hurdles) (Mogren et al. 2018) are some more recent innovations in this area (Table A2).

Treatments that are unlikely to be useful for pollen treatments are ultrasound, irradiation and high hydrostatic pressure, due to a detrimental effect on pollen viability. Those with no penetrant activity such as ultraviolet light, pulsed light or cold gas plasma are also unlikely to be effective.

A large number of liquid additives are also used to preserve food (Table A2). Application to pollen requires use of a liquid pollen medium rather than applying pollen as a dust. However, these products are unlikely to remove all contaminating microorganisms. They would also need to be tested for their effect on pollen viability (Table A2).

The most promising new technology is hurdle technology, which uses a combination of different treatments so that more microorganisms are killed than when treatments are applied singularly. For kiwifruit pollen the combination of ozone, heat and low humidity could be more effective than each treatment by itself.

3.3.3 Plant material for planting

Methods for removing pathogens from plant material such as corms, budwood, seeds, cuttings, and bulbs are reviewed and their applicability as pollen treatments is discussed. Methods used are similar to those used for treatment of fresh food products and include heat treatments (thermotherapy), fungicides, electron bombardment and application of biocontrol agents (see Appendix: Table A3).

Thermotherapy (heat treatment) is routinely used for the treatment of sugar cane setts (sugar cane stem cuttings), and has been since the 1930's (Nyland & Goheen 1969). It is also used for virus removal from strawberry, citrus, grape, apple and stonefruit propagation material, and in the flower industry to remove nematodes from bulbs (Turechek and Peres 2009). For grapevine cuttings it is used to remove the crown gall bacterium, *Agrobacterium tumefaciens* (Burr et al. 1989), as well as virus-like organisms (Ophel et al. 1990 ; Bianco et al. 2000; Bertaccini et al. 2001). Direct application of heat to dormant or semi-dormant propagating material may involve direct hot water treatment, hot air treatment, moist hot air treatment, and aerated steam treatments. As heat treatment can result in significant damage to plant tissues, commercial treatment protocols are normally very specific with respect to application temperatures, temperature tolerances, duration times, and heat delivery systems such as water circulation requirements and aeration of hot water baths.

Seed treatments are routine, and generally consist of fungicides applied to the seed as dusts, thick water suspensions, or the seed is soaked in a chemical then dried. Tubers, bulbs, corms and roots can be treated in similar ways (Agrios 2005). Chemicals used for these treatments in the past include inorganic copper and zinc compounds, but are more recently mostly organic compounds such as chlorothalonil, iprodione, thiram, boscalid, thiophanate methyl, tebuconazole, pyraclostrobin, trifloxystrobin and azoxystrobin (Mancini & Romanazzi 2013) (Table A3).

Seeds can also be treated with hot water, aerated steam, and electrons. Hot water treatments are having a resurgence in interest (Forsberg et al. 2002; Gilbert et al. 2005; Koch et al. 2010; Schmitt et al. 2009), and are extremely effective at killing pathogens yet retaining seed germinability. Aerated steam and electron bombardment are more recent innovations that are also effective (Schmitt et al. 2006).

Biopesticides have also been used as vegetable seed treatments, and are considered an attractive option to reduce the use of synthetic fungicides in agriculture. The most effective essential oil in vitro and in field tests was thyme oil (Mancini & Romanazzi 2013). Other

essential oils, such as tea tree, clove, peppermint, rosemary, laurel, oregano oils, show good anti-fungal activity in vitro, as do onion seed extracts. When applied to seeds, chitosan, which is a compound extracted from crab-shell chitin, can improve germination, both percentage and rapidity, increase shoot height, root length, and root and shoot weights. It can also protect seeds against several pathogens (Mancini & Romanazzi 2013). Biocontrol agents are also used for coating seeds, most particularly plant-growth promoting rhizobacteria (PGPR) which colonise the rhizosphere and produce substances that promote growth of the associated plant, such as hormones, vitamins and growth factors. These PGPR also reduce the populations of root and foliar pathogens in the rhizosphere (Mancini & Romanazzi 2013). PGPR include species of *Pseudomonas*, *Bacillus*, and *Streptomyces*.

Trichoderma species have also been shown to reduce pathogen populations such as *Pythium, Phytophthora*, *Rhizoctonia* and *Fusarium* spp. when applied to seeds (Harman 2006).

Mychorrhizal species also show promise as seed treatments. Application to roots has been shown to protect wheat and tomato from fungal and viral pathogens (Fakhro et al. 2010). Mycorrhizae are used routinely to inoculate seedlings in forestry nurseries, which is proven to enhance seedling establishment in the field (Chu-Chou & Grace 1990; Bowen 1965). Research has shown that mycorrhizal associations can increase the host’s resistance to pathogen attack (Smith & Read 2008).

Some treatments such as application of mycorrhiza are not suitable as pollen treatments. Thermotherapy is usually conducted by heating water, and pollen is likely to be killed by this treatment. Inducing heat shock proteins by placing plant material at a moderate heat before heating protects the plants against damage, and this technique could be explored as an option for pollen treatment. As discussed above, irradiation is likely to kill pollen. Application of fungicides, essential oils, biopesticides and biocontrol agents is not likely to remove the entire microbial population from treated pollen if that is required for exporting or importing fresh pollen.

3.3.4 Novel medical methods to treat human infections by fungi, bacteria and viruses

An extensive review was conducted of the mitigation measures used to treat human patients with fungal, bacterial and virus infections to identify treatments that could be used to treat pollen. A detailed review of the novel medical methods used to treat human diseases is in Appendix 3. In contrast to plants, humans have an immune system that relies on antibodies and production of T cells. Some drugs and vaccines stimulate the immune system, and therefore are not able to be used against plant pathogens. Because of the risk of antibiotic resistance, antibiotics developed for treating human diseases are not able to be used on plants.

Of the remaining treatments, there are several that are also used for treating plant propagation material and fresh food products, such as essential oils (see Appendix; Table A4).

Those treatments that could be applied to pollen include nitric oxide which disrupts biofilms and the natural products listed in Table A4.

Fungi

Fungal diseases of humans have not been important until the rise of immunocompromising epidemics such as HIV, use of cancer drugs that suppress the immune system, immunosuppressant drugs to treat autoimmune diseases and organ transplants, and for invasive procedures such as insertion of catheters during or after surgery. Fungal diseases of

humans are also not readily spread. Therefore, research efforts have been relatively modest (Casadevall 2018). No novel treatments were found that could be applied to pollen.

Bacteria

Bacterial diseases of humans have been treated since 1928 with antibiotics (Eickhoff 2008). Antibiotic resistance is now the factor most limiting treatment of bacterial infections, and despite intense research effort, only 12 new antibiotics have been registered in the 21st century (Kaufmann et al. 2018). However, none of these is available for treating pollen.

Other drug strategies are too specific to human pathogens to be useful for pollen treatments (Table A4).

However, although not commercialised, there are a large number of natural products that have been shown to have anti-bacterial effects that could be tested for plant bacterial control (Table A4).

Nitric oxide (NO) exhibited anti-bacterial activity in vitro (LuTheryn et al. 2020). Low dose (below 80 ppm) NO is used in the treatment of lung diseases of babies. Inhaled nitric oxide (NO; 160 ppm) delivered intermittently was shown to be well tolerated in phase 1 clinical trials and patients with bacterial lung infections improved. Lower doses (100 ppb and 40 ppm) showed no effect. Use of larger sample numbers are required to prove statistically significant improvements (Bentur et al. 2019). NO disrupts biofilms, and could be investigated for use in controlling plant bacterial disease because its use is not restricted to human medicine.

Because bacterial resistance to drugs is known to be worsened if they are used in agriculture as well as medicine, regulators are reluctant to allow their use on plants. However, novel compounds such as nitric oxide (NO), or therapies that use broad spectrum disinfectants such as chlorine or hydrogen peroxide, can potentially be used in agriculture. Any therapies that rely on mammalian immune systems are not directly applicable to control of bacterial infections of plants. However, conventional medicine does not utilise the antibacterial properties of the natural products listed in Table A3. This list is similar to the products that are used as food additives, and these should be of some use against phytobacteria. However, complete elimination of bacteria contaminating pollen is unlikely, and some are only effective in vitro.

Viruses

Approximately 90 new antiviral drugs have been approved in the past 50 years, of which 29 have been approved in the past 6 years. Most of these new antivirals have been developed to treat hepatitis C and human immunodeficiency virus (HIV). Antivirals are very specific to a distinct virus group, can only rarely cure infection, and there are no known broad spectrum antivirals currently available (Kaufmann et al. 2018).

New drugs are often specific to either the human host, by promoting the immune system, or to specific human viruses. They are therefore not able to be used on pollen.

Therefore drugs or anti-virals derived for human viral diseases that may be useful in plant systems are lacking, because of their specificity and because plants lack an immune system. Targeting the host defences or receptors is not likely to be directly transferrable from a mammalian system to a plant system, although plant virus research laboratories do conduct fundamental research likely to find targets for similar plant based therapies. The danger of resistance to human pathogens is enhanced if human applied drugs are used in plant production, and regulatory bodies are reluctant to approve plant use for this reason.

3.4 Summary

From the evidence considered for the preparation of this review, it is concluded that pollen is a substrate that has been shown to transmit bacterial, fungal, viroid and viral pathogens from diseased to healthy hosts. There is also some evidence for the transfer of phytoplasmas (e.g. citrus greening) to healthy plants on pollen.

A number of different types of pathogens (bacteria, viroids, viruses and fungi) have been detected on pollen, which may have been present as contaminants or on floral debris that is associated with milled pollen. Viruses can also infect pollen systemically and be inside the pollen grains.

Isolations of bacteria have been made from commercial pollen, which shows that these microorganisms can survive the pollen milling process. It is therefore likely that other microorganisms could also survive the pollen milling process, which is conducted at temperatures that are not likely to kill plant pathogens.

As yet there are no viroids reported to infect kiwifruit, but because viruses on kiwifruit were only recently discovered, it is possible that kiwifruit is a host for yet undiscovered viroids, and more viruses. The risk of pollen sourced from the geographic origin of kiwifruit (South-East Asia) spreading new virus or viroid diseases into healthy kiwifruit is high. Because the original germplasm imported into New Zealand was by seeds, only seed-transmitted viruses have been introduced. However, if other planting material or pollen is sourced, there is a high risk that other viruses and/or viroids will be introduced into New Zealand. Most kiwifruit viruses discovered so far have been novel, and are difficult to detect.

There is evidence for pollen transmission of bacteria from kiwifruit, other plants and bees. Bacterial cells of kiwifruit canker (*P. syringae* pv. *actinidiae*; Psa), bacterial blast (*P. syringae* pv. *syringae*), fire blight (*Erwinia amylovora*) and American foul brood (*Paenibacillus larvae*) were detected on pollen, and Psa, fire blight and *X. juglandis* were isolated from pollen.

There is evidence for pollen transmission of Psa, blast, fireblight, American foul brood, walnut blight, *Pseudomonas* used as a biological control and maize bacterial wilt (*Erwinia stewartii*). Only one reference was found supporting pollen transmission of maize bacterial wilt, and the evidence appears to be simply that bacterial cells were found contaminating pollen. Direct evidence for pollen transmission was obtained for Psa on kiwifruit, *X. juglandis* on walnut, and American foul brood.

Indirect evidence for pollen transmission was obtained for *Pseudomonas* strains and fire blight in apple orchards. Artificial pollination is not generally used in apple orchards, and bees could have acquired the *E. amylovora* cells from other sites in infected flowers. The source of the biological control *Pseudomonas* was contaminated pollen, and bees spread this bacterium onto new flowers. Because bacterial cells of *E. amylovora* contaminate pollen, and because the biological control on pollen was spread by bees, it seems likely that the source of at least some of the fire blight spread by bees was contaminated pollen.

When considering the risk of pollen transmission of Psa it should be noted that it can also be spread by wind and rain, and therefore the importance of pollen transmission in a region that is already infected by Psa is likely to be minor. Preliminary evidence obtained following application of Psa contaminated pollen to an already infected orchard supports this hypothesis (Vanneste et al. 2020).

There is also evidence for spread of fungal diseases by pollen. A number of fungi infect pollen: *Botrytis cinerea*, *S. sclerotium*, *V. albo-atrum*, *Peronoslerospora sacchari*, *Coniothyrium minitans* and *Gleocladium catenulatum*. As proposed by Phatak (1980), the presence of fungal propagules on pollen presents a very real risk that these pathogens are spread by pollen. *S. sclerotium* ascospores have been shown to be spread on infected pollen. Other fungi replace pollen with spores of an equivalent size and shape and are spread by pollinators, such as smut fungi and *Fusarium semitectum*. *Colletotrichum acutatum* sporulates on stigmas of infected Citrus, suggesting that its spores could also be spread by pollinators with pollen. A fungal bee disease, chalkbrood, was able to be spread by feeding bumblebees with contaminated pollen. Altogether there is both direct and indirect evidence that fungi can be spread on contaminated pollen.

Current methods used for removing pathogens from pollen either kill or damage the pollen, are not very effective, or are not commercialised.

Thermotherapy is used to remove viral, bacterial and fungal pathogens from cuttings and other propagation material, seeds and bulbs. Fungicides are applied to seeds to remove fungal pathogens, but also biopesticides such as thyme oil, plant-growth promoting rhizobacteria (PGPR), the biocontrol fungus *Trichoderma* and mycorrhizae. Electron bombardment is also used to treat seed. Conifer seedlings are inoculated with mycorrhizae in the nursery to aid establishment after planting in forests.

Human pathogens are mainly treated with drugs, and most research is investigating new drugs with different modes of action to overcome problems with fungal and bacterial resistance. Research on drugs that affect the host rather than the pathogen are also being studied as a method of minimising the development of pathogen resistance. The likelihood of newly developed drugs permitted to be used in plant systems is low, due to the risk of resistance negatively affecting their use in medicine. Novel controls include methods to utilise the human immune system, and are not applicable to plants which do not have an immune system. Nitric oxide is being used to treat lung diseases to disrupt biofilms, and could be used in plant systems.

Novel mitigation methods from human medicine and treatments used for plants for propagation could be investigated as treatment options for kiwifruit pollen. A number of the human medicine options require genetic manipulation, which may not complement Zespri marketing strategies. Other strategies require careful consideration due to the cost, or they may not work in a plant system (e.g. lactoferrin). Different alternatives to antibiotics such as are used to treat plant propagation material or fresh produce would need to be used with pollen in a solution, which may not fit with current practice. There are gaseous alternatives, such as ozone and nitric oxide, but further investigation would be required. Novel treatments such as cold plasma, ultrasound and modified atmospheres could be investigated. Heat treatments show promise, and warrant further investigation due to being compatible with Zespri marketing strategies, and could be used in combination with other nil residue treatments such as ozone to improve efficacy.

In conclusion, from the evidence considered for the preparation of this review, pollen is a substrate that has been shown to transmit bacteria, fungi, viroids, phytoplasmas and viruses from diseased to healthy hosts. There is no evidence for oomycetes being transferred on pollen, although there are three records of flower infections by *Phytophthora* spp. of orchids, camellias and areca nuts.

Isolations of bacteria have been made from commercial pollen, which shows that these microorganisms can survive the pollen milling process. It is therefore likely that other

microorganisms could also survive the pollen milling process, which is conducted at temperatures that are not likely to kill plant pathogens.

Because milled pollen also contains floral debris, there is a risk that propagules of pathogens that infect flowers will contaminate pollen. However, contamination does not necessarily result in transmission.

The evidence for pollen transmission is strong for bacteria, some viruses (including viroids), and some fungi. Some fungi and viruses (including viroids) infect pollen and are found inside pollen grains.

American foulbrood is a bacterial disease of bees caused by *Paenibacillus larvae* which has been detected on pollen. It is well recognised that contaminated pollen is an important means of spread of American foulbrood. There are also examples of pollen transmission for several plant bacteria including Psa, walnut blight, fire blight, a biocontrol *Pseudomonas*, and bacterial blast.

In conclusion, two kiwifruit fungal pathogens have been shown in other crops to be able to infect pollen (botrytis and sclerotinia). Pollen transmission has been shown for sclerotinia but not for botrytis. Sclerotinia control may be able to be improved by using clean pollen. However, pollen transmission of botrytis is not likely to be important.

Only two viruses cause economically damaging losses for kiwifruit, *Cherry leaf roll virus*; CLRV and *Pelargonium zonate ringspot virus*; PZRV. Both are pollen transmitted, and PZRV is not present in New Zealand.

There is a high risk of pollen transfer of PZRV and new strains of Psa with effectors that could overcome the tolerance of Gold3 into New Zealand kiwifruit from off-shore sourced pollen. Within New Zealand, there is a high risk that CLRV could be spread on pollen.

There is also a risk that viruses not yet discovered cause economic losses on kiwifruit and could be imported on overseas sourced pollen.

Application of Psa contaminated pollen to already infected orchards is unlikely to worsen Psa epidemics. However, it is likely that application of Psa contaminated pollen to uninfected orchards will introduce Psa to those orchards.

Current methods used for removing pathogens from pollen either kill or damage the pollen, are not very effective, or are not commercialised. Using several mitigation methods in tandem could be investigated, and are likely to be more effective than each method alone. The most promising novel methods used to mitigate pathogens for fresh food products, plant propagation material and human diseases for use on kiwifruit pollen to eliminate fungal and bacterial contamination are ozone, nitric oxide, modified atmospheres and thermotherapy. Fungicides and antibiotics could also be used to suppress fungal and bacterial populations. For production of virus-free pollen, it is best to source pollen from high health plants that are regularly tested. Usually high health plants are within insect exclusion enclosures to prevent insect vectored diseases infecting these plants, and may be necessary to prevent introduction of Psa, sclerotinia and botrytis on bee borne pollen. Because the recently discovered kiwifruit adapted viruses are not well researched, it is possible that some of these could also be insect vectored.

The greatest risk of economic damage from spread of pollen borne diseases within New Zealand is for *Cherry leaf roll virus*, and therefore the greatest need is for high health indexed plants to be established for virus-free pollen production.

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Appendix

Review of Pollen transmission of Psa

Kiwifruit canker, caused by the bacterial pathogen *Pseudomonas syringae* pv. *actinidiae* (Psa), was first detected in New Zealand in November 2010 (Everett et al. 2011). Although difficult to prove, the most probable pathway by which a virulent strain, first named Psa3 (Chapman et al. 2012) or PsaV (McCann et al. 2013), then later biovar 3 (Vanneste et al. 2013), entered New Zealand was via imported pollen (Froud et al. 2015; Mallon 2018). Psa went on to be the most devastating kiwifruit pathogen in New Zealand's history, costing the industry hundreds of millions of dollars in lost plants, re-grafting and decline in land values. It also led to the commercial extinction of *Actinidia chinensis* var. *chinensis* 'Hort16A', the first commercialised gold kiwifruit (Vanneste 2017).

There is evidence for one bacterium transmitted to healthy vines by kiwifruit pollen, and that is Psa. This has been controversial, with conflicting reports in the literature and criticisms of the methodology used in various studies which are catalogued and discussed in the Judgement for the Strathboss versus Attorney-General court case conducted in 2017 (also known as the Kiwifruit Claim) (Mallon 2018).

The first set of evidence is based on observation of incursions of Psa3 into New Zealand (Mallon 2018) and Korea (Kim et al. 2016) supported by genetic strain analysis.

In New Zealand on 24 June 2009, kiwifruit anthers were imported by Kiwi Pollen (a New Zealand pollen company) from Shaanxi, China. This consignment was processed into pollen at a mill in Te Puke township, and the epicentre of the New Zealand outbreak was 7 km away at an orchard (Kairanga). One Kiwi Pollen principal used pollen that was milled by Kiwi Pollen to test different pollen application systems on Kairanga, and the adjacent Olympos orchard (Mallon 2018). Psa3 was first detected at Olympos orchard, Mark Road, Te Puke on 5 November 2010 (Everett et al. 2011), and the symptoms were more advanced on Kairanga suggesting that as the true epicentre. Evidence was presented that the strain of Psa isolated from the centre of the epidemic was from China, but the evidence that it came from Shaanxi was not strong (Mallon 2018).

Examination of the scientific evidence by Judge Mallon during the Strathboss versus Attorney-General court case resulted in the following summation (referring to the consignment of anthers in June 2009); "Taken together, the strands of circumstantial evidence go beyond conjecture or coincidence and mutually support the overall inference that it is more likely than not that the anthers consignment contained Psa3 and that this ultimately caused the outbreak of the disease in New Zealand" p. 15. (Mallon 2018).

Both expert witnesses in the court case agreed that the most likely source of the New Zealand incursion, based on phylogenetic sequence analysis of whole genomes, was China (Butler et al. 2013; McCann et al. 2013). However, the use of integrative conjugative elements (ICEs), which are mobile genetic elements that can be horizontally transferred from other bacteria, or vertically transferred between the same species, as evidence that the New Zealand incursive strains were from Shaanxi was not supported by both experts. The New Zealand incursive strains uniquely contained PacICE1, which also occurred in a single strain from Shaanxi. Because of the instability of ICE and the ability to transfer from other bacterial genera, and the limited

strains that had been sequenced from Shaanxi, this was not considered to be a reliable indicator of strain relatedness (Mallon 2018).

Since then, more evidence has been compiled that examines the origin of the incursive strain(s) using different techniques. Ciarroni et al. (2015), using a technique called multilocus variable number of tandem repeats analysis (MLVA), compared 142 strains of Psa, 14 from before 1999 and 128 from 2008 on. In this analysis they showed that the isolates from Shaanxii were similar to those from the incursion in New Zealand in 2010.

A similar conclusion regarding the introduction of Psa3 by pollen was made following an incursion into South Korea (Kim et al. 2016; Kim et al. 2020). The evidence was initially mostly circumstantial; reports suggested the first occurrence of Psa3, in 2011, in Goheunggun, Jeonnam Province was due to imported seedlings from China in 2006, based on symptom development descriptions by the grower and historical records of planting and purchase of kiwifruit material. This region was not planted in kiwifruit prior to 2006. However, wider spread of Psa3 during 2014 and 2015 was attributed to Psa3-contaminated pollen imported from New Zealand and China (Kim et al. 2016). The evidence was based on PCR testing of imported pollen, which was positive for Psa3, and inoculation experiments with infected pollen applied during flowering and inspected for symptoms 3 weeks later. Bacteria were isolated from resulting symptomatic material and identified as Psa3, proving Koch's postulates (Kim et al. 2016).

Because the first orchard that was infected in South Korea in 2011 in Goheung gun, Jeonnam Province (Figure A1) was shut down on 11 September 2014, Kim et al. (2020) suggest that the subsequent epidemic starting in 2015 was due to the application of infected pollen. However, they go on to say that the epidemic, first noticed officially in 2015, probably started in the early summer after pollination in 2014, but was not reported, similar to the epidemic on Jeju Island (Figure A1) in 2014. The basis for this assertion is also genetic analysis of the strains found in South Korea (Kim et al. 2020). Pac-ICE1, unique to New Zealand and Chinese strains, was found in three of eight sequenced strains from Korea, including one from Jeju Island, one from Gwangyan, Jeonnam, and one from Boseong, Jeonnam (Figure A1). Different elements were found from the remaining five strains, which were from Jeju Island (Pac-ICE3, 2, 8 10 and 11). Pac-ICE10 has also only been found in New Zealand strains, and carries genes for copper resistance. Changes in the sequence that are specific to New Zealand strains (eight single nucleotide polymorphisms (SNPs), a specific insertion sequence element and a 22 bp deletion) were found in six isolates. Phylogenetic analysis of these strains and two more showed eight Korean strains in the same clade as New Zealand strains, but also with strains from China and Chile. Of the remaining four isolates, two were similar to isolates from Chile, and the last two from China. One of these two, from Jeju Island, was similar to two strains isolated from the original incursion in Jeonnam in 2011 (Kim et al. 2020).

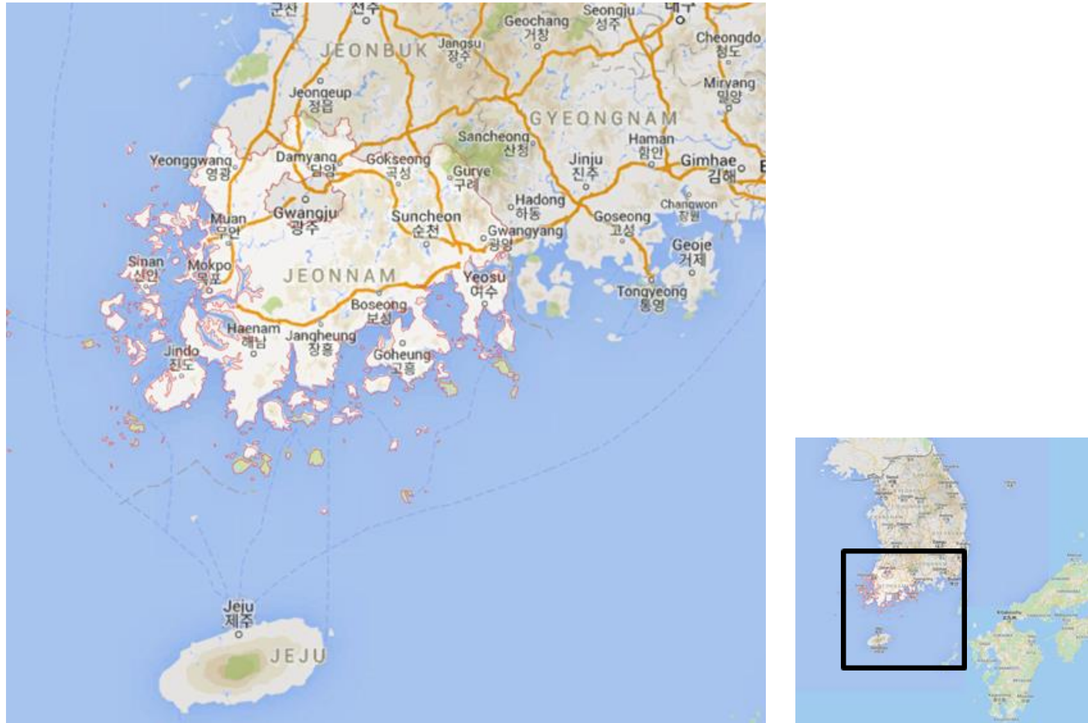

Figure A1. Map of South Korea and the southern tip of Japan (right). Box is enlarged southern part (left) showing Jeonnam Province and JeJu Island in more detail.

The description of the inoculation experiments with pollen from New Zealand was sparse. It is not clear how the experimental plot was separated from infected plots, or how the Psa3 status of the inoculated plants was determined prior to inoculations. Despite the author's assertion that because of the destruction of the orchard in Goheung gun in 2014 it was not likely to have caused the subsequent outbreak, there was one strain found on Jeju Island in 2015 that was similar to the strains isolated in 2011. It is also possible that all strains originated from China, which is the origin of the strains found in New Zealand and Chile (Butler et al. 2013). However, the similarity of 9/10 strains found in Korea to strains from China, Chile and New Zealand, and the dissimilarity of these strains to those imported on infected seedlings in 2011, points to the application of imported pollen from these three countries to Korean orchards as the likely origin of these strains of Psa3 now found in Korea.

There is a growing body of scientific evidence that Psa can be transmitted to healthy vines by contaminated pollen (Table A1). However, not all experiments were robust.

Stefani and Giovanardi (2011) conducted pollination experiments in the glasshouse and in the field. Although Koch's postulates were proven when plants in the glasshouse were pollinated with an aqueous suspension artificially contaminated by Psa3, it was not possible to re-isolate Psa3 when naturally contaminated pollen was applied by dusting. The trial was not set up in a randomised block design. Application of pollen in the field was solely by dusting, but the replication was not specified, beyond there being five plant plots, nor was it shown or demonstrated that the orchard that was used was not already infected with Psa3. Results from plants not pollinated with Psa3 positive pollen were not reported.

Tontou et al. (2014) planted a study orchard 100 km away from commercial kiwifruit production in Italy. Four treatments of four plots each, randomly distributed, were applied to 2-year-old 'Hayward' kiwifruit plants established 6 months prior (2.5-year-old kiwifruit plants) during

flowering using dry and wet application of naturally contaminated pollen, and compared with dry and wet application of control pollen. Naturally contaminated pollen contained c. 6 x 10^4 colony forming units (cfu)/g Psa3, and control pollen was Psa3-free as determined by PCR testing. The plots consisted of four adult plants, thus there were 16 plants per treatment. Each replicate was separated from neighbouring plots by an additional untreated adult plant.

Treated plants were monitored by observing symptoms and destructively sampling either flowers, fruitlets or fruits, and leaves, every 2 weeks during the following two growing seasons. Plant tissue was tested for Psa3 by PCR (Rees-George et al. 2010) and by isolation onto selective media. Resultant colonies that induced an hyper-sensitive (HR) response in tobacco were further analysed by PCR (Rees-George et al. 2010; Gallelli et al. 2011a) and rep-PCR (Versalovic et al. 1991; Scortichini et al. 2002; Vanneste et al. 2010).

During the 2 years (2012/2013) of sampling, no Psa3 was detected from flowers, leaves or fruitlets from plants pollinated with control pollen when sampled 24 h and 2 weeks after 90% flowering (when pollen was applied the first year). During the first season, Psa3 was re-isolated from flowers and leaves sampled 24 h and fruitlets sampled 2 weeks after pollination with both dry and wet naturally contaminated pollen. Leaves from plants pollinated with an aqueous suspension of naturally contaminated pollen yielded Psa3 detected by PCR and isolation after 2 weeks, but it was not detected from leaves from dry pollinated plants. In the second year, Psa3 was detected by PCR from leaves 2 weeks after flowering on plants wet pollinated with naturally contaminated pollen, but not from those that were dry pollinated. During the first and second season Psa3 was detected in leaves up to 8 weeks after flowering on both inoculated treatments (wet and dry contaminated pollen), and during the first season, in leaves from plants pollinated with wet pollen after 16 and 21 weeks. During the second season Psa3 was isolated from leaf spots on plants both wet and dry pollinated with contaminated pollen 6, 8 and 12 weeks after 90% flowering. Symptoms were erratic, and after 21 weeks no longer observed. However, 21 weeks after flowering would be late summer, when new leaf spots may be less likely to appear, especially in a dry season (Horner et al. 2013). Regrettably, Tontou et al. (2014) did not provide climate data. Psa3 was also isolated from a few control plants, but further details were not provided. These infections could be caused by secondary spread from inoculum produced on plants pollinated with contaminated pollen. Cankers were observed in autumn 2013, but further details were not provided. This study was intended to be preliminary to more extensive experiments (Tontou et al. 2014).

In New Zealand (Miller et al. 2015), although Psa3 was detected in pollen through the milling process, application of PCR positive pollen in an aqueous suspension to wounded and unwounded leaves of kiwifruit seedlings in the glasshouse did not result in detection of Psa3 when leaves were harvested and tested by PCR and isolated 3 weeks later. The replication and layout of the trial is not reported, neither is the method used to achieve the humid conditions described, nor the method used to wound the kiwifruit leaves. Spraying contaminated pollen onto leaves may not have been the best way of testing if PCR positive pollen was infectious. More reliable would have been applying pollen to flowers, as is normal practice.

However, a similar study in New Zealand (Everett et al. 2012b) using different methodology did induce leaf spots following inoculation of naturally contaminated pollen to kiwifruit seedlings. The stored kiwifruit pollen was supplied by the New Zealand Ministry for Primary Industries (MPI) and the source was not known to the authors when the experiment was conducted. Incubation of the pollen for 4 h in nutrient broth followed by plating on King's Medium B, DNA extractions from the resultant colonies and subsequent quantitative polymerase chain reaction (qPCR) analysis resulted in convincing positives for two New Zealand sourced pollen samples with both sets of Psa primers used (Rees-George et al. 2010; Andersen et al. 2018). A third

New Zealand pollen sample and three Chilean pollen samples were negative. This negates the possibility of laboratory contamination of the pollen samples, which were all treated in the same way.

The pollen with the highest amount of Psa was then used in inoculation experiments. The experiment was conducted twice, the first time without and the second with high humidity immediately after inoculation. Leaves were inoculated with this naturally contaminated pollen in a suspension containing carborundum (an abrasive used for virus inoculations) and applied by rubbing on leaves using latex gloves. When humidity after inoculation was not increased, lesions produced were not related to treatments, and Psa3 was not detected even in control plants inoculated with pure bacterial suspensions following sampling 12 days later.

When the experiment was repeated and plants were placed in conditions of high humidity immediately after inoculation, lesions were produced that were reasonably consistent with the treatments, Psa3 was re-isolated, and there were strong PCR positives for inoculated controls. During this second experiment, Psa3 was re-isolated from leaves inoculated with New Zealand sourced Psa3 contaminated pollen (Everett et al. 2012b) thus proving Koch's postulates when plants were sampled 10 days after inoculation. The author was concerned regarding weak Ct values obtained from uninoculated controls when tested with the Rees-George et al. (2010) primers during the second experiment. However, these uninoculated controls were negative when tested with the highly specific Andersen et al. (2018) primers, and were in the same range as the Ct values obtained for the first experiment, in which Psa3 did not establish even following leaf inoculations with a pure culture. One of these isolates was confirmed to be Psa3 by sequencing analysis (unpublished data).

It became apparent that the uncertainty around the weak positives detected by the Rees-George et al. (2010) primers, which were known to not be 100% specific to Psa, confused non-experts. If instead the authors had only presented the results of the Andersen et al. (2018) primers, the results would have been clear cut and easy to understand.

The inoculation method that was used by Everett et al. (2012b) was severe, and not likely to be replicated in nature. However, the aim of the experiment was to isolate Psa3 from what was understood at the time to be the batch of pollen that possibly contained the incursive strain. This pollen was collected by MPI from Kiwi Pollen in 2010, just after the first report of Psa3 in November 2010. Because of the possibility of a court case, it was difficult to obtain further information about the origin of pollen batch T10-06084 t 2. However, Froud et al. (2015) state that the pollen was harvested in spring 2009. MPI at the time could also detect Psa3 in these batches of pollen, but could not isolate Psa3 from it (Rob Taylor, pers.comm.). The six coded samples of pollen were given to The New Zealand Institute for Plant and Food Research Limited (PFR) by MPI with instructions to attempt to isolate Psa3.

According to Mallon (2018) Psa3 was introduced into New Zealand in a batch of anthers that were imported in June 2009 from Shaanxi, China. To explain the PCR positive New Zealand pollen samples, either the 1 kg of pollen that was milled from these imported anthers was mixed with other batches of pollen, or the 4.5 kg of anthers that were milled contaminated the milling equipment, which then contaminated further batches of pollen. Because there was no record of Psa3 in kiwifruit orchards in New Zealand when the pollen was harvested, probably during September/October 2009, it is not likely to have been contaminated in the field.

The extreme difficulty of isolating Psa3 from batches of PCR positive stored pollen at the time is belied by other reports of easily isolating Psa3 from stored pollen sourced from orchards where Psa3 was established (Gallelli et al. 2011b; Stefani and Giovanardi 2011; Vanneste et al. 2011;

Loreti et al. 2014; Tontou et al. 2014; Miller et al. 2015; Balestra et al. 2018a; Kim et al. 2018). One witness described isolating 10,000 cells of Psa3 per gram from pollen that was harvested in 2011 (Mallon 2018), and Miller et al. (2015) report $10\text{-}10^4$ cells per 0.2 g of pollen ($50 - 5 \times 10^4$ cells per gram).

It is possible that the 1 kg of pollen that was milled from the anthers imported from Shaanxi was added to other pollen, thus diluting the Psa3, explaining why the pollen was PCR positive but difficult to isolate. Mallon (2018) suggests this as one possibility of the fate of the pollen milled from the Shaanxi anthers (p. 396). Kiwi Pollen were later warned by the Commerce Commission because they were adding Chilean pollen to New Zealand sourced pollen without indicating this on the label (Fisher 2012); cutting New Zealand pollen with overseas sourced pollen was a common practice. Statements recorded in Mallon (2018) suggest that pollen was never thrown away by Kiwi Pollen. Mallon (2018) does not provide an explanation of how the epidemic in New Zealand was started by the anthers from Shaanxi, but she does conclude that this material was the most likely source of the introduced Psa3. It is possible that the pollen milled from the anthers from Shaanxi was applied on Kairanga and Olympos orchards during flowering in September/October 2009, also described as a possibility in Mallon (2018) on p. 397 during evidence discussing pollen experiments on these two orchards. Symptoms of leaf spots and flower wilting were first noticed in October 2010 on 'Hort16A' on Olympos. By November 2010, shoots showed blackening and terminal wilting and die-back. After a further 3 months, red ooze was observed as well as young cankers at the base of canes (Everett et al. 2011).

In a glasshouse study in Italy (Balestra et al. 2018b) with a fluorescent labelled strain of Psa3, bacterial cells had spread from styles to the flower stalk 10 days after inoculation with Psa3 contaminated pollen, and were observed to invade the vasculature. In some flowers visible bacterial ooze was produced with the nectar (Balestra et al. 2018b), presumably within the 11 days before ovules are fertilised. Leaf spots were observed after 2 months, and 4 months after inoculation the bacteria was distributed throughout the plant, including roots. Certainly this time frame is consistent with kiwifruit on Kairanga and Olympos being infected in spring 2009, with severe symptoms expressing a year later. It also aligns with the timeline described in Tontou et al. (2014), where they report the appearance of cankers in the second season after application of Psa3 contaminated pollen to kiwifruit flowers.

It is not known what year the Chilean pollen was harvested, but it was given to PFR by MPI, along with the New Zealand pollen samples collected from Kiwi Pollen, shortly after the first report of Psa3 in New Zealand in November 2010. The statement in Mallon (2018) from Kiwi Pollen was "pollen used in October had generally been bought the season before", and "a New Zealand customer might be given Chilean pollen, or that pollen might be used for export". … "for us, pollen was just all the same" p. 394. Psa3 was first reported from Chile in 2011 (EPPO 2011).

Microscopical examination showed that infection of anthers by inoculating flowers with bacterial suspensions resulted in pollen contaminated on the outside with Psa, although distribution was sporadic with only a small number of pollen grains being contaminated (Balestra et al. 2018b). Psa was never found inside pollen grains. However, examination of naturally contaminated pollen showed that 4–5% of pollen harvested from closed flowers and 10–80% of pollen from open flowers was contaminated with Psa. The authors suggested that bumble bees used to pollinate kiwifruit orchards were contaminating pollen in open flowers, as well as natural spread from inoculum sources in the orchard facilitated by rain and wind.

Exposure of female kiwifruit plants ‘Hort16A’ to naturally infected male vines resulted in 100% contaminated flowers after 10 days, with 48% leading to systemic infection, and in ‘Hayward’ 44% of flowers became infected leading to 25% systemic infections (Balestra et al. 2018b).

Table A1. Inoculation studies with pollen naturally contaminated by *Pseudomonas syringae* pv. *actinidiae* (Psa) biovar 3.

Koch’s postulates proven	Method	Replication	Glasshouse or field	Reference
- +	1. dusting during flowering (naturally contaminated and control pollen) 2. spray aqueous suspension (artificially contaminated pollen)	3 plots of 3 plants-each plot pollinated using a different method.	GH	Stefani and Giovanardi (2011)
-	1. dusting during flowering (naturally contaminated and control pollen)	Two orchards Five plant plots	F	Stefani and Giovanardi (2011)
+	1. dusting and spray aqueous suspension	16 plants per treatment, 4 plots of 4 plants. Guard plants between treatments.	F	Tontou et al. (2014)
-	1. spray aqueous suspension on leaves - half of which were wounded	?	GH	Miller et al. (2015)
+	1. incubate pollen for 4 hours in nutrient broth followed by rub inoculation with carborundum	3 replicate plants, 3 inoculated leaves per plant.	GH	Everett et al. (2012b)
+	dusting with a wet brush?	12 potted plants	GH	Balestra et al. (2018b)

In summary, Psa3 has been conclusively proven to contaminate kiwifruit pollen by a number of different authors, either by PCR only or by inoculations and re-isolation, thus proving Koch’s postulates (Table A1) (Gallelli et al. 2011, Vanneste et al. 2011, Stefani and Giovanardi 2011, Everett et al. 2012b, Loreti et al. 2014, Tontou et al. 2014, Miller et al. 2015, Balestra et al. 2018). The time scale of infection of Olympos and Kairanga orchards matches the symptom progression described by Donati et al. (2018) and Tontou et al. (2014), but the symptoms were more severe. Progression could also be related to inoculum dose and climatic conditions. The judgement following evidence presented by a number of expert witnesses and examination of the scientific literature was that Psa3 was introduced into New Zealand from infected anthers that were imported from Shaanxi, China in June 2009 (Mallon 2018). Similarly, the evidence based on observations and genetic analysis of the incursion in South Korea showed the incursion was probably due to the application of infected pollen (Kim et al. 2016; Kim et al. 2020). Tontou et al. (2014) also conclusively showed that application of contaminated pollen to a Psa3 free orchard 100 km away from other kiwifruit orchards resulted in infection by Psa3. The laboratory experiments of Donati et al. (2018) conclusively showed that Psa3 could

infect kiwifruit systemically after application of contaminated pollen to flowers. Koch's postulates were proven by several authors (Stefani and Gionvanardi 2011, Tontou et al. 2014, Everett et al. 2012b, Donati et al. 2018). The evidence is conclusive; Psa3 on contaminated pollen can transmit Psa3 to previously uninfected kiwifruit vines.

Detailed review of novel medical methods to treat human infections by fungi, bacteria and viruses

Fungi

Fungal diseases of humans have not been important until the rise of immunocompromising epidemics such as HIV, use of cancer drugs that suppress the immune system, immunosuppressant drugs to treat autoimmune diseases and organ transplants, and for invasive procedures such as insertion of catheters during or after surgery. Fungal diseases of humans are also not readily spread. Therefore, research efforts have been relatively modest (Casadevall 2018).

Currently there are no vaccines or immunotherapies for fungal diseases, but many are in development. For example, recently a vaccine for vaginal candidiasis was proven safe and effective (Casadevall 2018). Several animal models have shown that immunotherapy based on monoclonal antibodies was effective (Casadevall 2018), and trials are commencing using monoclonal antibodies for human fungal infections, e.g. Efungumab (Schwarz et al. 2018).

Drugs designed for other purposes have recently been found to be antifungal – such as sertroline, an antidepressant, and anti-malaria drugs are also showing some anti-fungal activity against yeasts such as *Cryptococcus neoformans* and *Candida albicans* (Mayer & Kronstad 2017; Jung et al. 2018).

The study of the mycobiome also offers potential opportunities for purposeful shifting of population dynamics to favour healthy outcomes (Casadevall 2018). For example, faecal implants from patients with healthy guts have been successfully used to treat diseases such as *Clostridium* associated diarrhea (Vemuri et al. 2020). Only very recently has the role of fungi in the health of the microbiome been investigated (Venuri et al. 2020).

Most human fungal diseases are treated with azoles, but there is increasing fungal resistance due to their use in agriculture. The other major drug group is the echinocandins. Delivery of antibiotics by inhalation has been shown to increase drug concentration in localized regions and reduce the risk of resistance (Dhand 2019; Elborn et al. 2016; Hamed et al. 2017; Klodzinska et al. 2016; Sala et al. 2019; Vazquez-Espinosa et al. 2016).

New drugs with different modes of action are being developed, such as E1210, a glycosylphosphatidylinositol (GPI) anchor biosynthesis inhibitor, T-2307- an arylamidine derivative, for which the mode of action is unknown. A new antiseptic drug belonging to chloramins and named N-chlorotaurine is promising (Perfect 2017; Schwarz et al. 2018).

Otherwise novel uses of the human immune system are leading to new therapies based on T-cells, which are manufactured using recombinant techniques (Schwarz et al. 2018).

Bacteria

Bacterial diseases of humans have been treated since 1928 with antibiotics (Eickhoff 2008). Antibiotic resistance is now the factor most limiting treatment of bacterial infections, and despite intense research effort, only 12 new antibiotics have been registered in the 21st century (Kaufmann et al. 2018).

However, new strategies are being sought – including genetically engineered saprotrophic bacteria such as a benign strain of *E. coli*, (EcN) which can express effectors under specific conditions. Several have already been tested in preclinical models including the EcN-based gastrointestinal delivery of anti-biofilm enzyme, dispersin B (DspB) which reduced *Pseudomonas aeruginosa* abundance, and another EcN producing antimicrobial peptides to successfully reduce populations of Enterococcal species or *Salmonella typhirmurium*. Probiotic bacterium *Lactococcus lactis* has also been engineered to secrete effectors in the gut, and three strains are under clinical development (Charbonneau et al. 2020).

Natural antimicrobial peptides (AMPs) and their synthetic derivatives are considered to be promising alternatives to antibiotics, and can be administered as antimicrobial random peptide cocktails to increase their efficacy (Moravej et al. 2018; Amso & Hayouka 2019).

Bacteriophages were developed over a century ago for bacterial control, but were superseded by antibiotics due to their very specific activity, often against only one bacterial pathogen. Their use continues in east Europe and they have proven efficacy against *P. aeruginosa* when inhaled as a powder, or in combination with antibiotics (Horcajada et al. 2019). A synergistic effect was noted when phages and antibiotics were used together.

Bacteriocins, which are substances with anti-bacterial activity produced by some bacteria, show some promise in clinical trials for treatment of *P. aeruginosa* infecting lungs of cystic fibrosis patients (Horcajada et al. 2019).

The latest conventional treatments for combatting *P. aeruginosa* infections recommend drug cocktails designed to minimise antibiotic resistance by using drugs with differing modes of action (Shaw & Wuest 2020). To that end, acyl homoserine lactones (AHL) are a group of signalling molecules that can regulate biofilm growth (Srivastava and Sivashanmugam 2020). Also described is the use of conventional antibiotics at low dosages to reduce biofilms (Olivares et al. 2020).

Novel drugs are also being developed, with Ceftolozane/tazobactam(C/T) the first of a new class of β–lactam/β-lactamase inhibitors targeting Gram-negative bacteria (Frattari et al. 2018; Garazzino et al. 2020; Frattari, 2018; Maraolo et al. 2020) that overcomes multiple drug resistance (Horcajada et al. 2019). Motility regulators that disrupt the Quorum Sensing (QS) pathway have been developed. These include (z)-5-octylidenethiazolidine-2,4-dione and lipoic acid, phenylalanine arginyl β-naphthylamide, anteiso-C15:0, doxycycline antibiotic, 2,5-piperazinedione, 1037 cationic peptide, and 1-naphthol (Khan et al. 2020).

Natural products derived mainly from plants have also been shown to disrupt motility of *P. aeruginosa* including 7-hydroxyindole, ginseng extract, carvacrol, eugenol, Ag-TiO2, TiO2-Ag, Ag-Cu and Cu-Ag nanocomposites, cinnamaldehyde, lactic acid, 3-phenyllacetic acid, equisetin, flavone derivatives, cucumin, azithromycin and gentamicin, a formulation of resveratrol, stilbenoids (plant phytoalexins), sodium houttuyfonate, curcumin, zingerone, 7-fluoroinole, ellagic acid, caffeic acid, cinnamic acid, ferulic acid and vanillic acid, tea polyphenols, baicalin, methyl gallate. Diallyl disulphide (garlic oil), hordenine, fenaclon and 2, 4 di-tert-butylphenol, reserpine, 3,5,7-trihydroxyflavone, glycoprotein DNBT1 (tear fluid),

curcumin, ceftazidime and ciprofloxacin, quercetin, D3112 protein gp05 (phage protein), adenosine triphosphate, chlorogenic acid, Vitexin, caffeine, 2,5-piperazinedione, 3-benzyl-hexahydro-pyrrolo(1,2-a)pyrazine-1,4-dione and AiiAs1-5 (Khan et al. 2020). A traditional herbal medicine, S*ophora flavescens*, was shown to reduce lung inflammation induced by the body's defence against *P. aeruginosa* (Lee et al. 2014), and extracts from *Crataegus pinnatifida* fruit were shown to have antibiotic activity in vitro (Ryu et al. 2010). Also effective are furanones and Mānuka honey (LuTheryn et al. 2019).

Lactoferrin impedes bacterial attachment to surfaces by sequestering iron needed for bacterial motility, and directly lyses bacterial cell walls (LuTheryn et al. 2019)

Inhalation as a delivery system is proving effective at overcoming resistance issues, leading to delivery of higher doses. One antibiotic showing promise is ciprofloxacin as a dry powder delivered by inhalation to lungs of cystic fibrosis patients (Chorepsima et al. 2018; Dhand 2018).

Nitric oxide (NO) exhibited anti-bacterial activity in vitro (LuTheryn et al. 2020). Low dose (below 80 ppm) NO is used in the treatment of neonates with persistent pulmonary hypertension. Inhaled nitric oxide (NO; 160 ppm) delivered intermittently was shown to be well tolerated in phase 1 clinical trials and patients with bacterial lung infections improved. Lower doses (100 ppb and 40 ppm) showed no effect. Use of larger sample numbers are required to prove statistically significant improvements (Bentur et al. 2019).

Gas-filled microbubbles have been developed for improved drug delivery (LuTheryn et al. 2020). Their structure varies, and usually consists of a surfactant, polymer, protein or phospholipid shell, which encapsulates a gaseous core. Acoustically activated microbubbles carrying biologically active gases as a novel means of drug delivery shows promise as an emerging treatment option for chronic wounds (Vyas et al. 2019). Ultrasound alone can also disrupt biofilms (LuTheryn et al. 2019).

A novel method has been developed to disrupt biofilms based on diatoms, hydrogen peroxide and nanosheets of manganese oxide. A chemical reaction between hydrogen peroxide and manganese oxide takes place inside the hollow interior of the diatoms resulting in tiny microbubbles that propels the diatoms forward with enough force to break up the surface and internal structure of biofilms. Once the diatoms have penetrated the biofilm, they continue to release hydrogen peroxide which is an effective disinfectant against bacteria and fungi (Seo et al. 2018).

Microbubbles have also been investigated for the improved delivery of nitric oxide for disrupting biofilms (LuTheryn et al. 2019), but this is a largely unexplored field to date.

Investigation of the function and cassette formation by integrons (bacterial genetic elements that can capture, rearrange and express mobile gene cassettes, such as spreading antibiotic resistance between strains) is expected to lead to new therapeutic molecules (Ghaly et al. 2020). ADP-ribosylation is also suggested as a target for new drugs for controlling bacteria (Catara et al. 2019).

Because bacterial resistance to drugs is known to be worsened if they are used in agriculture as well as medicine, regulators are reluctant to allow their use on plants. However, novel compounds such as NO, or therapies that use broad spectrum disinfectants such as chlorine or hydrogen peroxide, can potentially be used in agriculture. Any therapies that rely on mammalian immune systems, such as manufactured T cells, are also not directly applicable to control of bacterial infections of plants. However, conventional medicine does not utilise the antibacterial

properties of the natural products such as are listed above. This list is similar to the products that are used as food additives, and these should be of some use against phytobacteria, but some are only effective in vitro.

Viruses

Approximately 90 new antiviral drugs have been approved in the past 50 years, of which 29 have been approved in the past 6 years. Most of these new antivirals have been developed to treat hepatitis C and HIV. Antivirals are very specific to a distinct virus group, can only rarely cure infection, and there are no known broad spectrum antivirals currently available (Kaufmann et al. 2018).

One new strategy for virus control is host-directed therapy (HDT) which uses biologics or small molecules. HDT interferes with host mechanisms essential for viral replication or persistence, enhances host defence mechanisms, reduces inflammation induced by the pathogen or modulates over-stimulation of host defences (Kaufmann et al. 2018). Because host responses are targeted, pathogen resistance is less likely to evolve. HDT drugs that are in use or in clinical trials are listed in the public domain supplementary material provided by Kaufmann et al. (2018).

An example of a drug that blocks the virus particle binding to host cell receptors is a CCR5 inhibitor (Maraviroc) which is in clinical use (Kaufmann et al. 2018). Virus controls also include engineered T cells, immunotherapy, monoclonal antibodies and antiviral cytokines (Kaufmann et al. 2018). Other examples of antiviral agents also include neuraminidase inhibitors such as zanamivir and oseltamivir (Jagannath et al. 2016).

However, drugs or anti-virals derived for human viral diseases that may be useful in plant systems are few, because of their specificity and because plants lack an immune system. Targeting the host defences or receptors is not likely to be directly transferrable from a mammalian system to a plant system, although plant virus research laboratories do conduct fundamental research likely to elucidate possible targets for plant based HDT. The danger of resistance to human pathogens is enhanced if human applied drugs are used in plant production, and regulatory bodies are reluctant to approve plant use for this reason.

Detailed review of mitigation treatments for plant material

Dormant budwood destined for use as grape scions are treated with hot water at 50°C for 30 min (Bazzi et al. 1991). For sugarcane, the protocol can differ depending on the pathogen that is being treated. Applications can be by hot water, hot air, moist hot air and aerated steam applied at temperatures from 50–54°C for 1–8 min. Apples are not always hot water treated, but hot water treatment of budwood at 45°C for 3 h was recommended for killing *E. amylovora* (fire blight) (Keck et al. 1993) and *Neonectria ditissima* (Amponsah et al. 2016). Hot water baths and drenches have also been experimentally proven to reduce expression of Bull's eye rot (*Neofabraea* spp.) and other fungal pathogens of apple fruit during coolstorage (Maxin et al. 2012).

Pre-conditioning can improve tolerance of the plant material to heat, as was shown by storing pineapple crowns at 30–40°C for 30 min before treatment at 50°C (Nyland & Goheen 1969).

Seed treatments are routine, and generally consist of fungicides applied to the seed as dusts, thick water suspensions, or the seed is soaked in a chemical then dried. Tubers, bulbs, corms

and roots can be treated in similar ways (Agrios 2005). Chemicals used for these treatments in the past include inorganic copper and zinc compounds, but are more recently mostly organic compounds such as chlorothalonil, iprodione, thiram, boscalid, thiophanate methyl, tebuconazole, pyraclostrobin, trifloxystrobin and azoxystrobin (Mancini & Romanazzi 2013).

Seeds can also be treated with hot water, aerated steam, and electrons. Hot water treatments are having a resurgence in interest (Forsberg et al. 2002; Gilbert et al. 2005; Koch et al. 2010; Schmitt et al. 2009), and are extremely effective at killing pathogens yet retaining seed germinability. Aerated steam and electron bombardment are more recent innovations that are also effective (Schmitt et al. 2006).

Biopesticides have also been used as vegetable seed treatments, and are considered an attractive option to reduce the use of synthetic fungicides in agriculture. The most effective essential oil in vitro and in field tests was thyme oil (Mancini & Romanazzi 2013). Other essential oils, such as tea tree, clove, peppermint, rosemary, laurel, oregano oils, show good anti-fungal activity in vitro, as do onion seed extracts. When applied to seeds, chitosan, which is a compound extracted from crab-shell chitin, can improve germination, both percentage and rapidity, increase shoot height, root length, and root and shoot weights. It can also protect seeds against several pathogens (Mancini & Romanazzi 2013). Biocontrol agents are also used for coating seeds, most particularly plant-growth promoting rhizobacteria (PGPR) which colonise the rhizosphere and produce substances that promote growth of the associated plant, such as hormones, vitamins and growth factors. These PGPR also reduce the populations of root and foliar pathogens in the rhizosphere (Mancini & Romanazzi 2013). PGPR include species of *Pseudomonas*, *Bacillus*, and *Streptomyces*.

Trichoderma species have also been shown to reduce pathogen populations such as *Pythium, Phytophthora*, *Rhizoctonia* and *Fusarium* spp. when applied to seeds (Harman 2006).

Mychorrhizal species also show promise as seed treatments, e.g. *Piriformospora indica*, which is a recently discovered arbuscular fungus in the Basidiomycetes. Application to roots has been shown to protect wheat and tomato from fungal and viral pathogens (Fakhro et al. 2010). Mycorrhizae are used routinely to inoculate seedlings in forestry nurseries, which is proven to enhance seedling establishment in the field (Chu-Chou & Grace 1990; Bowen 1965). Research has shown that mycorrhizal associations can increase the host’s resistance to pathogen attack (Smith & Read 2008).

Table A2. Treatments used for preservation of fresh food products.

Type of treatment	Wet	Dry	Commercialised	Options
antioxidants	yes	no	yes	lactic, citric, L-ascorbic, acetic, tartaric, malic, sorbic, malic and peroxyacetic acids
essential oils	yes	no	yes	vanillin, citral and citron, hexanal, hexyl acetate, carvacrol, cinnamaldehyde, cinnamon, eugenol, thymol, eucalypthol and menthol
derivatives of essential oils	yes	no	yes	terpenoids, polyphenols and thiols
secondary metabolites from plants	yes	no	yes	rutin, gallic acid, chlorogenic acid, caffeic acid, catechin and ferulic acid
elicitors	yes	no	no	Jasmonic and salicylic acid, acibenzolar-s-methyl and methyl jasmonate
electrolysed water	yes	no	yes	
heat	yes	yes	yes	blanching, heat shock, infrared radiation, electric heating (dielectric and microwave)
irradiation	no	yes	yes	x-rays, gamma rays
ultraviolet light	yes	yes	yes	
ultrasound	yes	no	?	
photodynamic inactivation (pulsed light)	no	yes	?	
cold gas plasma	yes	yes	no	
high pressure homogenisation	yes	no	yes	
quorum sensing inhibitors	yes	no	?	
bacteriophages	yes	no	no	
biological control agents	yes	no	yes	
zinc oxide nanoparticles	yes	no	?	
RNA interference (RNAi)	yes	no	no	
modified packaging/atmosphere	no	yes	yes	moderate-vacuum packaging, active or intelligent packaging, nanocomposite materials, edible films
low humidity	no	yes	yes	
chilling	no	yes	yes	
fungicides	yes	no	yes	fenhexamid, bicarbonate, fenbuconazole, triforine, prohexadione-ca
antibiotics	yes	no	yes	levomycetin, ocytetracycline
calcium-based solutions	yes	no	yes	calcium carbonate, calcium citrate, calcium lactate, calcium chloride, calcium phosphate, calcium propionate and calcium gluconate

Table A3. Treatments used to mitigate plant pathogens from propagation material.

Type of treatment	Wet	Dry	Commercialised	Material treated	Options
thermotherapy	yes	yes	yes	cuttings, bulbs	hot water, hot air, moist hot air and aerated steam
fungicides	yes	yes	yes	tubers, bulbs, corms, roots	inorganic copper and zinc compounds, chlorothalonil, iprodione, thiram, boscalid, thiophanate methyl, tebuconazole, pyraclostrobin, trifloxystrobin and azoxystrobin
electron bombardment	no	yes	?	seeds	X-rays, gamma irradiation
essential oils	yes	no	no	seeds	thyme oil, tea tree, clove, peppermint, rosemary, laurel, oregano oils, onion seed extracts.
biopesticides	yes	no	no	seeds	chitosan
biocontrol agents	yes	no	yes	seeds, roots, seedlings	Plant-growth promoting rhizobacteria (PGPR), trichoderma, mychorrhiza (e.g. *Piriformospora indica*)

Table A4. Novel treatments used for controlling human bacterial infections.

Type of treatment	Wet	Dry	Commercialised	Available for pollen treatments	Options
new drugs	yes	inhaled	yes	no	effectors, anti-biofilm enzymes (dispersin B), anti-biofilm signallers (acyl homoserine lactones), antimicrobial peptides, bacteriophages, B-lactam/B-lactamase inhibitors
Quorum sensing disruptors	yes	no	yes	no	(z)-5-octylidenethiazolidine-2,4-dione and lipoic acid, phenylalanine arginyl β-naphthylamide, anteiso-C15:0, doxycycline antibiotic,2,5-piperazinedione, 1037 cationic peptide, and 1-naphthol
natural products	yes	no	no	yes	7-hydroxyindole, ginseng extract, carvacrol, eugenol, silver nanocomposites, cinnamaldehyde, lactic acid, 3-phenyllacetic acid, equisetin, flavone derivatives, cucumin, azithromycin, gentamicin, resveratrol, 2, 4 di-tert-butylphenol, reserpine, 3,5,7-trihydroxyflavone, glycoprotein curcumin, ceftazidime and ciprofloxacin, quercetin, D3112 protein gp05 (phage protein), adenosine triphosphate, chlorogenic acid, Vitexin, caffeine, 2,5-piperazinedione, 3-benzyl-hexahydro-pyrrolo(1,2-a)pyrazine-1,4-dione and AiiAs1-5 stilbenoids,7-fluoroinole, ellagic acid, caffeic acid, cinnamic acid, ferulic acid, vanillic acid, tea polyphenols, baicalin, methyl gallate. Diallyl disulphide (garlic oil), hordenine, fenaclon and
lactoferrin	yes	no	yes	no	
nitric oxide	no	yes	yes	yes	
microbubble	yes	no	no	no	

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