Quarantine Regulations and the Impact of Modern Detection Methods

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Abstract

Producers worldwide need access to the best plant varieties and cultivars available to be competitive in global markets. This often means moving plants across international borders as soon as they are available. At the same time, quarantine agencies are tasked with minimizing the risk of introducing exotic pests and pathogens along with imported plant material, with the goal to protect domestic agriculture and native fauna and flora. These two drivers, the movement of more plant material and reduced risk of pathogen introduction, are at odds. Improvements in large-scale or next-generation sequencing (NGS) and bioinformatics for data analysis have resulted in improved speed and accuracy of pathogen detection that could facilitate plant trade with reduced risk of pathogen movement. There are concerns to be addressed before NGS can replace existing tools used for pathogen detection in plant quarantine and certification programs. Here, we discuss the advantages and possible pitfalls of this technology for meeting the needs of plant quarantine and certification.

INTRODUCTION

The best approach to minimize the impact of pests and diseases is to contain the organism at the center of origin. This is true independent of host, geography, or timing, from the Ebola viruses in Africa (10) to bovine spongiform encephalopathy in Europe (63) and late blight in Mexico (45, 89). Such cases affecting humans, livestock, and plants provide excellent examples as to why quarantine regulations have been developed across the globe. According to the Merriam-Webster Dictionary, quarantine is "a state of enforced isolation or a restraint upon the activities or communication of persons or the transport of goods designed to prevent the spread of disease or pests." Still, quarantine will never be absolute, and this should always be taken into consideration when implementing rules and laws, because, unless eliminated, the disease agent will eventually escape and move to unaffected areas. This is more true than ever before, as climate change alters the distribution and prevalence of pathogen vectors and globalization has allowed for the greatly expanded flow of goods and people, which themselves can be excellent carriers of animal and plant pathogens (6, 7, 12, 17, 43, 71, 73, 111, 114).

A primary example of a commodity for which the global distribution has increased dramatically in recent years is planting material that supports improvement and expansion of agricultural and horticultural industries in developed and developing nations (50, 98). Such material should be thoroughly tested and found free of pathogens of concern. Whereas most entities involved in movement of plant material comply with regulations, illegal introductions can cause major mayhem, as was, for example, the case of the introduction of *Plum pox virus* into the United States, which cost more than 53 million US dollars to eradicate from a relatively small area in the state of Pennsylvania (127). Still, a very important factor that should be taken into consideration when testing plant material distributed at the global, national, or even local level is the unknown, i.e., pathogens that have not been reported before and for which there are no methods of detection.

As detection technologies evolve, becoming more sensitive and broader in scope (61, 81, 119), there has been a clearer view of how diseases emerge. This is especially true for diseases of perennial crops, which are often caused by pathogen complexes. The individual components of these complexes may be asymptomatic, but when they come together they may cause dramatic symptoms and losses (27, 78, 79, 97). This is where the incorporation of new technologies that provide a greater capacity for unbiased detection of any disease agent, known or unknown, will play a significant role in minimizing the possibility of such epidemics.

In this review, we discuss the challenges facing quarantine regulations in the era of metagenomics. New technologies can detect an array of uncharacterized pathogens, which are a major challenge for quarantine standards, as it is impossible to differentiate between agents that may appear pathogenic but are benign in agricultural and ecological terms and those that are truly harmful on the local, national, and global scale. The importance of biological characterization of new agents is addressed with emphasis on the population structure and epidemiology of the potential pathogens.

THE ROLE OF QUARANTINE

The aim of plant biosecurity is to protect our way of life from important plant pests that can harm the economy through loss of crop production and market access, to ensure our food security, and to protect our environment. The term plant pests most commonly refers but is not limited to insects, viruses, viroids, bacteria, fungi, oomycetes, and nematodes. As noted before, one of the primary threats to plant biosecurity occurs through trade and movement of contaminated plants and plant products.

Plant quarantine is a biosecurity measure designed to reduce the introduction and spread of economically important pests of plants or plant products that are not yet present in an area or that are present but do not widely occur and are under official control [Food and Agriculture Organization of the United Nations (FAO)] (41, 42). Plant quarantine regulations can be implemented nationally at the border and regionally within a country. All countries have a sovereign right to impose plant quarantine policies and regulations to protect production and market access for plant industries and to protect the natural environment. This approach to pest control may also be applied regionally to protect local industries and ensure containment of a regulated pest.

In reducing the risk of introduction of plant pests, plant quarantine policies also have the capacity to prevent trade of plants and plant products, and therefore limit movement of plant germplasm. However, movement of germplasm is important for not only the economic development of the export country but also the capacity of the importing country to develop markets and trade competitively at regional, national, and international levels and to ensure food security (11, 114). For the countries that are members of the World Trade Organization (WTO), the WTO Agreement on the Application of Sanitary and Phytosanitary (SPS) Measures requires that, where appropriate, these quarantine policies and measures be consistent with the rules and standards developed by the International Plant Protection Convention (IPPC) and that plant quarantine should not be used unreasonably as a technical barrier to trade (41, 42, 129). It is also important to recognize that no country or region within a country can operate on a no-risk basis; if this were to occur, there would be no trade or movement of plant germplasm into that area (11). Instead, it is important to undertake a policy of manageable risk, which is based on science, and that all quarantine measures be commensurate with the risk related to the specific pest (85, 88).

Countries can undertake a pest risk analysis (PRA) to assess the potential for entry, establishment, and spread of a pest and the potential biological and economic consequences of such an entry on plants or plant products [International Standards for Phytosanitary Measures (ISPM) No. 2 (42) and ISPM No. 11 (41)]. This is then used to determine the biosecurity measure required to minimize the risk of introduction and to mitigate the impact of introduction should it happen. The plant quarantine import conditions depend on the genus and species of the plant and the type of imported plant material. In undertaking a PRA, the first step is to determine whether a pest is present in a region, and if so, whether it has a restricted distribution and is under official control. Surveillance should be undertaken within a region to determine the presence or absence of a pest before imposing plant quarantine. The potential for establishment and spread and the economic impact should also be considered in determining the quarantine status of a potential pest.

When plants or plant products arrive at the country border, they may be held in quarantine for a length of time dependent on the commodity and its risk of introducing a quarantine pest into the importing country. Some plant propagation material and seed can be inspected, treated, and/or pathogen-tested upon arrival, which can provide a rapid and safe release. However, many plant propagation materials require a period of growth and disease screening and/or pathogen-testing in a post-entry quarantine (PEQ) facility to ensure the absence of pests and diseases. This process is undertaken because some pests, especially viruses and vascular-limited bacteria, exist in low concentration and can take time to reach detectable levels or symptoms can take time to develop (32). In some instances, PEQ has been imposed for several years to minimize the risk of not detecting a pathogen, which can represent a significant delay in germplasm reaching new markets and reduce competitiveness.

Biosecurity measures to expedite the movement of germplasm can begin offshore. Many countries ask that the exporting country provide evidence of absence of pests within the regions from which the material originates. Importing material from approved suppliers who have processes

in place to minimize the incidence of pests and diseases in propagation material can reduce PEQ periods in some countries (33). For example, Australia recognizes two approved sources for *Fragaria* × *annassa* (strawberry) that can provide meristem-cultured plantlets produced from mother plants that have been maintained in an insect-proof environment and tested using acceptable procedures. This information must be declared on the accompanying phytosanitary certificate, and the plants must be found free of several viruses (**https://bicon.agriculture.gov.au/BiconWeb4.0**). The material must still undergo some required pathogen-testing for quarantinable pests in PEQ, but the time to release can be reduced.

Some offshore testing and treatment of propagation material may even allow immediate entry of material after inspection of phytosanitary certificates and inspection of the material. *Candidatus* Liberibacter solanacearum is one of the most serious threats to potato production and market access worldwide and is a high priority quarantine pest for Australia (91). Studies indicate that it may be seedborne in carrot (14), and contaminated seed is a potential pathway for the introduction of this bacterium into Australia. Therefore, in 2015, Australia introduced phytosanitary measures for import of carrot seed that included onshore or offshore heat treatment prior to entry to minimize the risk of the pathogen in the seed, or onshore or offshore testing to provide evidence of absence of the bacterium in the seed. By offshore treatment or testing for *C.* L. solanacearum, carrot seed arriving at the Australian border should be allowed immediate release as long as it complies with all other conditions, meaning faster access to seed for growers to meet planting deadlines.

CERTIFICATION PROTOCOLS: TWENTIETH CENTURY SCIENCE FOR TWENTY-FIRST CENTURY NEEDS

Most phytosanitary certification protocols are targeted toward specific pests and diseases, especially those that are considered significant threats to production and the ecosystem. They tend to target pests, such as viruses, viroids, vascular-limited bacteria, and endophytic fungi, that are spread in propagation materials and cannot be easily eradicated through chemical or physical control treatments, such as chemical dips, sprays, fumigation, or heat treatments. For many crops, testing is required for multiple pests and pathogens. Detection methods for pests in quarantine and certification programs include visual inspection of germplasm, isolations onto growth media, bioassays in which a sensitive indicator is inoculated and inspected for specific symptoms, microscopy, serology, and molecular methods such as polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), quantitative-PCR (q-PCR), or nucleic acid blot assays.

Central to any phytosanitary certification protocol is diagnostic reliability: Protocols should have repeatability, reproducibility, and diagnostic sensitivity to minimize false results (39). However, the detection methods used in certification protocols vary greatly in their process and analytical sensitivity, and it is important to remember that no single method is completely reliable for pathogen detection. They are all impacted by the biology of the pest and its interaction with the host and the environment, which can affect symptom expression and pathogen titer. Many of the methods listed above were designed for the specific detection of a target pest or disease (131). Most were developed using the best available knowledge of the pest biology and genetics, which may have been limited at the time of test development, and therefore they are at risk of being limited in their capability for pest detection. Additionally, most were developed using plant material grown under greenhouse conditions in which pathogen titer is often higher than that found in plants grown under field conditions. Additionally, virus titers can be greatly impacted in plants having mixed infections, which is the norm in perennial plants. All detection methods are sensitive to pest concentration, genetic variability within a target pest, and similarities between the target and other organisms, and they may not be suitable for discovery of unknown pests (22).

Visual inspection of plant germplasm and subsequent selection of healthy material (no obvious symptoms of specific disease) is one of the simplest methods of plant pest detection and was the basis for early certification programs (44). However, visual inspection for disease on germplasm or in a bioassay is impacted by pathogen concentration in the host and by environmental and biological factors that affect symptom expression (32, 33, 79, 108). Some serious pests that are less virulent, asymptomatic, or symptomless in some plant varieties, clones, or cultivars can escape visual detection, e.g., many host plants persistently infected by *Xylella fastidiosa* remain symptomless (54, 95). Visual inspection of germplasm and bioassays may not differentiate between species and strains of pathogens that are associated with similar symptoms, e.g., all *Grapevine leafroll virus* species are associated with nearly identical leafroll disease symptoms in many cultivars and in the sensitive Cabernet franc and Pinot noir indicators. Detection by visual inspection of germplasm and bioassays is also dependent upon the skill in identifying symptoms and differentiating them from those caused by other factors (100). Bioassays, particularly indexing for viruses on woody indicators for crops such as grapevine, stone fruits, and pome fruits, are dependent on successful inoculation (32). Bioassays can take from several weeks to several years to complete (107). Therefore, more active diagnostic methods improve the chance of detection of a pest and can reduce the time taken for diagnosis.

Microscopy methods may be the frontline for diagnosis of some pests based on morphology, such as fungi and insects, and a useful backup to prove the presence of some viruses, but they require a high level of skill and knowledge. For some pests, such as viruses of the same family, genus, and species with nearly identical particle morphology, microscopy can be nonspecific. It can also lack sensitivity for pathogens detected in planta that occur in low titer, such as some viruses and phytoplasmas.

Serological methods such as enzyme-linked immunosorbent assay (ELISA) use antibodies to detect the presence of an antigen, such as a viral coat protein, in a sample (28, 29). ELISA is a simple technique and is useful for high-throughput testing. The reliability of polyclonal antisera is compromised by poor quality purifications that contain contaminating proteins of the plant host or coinfecting organisms, leading to nonspecific detection and false positive results (55). Polyclonal and monoclonal antisera may be affected by the degree of conservation within a protein sequence, and it is possible that they cross react with other species that produce a similar protein (51). Occasionally significant variation in the structure of the protein between closely related and targeted organisms can result in nonrecognition of the target, leading to a false negative result (13, 40). ELISA can also lack the sensitivity of methods, such as PCR and some bioassays, that multiply the target nucleic acid or the target pest to a level where they are easily detected (25, 57, 109).

Molecular methods, especially PCR, have become the method of choice for plant pest detection. PCR tests can be highly specific, targeting individual strains or species, or generic, targeting groups of pests (e.g., 26, 49, 74, 134). They can be used in combination with sequencing technology to confirm the identity of the amplified target (e.g., 77, 82, 126). However, genetic variability can impact test reliability, reducing the binding capacity of the primers, even in conserved regions of the genome, or mispriming as a result of genetic similarity to other organisms at primer and probe binding sites (94, 124). Reverse transcription and polymerase enzymes are sensitive to compounds that may be coprecipitated with extracted nucleic acids, resulting in false negative results (72, 128). PCR methods generally have greater sensitivity than other pest detection methods; however, there is still a lower limit of detection for any pathogen, and its reliability can depend upon appropriate sampling strategies.

QUARANTINE, DISEASE, AND DETECTION CHALLENGES

The biosecurity of many crops can be challenged by natural dispersal of pests and disease, which can thwart quarantine measures. The dispersal of airborne pests and diseases is influenced by multiple factors, the weather patterns being the most significant and yet uncontrollable. Such are the cases of the emergence of soybean rust in North America (58, 67) and dispersal of wheat stem rust lineage Ug99 around the globe (113).

The human factor, as seen in ongoing climate change, affects the emergence and reemergence of pests and diseases and limits production potential in several areas across the globe (23, 34, 123). Still, there are myriad examples of disease agents and/or vector dispersal, plant or other, correlated with human activities at the individual level (48, 65).

For quarantine purposes, human error can also have detrimental effects on pathogen distribution. An excellent example comes from the *Rubus* virus indicator *Rubus occidentalis* "Munger." "Munger" has been the standard indicator for virus screening, although many of the viruses identified in recent years do not induce symptoms on the cultivar (78). A study focusing on the genetic diversity of black raspberry in North America revealed that four major cultivars, including "Munger," are virtually indistinguishable based on phenotype and were all being sold as "Munger" (38). The intentional or unintentional mislabeling of "Munger" may have greatly affected the *Rubus* virome. *Blackberry yellow vein associated virus* and *Blackberry virus Y*, two asymptomatic viruses on "Munger" and major components of the blackberry yellow vein disease complex (118), were circulating at high incidences in a significant number of nurseries across the United States prior to the development of specific tests for these viruses (117). If the true "Munger" developed symptoms from the newly identified viruses and these viruses were detected in bioassays, it is conceivable that producers would have avoided the better part of the blackberry virus epidemic in the southeastern United States. Given that grafting onto susceptible indicator plants is the gold standard for virus detection in quarantine facilities, it is possible that a mix-up in a plant cultivar used as a virus indicator, leading to false negative results, could lead to the distribution of important pathogens across the globe.

Advances in technology that have allowed for major expansion in the list of pathogens affecting crops provide another challenge to the maintenance of quarantine and certification pathogen lists and detection protocols. For example, in the 2014 release of the International Committee on Virus Taxonomy there were more than 1,200 plant viruses formally recognized compared with 980 in 2005 and 380 in 1991, and most are associated with disease (**http://ictvonline.org/**). Many recent virus discoveries have occurred through new technologies to identify viruses associated with disease (2, 9, 73). Ecological prospecting projects suggest that the number of plant viruses is likely to be far greater, with many being cryptic or possibly conferring a benefit to the plant hosts (102–105, 130). The number of formally recognized plant-pathogenic bacteria is also increasing, especially because of the recognition of new species of uncultivable bacteria such as *Candidatus* Liberibacter and *Candidatus* Phytoplasma species, pathovars, and strains (18–20). As with viruses, there are many unique uncultured bacteria observed in environmental samples, especially soils, based on genome fragments such as the 16S rRNA gene, for which no biology is known.

Many plant-inhabiting pests, such as viruses, viroids, and phytoplasmas, can be unevenly distributed and in low titer, making their detection difficult (31, 33, 72). It is a prerequisite for any technology discussed herein for the user to understand sampling strategies to improve the chance of detection and reduce the risk of false negative results. Regardless of how sensitive a test is, sampling strategy is paramount to developing a reliable assay.

As technology evolves, so do detection methods. In general terms, methods are becoming faster and more sensitive, allowing for monitoring and detection in real time (16, 52, 77, 110). If we look

at the most common detection methods used by quarantine facilities, it becomes evident that the newer technologies are more specific than the ones they replace (e.g., PCR is more specific than ELISA, which is more specific than grafting or mechanical inoculation onto indicators) (77).

There are, of course, exceptions to this rule, e.g., group-specific PCRs (74, 101, 121, 134), but in most of those cases, sensitivity tends to be lower than the specific counterparts. Whereas all agree that those technologies are superior to the ones they replace, pathogen diversity is a major concern.

It is natural law: The pathogen evolves faster than the host or else it perishes. Viruses provide an excellent example as they evolve to overcome resistance, improve vector transmissibility, or extend their host range using both micro- and macroevolution from genetic drift via the quasispecies distribution of mutations to recombine and reassort in multipartite viruses (56). It becomes evident that the definition of a virus isolate or even species is a fluid concept.

The population structure and diversity of most pathogens are grossly understudied. One or a few isolates are characterized at the molecular and biological level and are used for the development of laboratory tests. Still, this is only a snapshot of the population of the species, whereas the studied isolates may, for all intents and purposes, be the outliers rather than representative of a species. In such a case, the newer tests may provide the least reliable results because they are too specific and lack recognition of many isolates of the pathogen. There are several examples to that effect. Monoclonal antibodies recognize a single epitope, which results in a test with minimal background because they are screened for reaction to the pathogen and nonreaction to the host. In the case of *Raspberry bushy dwarf virus*, three monoclonal antibodies developed against the R15 strain of the virus have reacted with all isolates from red raspberry tested to date, whereas only two of the three reacted to an isolate from grapevine (92) and an isolate from *Rubus multibracteatus* from China (24). A more extreme example is the development of monoclonal antibodies against *Blueberry red ringspot virus*, which reacted only with the isolate of the virus in the field from which it was purified (R.H. Converse, personal communication). A potyvirus genera-specific monoclonal antibody has been developed that is very useful for a first screen when examining an unknown; however, it must be cautioned that it does not react with all potyviruses (62). Another example involves *Blackberry chlorotic ringspot virus* (BCRV), reported almost simultaneously in the United Kingdom and the United States (60, 120). A population structure study based on US isolates obtained from different hosts was conducted. It became apparent that the US isolates are homogeneous but distinct when compared with the UK isolate, with nucleotide identities of the UK isolate being as low as 66% in parts of the genome when compared to the US isolates (93). If someone were to use the sequence data from the UK isolate for development of detection tools to screen US material, it is probable that much of the material tested would be categorized as BCRV-free, allowing for the dispersal of the virus or US isolates of the virus around the globe.

Although current diagnostic techniques provide opportunities for disease diagnosis and pathogen detection, a further challenge for plant quarantine is the ability to determine the cause of a disease or symptoms that might be observed during quarantine inspections, especially those that are unfamiliar or unusual or for which a pathogen is not known. If a pest cannot be detected by any one of these methods, should that diseased material be released? A plant protection organization is often conservative in this situation and stops the release of germplasm exhibiting unusual symptoms for which a cause cannot be determined using the test methods that are available.

This is where new sequence-independent, unbiased detection technologies could play a significant role in eliminating such problems, as they have the ability to identify all known and, most importantly, unknown agents in a timely fashion, eliminating largely the human and pathogen factors as described above.

New Detection Technologies

Since the discovery of pyrosequencing (87) and sequencing of the *Mycoplasma genitalia* genome in 2005 (76), there has been a revolution in biology associated with what is referred to as large-scale or next-generation sequencing (NGS). As there have been several excellent reviews on the mechanics of the different NGS platforms (75, 99, 122), they are not discussed here. Instead, this review focuses on the potential applications of the NGS technologies in detection and quarantine.

NGS technology is being embraced in plant pathology and is likely to be used in the future as a fast, accurate, and routine diagnostic tool for plant quarantine and certification protocols, especially as the cost is reduced and pipelines for analysis improve (1, 16, 22, 64). It is a method that can be used to detect knowns, unknown knowns, known unknowns, and unknown unknowns, and it has a greater capacity to detect multiple pests in different kingdoms and down to the strain level in a single test (116, 131). It can also be used to detect very low titer organisms (116).

As many of the technologies are offered as services by several commercial entities and university central laboratories, the end user is often provided with sequence data. Yet, analysis of massive amounts of sequence data presents a major bottleneck. For the average user, the process is time consuming in the absence of bioinformatics pipelines tailor-made to the analysis required. Those pipelines identify signature motifs or structures of known and putative new agents without much involvement from the end user other than the choice of appropriate parameters for the analysis (15, 53, 96, 106, 115, 125, 132). Although bioinformatics tools are constantly improving, there are several points that need to be taken into account before moving to a full adaptation of NGS in quarantine-related diagnostics.

Bioinformatics pipelines use existing sequence databases, which by all standards only provide a snapshot of the biosphere diversity. If a protein sequence does not have an identifiable ortholog as determined by algorithms such as blocks substitution matrix (BLOSUM) (5) or partitioning around medoids (PAM) (36), which, incidentally, use preset matrices and probability theory, it is marked as unknown. Depending on the depth of knowledge for any given system, the unknowns may compose the majority of the putative protein coding sequences. For example, less than 16% of the ~2,500 predicted gene products of a pandoravirus showed any significant similarity to other proteins when compared using BLASTx (90). Granted, pandoravirus may be considered exotic, having been discovered in amoebas in long frozen tissue preserved in permafrost, but it is possible that exotic pathogens with minimal identifiable similarities to studied organisms exist and cause disease. In such a case even the best pipeline would fail to identify anything of significance.

As with all other diagnostic technologies, it is possible that NGS technologies may not offer the diagnostic sensitivity desired in quarantine situations. Was tissue sampling adequate? Was the nucleic acid of adequate quality? Does the detection of only a partial genome of an organism, with limited coverage and depth, during NGS truly represent a positive result? Such a result could easily be obtained from a nonviable nucleic acid from a pathogen that has been inactivated by some means and poses no quarantine threat. Horizontal gene transfer can occur from viruses and bacteria to plants, where pathogen genes can be integrated into the plant genome, and their detection may result in prevention of entry of germplasm into a region (68, 116). Therefore, diagnostic technologies for pathogen detection and discovery need to be well developed and validated to enable an accurate interpretation of the results and minimize the risk of false positives or negatives.

NGS and metagenomics have already opened new horizons when it comes to quarantine and movement of planting material across state or country lines. This is where biology and epidemiology of the newly identified agents have to be studied in depth, as discussed in the next section, as

a sequence without biological data can have an immense negative effect on the global movement of propagation material.

The Importance of Biology

Improved knowledge of the genetic diversity of many pathogens and detailed phylogenetic analyses can result in the new classification of many viruses, bacteria, and fungi, which can further complicate quarantine regulation (21, 35, 112). However, ISPM No. 11 states that the identity of the pest should be well understood and recognizable, and one of the challenges for plant quarantine regulation is being able to determine the measures required for safe importation of plant germplasm in the face of limited biological information and verified scientific data for newly described plant-inhabiting or plant-associated organisms. Plant protection organizations may take a conservative approach and list an organism as quarantine pest even when an association between the organism and a disease has not been or cannot be proven. Therefore, it is important that when new organisms are detected the biology of the pathogen and its association to disease is determined in order to inform decisions about safe movement of propagation material. It may be that some plant-inhabiting organisms are latent and symbiotic rather than pathogenic and disease causing (116) and should not be placed on quarantine lists.

With the advent of NGS for the discovery of new organisms, there has been a plethora of novel viruses described over the past two decades and this will likely continue into the foreseeable future (9). This technology is very useful in identifying organisms associated with diseases and the presence of mixed infections. However, the leaps forward in obtaining sequence information have resulted in the identification of new agents but without any associated biological information. In many cases, these newly described entities, primarily viruses and viroids, have not been shown to be graft transmissible, which is a very basic test that demonstrates the ability of a virus, viroid, or phytoplasma to move within a plant. There are several virus genera with members that are known to infect plants but that are not graft transmissible (endornaviruses, alpha-, beta-, and delta-partitiviruses, and amalgaviruses). These viruses are transmitted to the seed via pollen or ova, and seed transmission efficiency is at or near 100%. No other means of transmission has been identified with these viruses; thus, horizontal transmission does not occur. Very few viruses of this group have been shown to cause disease in plants in either single or mixed infections, the exception being *Vicia faba endornavirus*, which has been associated with a cytoplasmic male sterility (70). There have been multiple reports of viruses in these four groups from plants in recent years (3, 30).

As an example, we use *Blueberry latent virus* (BBLV), which has appeared on several quarantine lists shortly after information on the virus was published in a peer-reviewed journal (80). The publication clearly states that the BBLV was not graft transmissible, did not cause any disease in blueberry in single infections, did not cause any synergistic effects when coinfecting with other viruses, and was seed transmitted at 100%. The virus was described as a hybrid between members of the *Partitiviridae* and *Totiviridae* and subsequently approved as a member of a new virus family, *Amalgaviridae*. Even though the paper clearly stated the aforementioned information, it took additional intervention and explanation to quarantine agencies to remove the virus from their lists (R.R. Martin, personal experience). There should be a mechanism to avoid this type of response to new organisms in the future, in which information on the biology of known members of the genus is considered before decisions are made to add that organism to quarantine lists.

Care must be taken to obtain biological information for newly discovered viruses before they are added to quarantine lists and have an unnecessary impact on trade. At a minimum, graft transmissibility should be confirmed, as this is an important means of transmission in vegetatively

propagated crops. Viruses that are not graft transmissible do not pose a threat to native vegetation or existing agricultural production. In contrast, there are several examples of identifying new members in the family *Luteoviridae*, namely Strawberry polerovirus-1 (133) and Nectarine stem pitting–associated virus (8). Viruses in this family are known to cause disease in a wide range of plants (37) or serve as helper viruses for the transmission of viruses that cause disease (84). Members of the *Luteoviridae* are transmitted by grafting and aphids and, in the case of the enamoviruses, are also mechanically transmitted.

With the *Luteoviridae* or other families or genera of plant pathogens in which there are members known to cause disease, the prudent action would be to add these organisms to quarantine lists pending development of biological information. Whereas for pathogens in families in which members are not known to cause disease or be transmitted horizontally, the reasonable action is to leave them off quarantine lists until shown to cause disease. In a study of the virome of a vineyard in South Africa that used dsRNA pooled from 44 diseased grapevines, there were multiple viruses identified that belong to the *Totiviridae* and *Chrysoviridae*, families that are composed primarily from members that infect fungi, in addition to several known viruses of grapevine (30). In this study, identification of a novel virus in the *Chrysoviridae* had more sequence reads than two of the three grapevine viruses known to be present in the samples. It was not clear whether the chryso- or totiviruses were infecting grapevines or fungi that were colonizing grape tissues.

Another potential concern with interpreting NGS data is that incomplete sequences may be the remnants of incorporation of pathogen sequences into host genomes, as has been reported for members of the families *Caulimoviridae* (46, 69) and *Geminiviridae* (83). Kreuze et al. (66) evaluated whether NGS of small RNAs could be used for virus detection. They were able to detect the viruses that had been grafted into the test plants but also identified partial sequences of two badnaviruses (dsDNA viruses in the family *Caulimoviridae*) and a *Mastrevirus* (ssDNA viruses in the family *Geminiviridae*). Given that there are reports of less than full-length genomes of badnaviruses being incorporated into host genomes (47), the presence of virus sequences does not confirm virus infection or the risk of virus infection from activation of incorporated endogenous viral sequences. Incorporated viral sequences of *Banana streak virus* have been shown to be quite diverse, and it is likely that many of the inserted sequences are no longer able to contribute to the activation of infectious viruses (47). Also, confirmation of sequence by PCR would not prove the presence of intact, transmissible, and infectious virus particles. Rolling circle amplification (RCA) or RT-PCR can be used to differentiate between incorporated or endogenous viral sequences and episomal intact and actively transcribing circular viral genomes (59, 69). However, even if RCA is negative and the viral sequences are endogenous rather than episomal, it is possible that endogenous sequences can lead to reactivated virus, as has been shown for *Banana streak virus* (86).

To publish information on a new organism, it is now a standard requirement for most journals that sequences be deposited in GenBank before or at the time of publication. Should there be a related requirement for minimal amount of biological information on newly discovered organisms before they are incorporated in quarantine lists? A minimum of graft transmissibility seems reasonable for viruses, viroids, and phytoplasmas to demonstrate the presence of a biologically active organism for genera of viruses expected to be graft transmissible. A test to determine pathogen viability should be used to demonstrate that therapy was effective and the pathogen inactivated. It is quite possible that NGS, as well as PCR and ELISA, would give positive results for phytoplasma, bacteria, fungi, or viruses that have been inactivated by treatment. Thus, there is a need to follow up NGS information with biological testing rather than another test that could detect inactive pathogens, such as PCR or ELISA.

NEXT-GENERATION SEQUENCING AS THE NEW STANDARD FOR QUARANTINE TESTING

Recent work suggests that NGS can be as sensitive as graft indexing onto indicator plants (4) for detection of known viruses of grapevine. Similar results have been reported during the latter part of 2015 at three major meetings: The 23rd International Conference on Virus and Other Graft Transmissible Diseases of Fruit Crops, the 18th Congress of the International Council for the Study of Virus and Virus-Like Diseases of Grapevine (ICVG), and the 2015 meeting on The Virus and Virus-Like Diseases of Berries, Fruit and Nut Trees, and Grapevines. The results from these and other ongoing studies need to be carried through to peer-reviewed publications. More peer-reviewed publications on the detection efficiency of NGS are required before quarantine agencies, certification agencies, or the International Plant Protection Convention (IPPC) can develop policies on the acceptance of NGS data for documenting the health status of plants. The real advantage of NGS over biological indexing is speed, especially for perennial plants such as grapevines and tree fruits, where biological indexing using woody indicators can take three or more years to get results and may return false negative results because of pathogen-host interactions and/or environmental conditions. Still, no technology is absolute, and we should remember that NGS data analysis is only as good as the bioinformatics mining tools and the data depth of the sequence databases. It may be that biological indexing can pick up an agent that bioinformatics analysis of NGS data does not, simply because it is not represented in genome or nucleotide databases.

We must be cognizant with NGS, as with any detection technology adopted in the past, including ELISA, PCR, qPCR, microscopy, etc., that what constitutes a positive or negative result must be agreed upon. Standardized protocols need to be in place to minimize the risk of contamination in the purification of nucleic acids from sampled tissues and in the processing of the nucleic acids. Once the NGS technologies, from sampling and nucleic acid extraction through to final analysis of the data, are validated, can we afford to keep biological testing and NGS methods running side-by-side? Or will NGS technologies become the new gold standard for pathogen detection and allow for efficient and cost-effective testing of quarantine material that facilitates trade and at the same time safeguards agriculture and native fauna and flora?

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Contents

Errata

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