

# Forewarned is forearmed: harmonized approaches for early detection of potentially invasive pests and pathogens in sentinel plantings

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## Abstract

The number of invasive alien pest and pathogen species affecting ecosystem functioning, human health and economies has increased dramatically over the last decades. Discoveries of invasive pests and pathogens previously unknown to science or with unknown host associations yet damaging on novel hosts highlights the necessity of developing novel tools to predict their appearance in hitherto naïve environments. The use of sentinel plant systems is a promising tool to improve the detection of pests and pathogens before introduction and to provide valuable information for the development of preventative measures to minimize economic or environmental impacts. Though sentinel plantings have been established and studied during the last decade, there still remains a great need for guidance on which tools and protocols to put into practice in order to make assessments accurate and reliable. The sampling and diagnostic protocols chosen should enable as much information as possible about potential damaging agents and species identification. Consistency and comparison of results are based on the adoption of common procedures for sampling design and sample processing. In this paper, we suggest harmonized procedures that should be used in sentinel planting surveys for effective sampling and identification of potential pests and pathogens. We also review the benefits and limitations of various diagnostic methods for early detection in sentinel systems, and the feasibility of the results obtained supporting National Plant Protection Organizations in pest and commodity risk analysis.

## Keywords

alien invasive pests and pathogens, commodity risk analysis, early warning, sampling techniques, sentinel plants, pest risk analysis, prediction

## Introduction

Invasive alien species (IAS) are amongst the leading global threats to biodiversity, economy and human health (Sarukhan et al. 2005; Early et al. 2016). The number of alien species accumulating worldwide shows no signs of saturation (Seebens et al. 2017). Globalization and international trade have largely facilitated the unintentional long-distance movement of alien plant pests and pathogens into regions outside their

native distribution ranges (Seebens et al. 2017). Climate change is also causing natural shifts in the geographic ranges of species, enabling species to migrate and establish in new locations and possibly on new hosts (Musolin 2007; Battisti and Larsson 2015). In the last 200 years, the number of alien invasive forest pathogens has increased exponentially (Santini et al. 2013) and the rate of establishment of alien insect species has nearly doubled over the last 30–40 years in Europe alone (Roques et al. 2016). Relatively recent examples of devastating plant pests and pathogens distributed with live plants include the citrus long-horned beetle (*Anoplophora chinensis* Foster), the box tree moth (*Cydalima perspectalis* Walker), box blight (*Calonectria pseudonaviculata* (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous), ash dieback (*Hymenoscyphus fraxineus* T. Kowal), sudden oak death and ramorum leaf blight (*Phytophthora ramorum* Werres, De Cock & Man in 't Veld) (Santini et al. 2013; Prospero and Cleary 2017; Kenis et al. 2018).

Global trade of plants for planting is recognised as the principal pathway for accidental introductions of alien invasive forest and agricultural pests and pathogens worldwide (Kenis et al. 2007; Brasier 2008; Liebhold et al. 2012; Santini et al. 2013; Santini et al. 2018). Once an IAS becomes established and widespread, eradication becomes nearly impossible, the resulting impact and societal costs increase substantially, and only mitigation measures are feasible to help minimise the long-term impact to resource assets. Measures aimed at improving the knowledge base for better prevention of potentially harmful organisms to plants before they are traded will help reduce the risk of new invasions.

Most National Plant Protection Organizations (NPPOs) perform inspections and follow diagnostic protocols of plants for planting and commodities e.g., the European and Mediterranean Plant Protection Organization (EPPO) standards based on lists of known organisms described as invasive and harmful elsewhere (Vettriano et al. 2015). However, alien pests and pathogens often enter in new countries on either non- or unknown hosts, on infected but asymptomatic hosts (e.g. as endophytes, latent infections) or on associated commodities (e.g. soil, wood packaging) (Roques et al. 2015; Vettriano et al. 2017). Thus, there is a need for better tools and strategies to improve early detection of potentially harmful species before they are introduced.

In principle, an early warning system is a major element of disaster risk reduction (Wiltshire and Amlang 2006) developed, for example, to prevent loss of life and/or reduce the economic and adverse effects from a potential disaster. The use of sentinel species, i.e. organisms used to provide an advanced warning of a risk or danger to humans, has a long history in various cultures. One of the earliest uses of sentinel species as an early warning system is from the early 20<sup>th</sup> century when canary birds (*Serinus canaria* L.) were used in coal mines to warn of carbon monoxide hazards for workers. Sentinel plants in early warning systems are used too as indicators of potential risk associated with damage caused by pests or pathogens based on regular inspections of the plants for signs and symptoms of insect attack or disease (Wylie et al. 2008; Paap et al. 2017; Eschen et al. 2018). For example, sentinel plants have been used to provide adequate warning for damage downy mildew on cucurbit crops, and roses planted at the

end of vineyard rows can give an early warning for problems with powdery mildew. In addition, some sentinel plants, are used as indicators of air pollutants (Nouchi 2002).

Two main strategies apply to the sentinel planting concept: sentinel plantations and sentinel nurseries (Figs 1, 2). A sentinel plantation (“*ex-patria*” plantings sensu Eschen et al. 2018) can be defined as a plantation of non-native plants grown in an environment and monitored to identify biotic agents that affect the growth and vitality of those plants (Roques et al. 2015; Vettraiño et al. 2015). A sentinel nursery (“*in-patria*” plantings sensu Eschen et al. 2018) is defined as a site where native traded plants are planted without phytosanitary treatments in their region of production (exporting country) and monitored to identify pests and pathogens which could be spread with the trade of those plants outside of their native range (Vettraiño et al. 2017; Kenis et al. 2018). In this paper, we also consider the sentinel arboretum (Fig. 3) (included as “*ex-patria*” plantings sensu Eschen et al. 2018). Though not specifically designed as an early warning tool to detect potential plant pests or pathogens, arboreta and botanical gardens can offer another opportunity for sentinel research and contribute valuable information about novel pest–host associations (Britton et al. 2010; Tomoshevich et al. 2013). Procedures for sampling and appropriate protocols for detection and identification of pests and pathogens require standardization for all sentinel systems.

The aim of this paper is to provide an overview of the protocols and techniques useful in sentinel plantings with a focus on: 1) the capacity for sentinel systems to provide useful information to NPPOs for pest and commodity risk analyses, 2) the description of the harmonized diagnostic approach in sentinel plantings, its potential and its relation with the PRA and CMA and 3) sampling, diagnostics and the utility of different techniques in increasing our ability to accurately detect and identify new threats.

## **Sentinel plants supporting National Plant Protection Organizations**

The Food and Agriculture Organization of the United Nations (FAO) defines pest as “any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products” (FAO 2016). However, in the literature plant damaging organisms are frequently divided into “pests” (i.e. invertebrates: arthropods, gastropods, nematodes, etc.; in some cases, also vertebrates) and “pathogens” (i.e. fungi, bacteria and other agents causing plant diseases). Despite the harmful connotation implicit in these terms, it is important to note that not all organisms present in sentinel plantations should be considered injurious. But non-harmful organisms can become so when they change host or their natural environment. As sampling methods and identification protocols differ depending on the organism in question, pests and pathogens will be considered separately as two distinct groups in this work.

Pest risk analysis (PRA) is the process of evaluating biological and economic evidence to determine whether an organism is a pest, whether it should be regulated, and the strength of phytosanitary measures to be taken to reduce the risk of introduction (FAO 2018). PRA is increasingly being replaced by commodity risk analysis (CRA),

which instead of focusing on an organism considers a particular commodity (e.g. a plant species) (USDA 2012). The sentinel planting approach is well suited to support such risk analyses: sentinel plantations are focused on identifying potential pests and pathogens that should be the target of PRA, and sentinel nurseries allow identification of pests that may be imported on live plant targets of CRA (Eschen et al. 2018). Moreover, sentinel plantings can also provide information on the extent of damage caused by pests and pathogens, and their biology and ecology (Roques et al. 2015; Fries 2017), all of which are important for PRA.

Despite the great amount of data that can be derived from sentinel plantings, there are several issues that the scientific community and plant health regulators need to address in order to best optimize the use of these data:

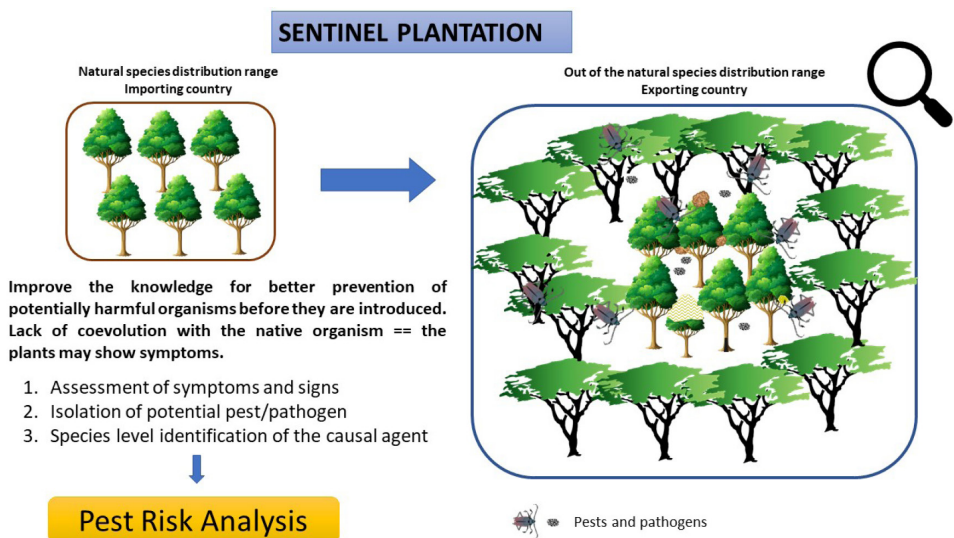
1. There is currently a mismatch between the systems of identification and classification of pests and pathogens used by scientists (e.g. pathogen lineages, molecular OTUs, taxon) and those used by regulators (usually formal species). How data on higher or lower taxonomic levels could be used in plant health regulations or specifically PRA has not been thoroughly examined, although Eschen et al. (2015) suggested that PRAs could target groups of potentially harmful organisms at a higher taxonomic level than species in order to improve plant health protection.
2. The number of unidentified taxa and new pest/pathogen-host relationships in recent sentinel planting studies remains high (Eschen et al. 2018). One main problem is that a PRA is normally only conducted once a pest or pathogen is formally described (FAO 2016). Time limitations and logistical issues restrict the ability of researchers to formally describe unknown taxa in sentinel plantings (Roques et al. 2015; Vettraino et al. 2015; Kirichenko and Kenis 2016). To alleviate this issue taxonomists based in the exporting countries need to be engaged through networking activities.
3. Reliance on DNA methods for detecting a pathogen does not reveal any indication of the viability of that particular organism. Hence, a limitation of high throughput sequencing (HTS) techniques, as suggested by Vannini et al. (2013), is that the risk to plant health remains unproven without a living sample of the pathogen.
4. If numerous potential pests and/or pathogens are detected, the limited resources available for carrying out the labour-intensive PRA process make it necessary to rank potential pests and pathogens according to their perceived risk. Ranking of potential pests that are detected in sentinel plantings need to be based on the biology and abundance of the pest, known substrates or hosts, frequency and severity of symptoms, or damage or known pathogenicity. Expertise or specialist knowledge from different fields (pathology, entomology, forestry) are essential to gain a holistic view.
5. Currently, the sharing of occurrence and disease data from existing sentinel plantings is rare, but a centralized database, as suggested by Britton et al. (2010), needs to be used by NPPOs to identify pests and pathogens for PRA. There are ongoing efforts as a part of the International Plant Sentinel Network (<http://www.plantsen->

tinel.org) to develop a database to store and share information related to sentinel plantings. This database should be updated with data from regular surveys and have some form of curation. Before data are added to the database, the records should be discussed with the NPPO of the exporting country. In some countries, it is obligatory to notify the NPPO of new findings of pest and pathogens whereas in all cases it is good practice to keep the NPPO duly informed (Eschen 2017). Fostering good relations with the NPPO is vital to enable the establishment and maintenance of the sentinel plantings (Roques et al. 2015). In many cases, NPPOs might also assist in pest/pathogen identification, data provision and further research.

## Diagnostic approach in sentinel plantings

### Sentinel plantations

In sentinel plantations, non-native plants are grown in a country out of their natural distribution range (e.g. native European trees planted in China) and monitored for potentially damaging agents which may provide useful data for PRA (Fig. 1). If novel pest/pathogen-host plant combinations occur, the plants are likely to develop symptoms due to a lack of coevolution with the native organism (Parker and Gilbert 2004; Vettraino et al. 2015). The assessment of symptoms and signs, along with sampling of symptomatic tissues, and the isolation of potential pest/pathogen organisms, should be prioritized. Therefore, methods and protocols used in sentinel plantations should aim



**Figure 1.** Schematic representation of the sentinel plantation concept. Tree species native to the importing country are planted in the exporting country. Being exposed to the resident pest and pathogens, they should develop visible symptoms.

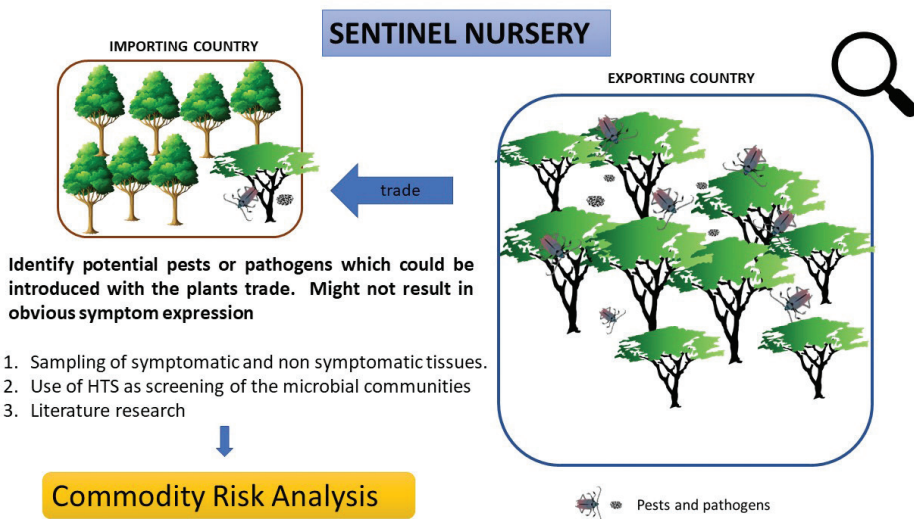


to characterize damage morphotypes, followed by isolation or collection and species level identification of the causal agent(s) (Roques et al. 2017).

It is necessary to carry out HTS analysis of a representative sample of the propagation material (e.g. seeds) intended to be used before export to the country where the sentinel planting will be located. Knowledge of the plant's endophytic community in its native range can give a baseline for interpretation of, for example, fungi contributing to disease. In sentinel plantation trials in China, absence of controls in the propagation material did not allow confirmation of the Asiatic origin of detected OTUs (Vettraiño et al. 2015).

### Sentinel nurseries

In a sentinel nursery, native plants are grown in their natural distribution range to identify potential pests or pathogens which could be spread with the international trade of these plants (Fig. 2). In this case, the results obtained will be helpful in CRA (Kenis et al. 2018). Assuming that host-parasite co-evolution of native species might not result in obvious symptom expression, a host shift to a taxonomically similar plant species in the final location of the plant may give rise to novel host-parasite interactions. Therefore, diagnostic methods that can detect endophytic or latent pathogens must be employed (Vettraiño et al. 2017) in addition to standardized diagnostics for symptomatic tissue. Thus, sampling must be oriented to both symptomatic and non-symptomatic material. In this system, the use of HTS is useful for screening of the



**Figure 2.** Schematic representation of the sentinel nursery concept. Tree species native to the exporting country and traded with the importing country are regularly inspected for resident pest and pathogens. Because of host-parasite coevolution, visible symptoms may not necessarily develop.

microbial communities even in the absence of symptoms. One possible way to filter large datasets arising from HTS is to group the OTUs according to their functional guild, focusing the sampling and identification on what are grouped as pathogens or opportunistic pathogens. In the case of fungi, online applications, such as FUNGuild (<http://www.stbates.org/guilds/app.php>), can be used for this purpose as a base for downstream analysis (Nguyen et al. 2016).

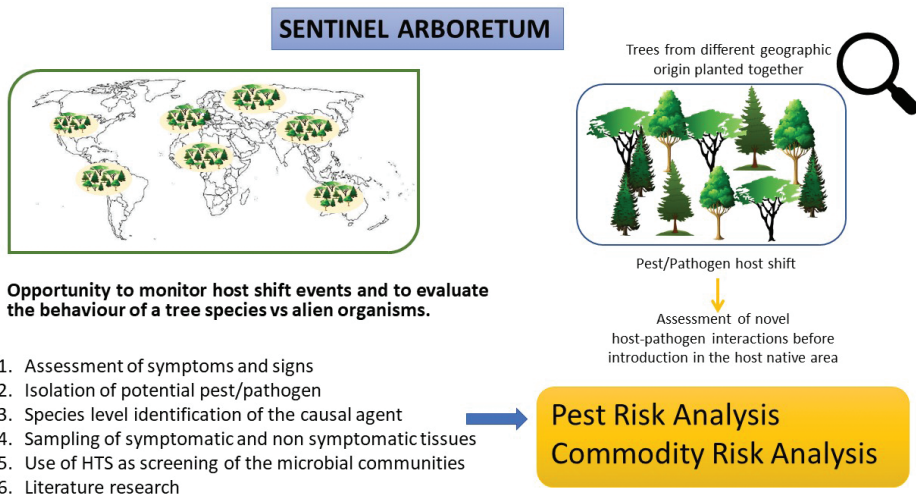
Previous fungal studies in sentinel nurseries have not provided conclusive evidence of identified risks but rather provided information that must be analyzed to arrive at a selection of taxa for further study of whether these organisms pose a threat if introduced in a naïve habitat (Vettraino et al. 2017). Information including a collection of isolates, with molecular barcoding and, eventually, taxonomic positions and a database of OTUs resulting from HTS analysis, would greatly strengthen further analyses. Large data sets can be difficult to interpret and require appropriate databases of molecular data and plant pathogens and, certainly, the scientific literature, to make full use of their potential. A limit to data interpretation is the fact that only a small percentage of global microorganism diversity is so far present in the databases. A positive aspect is that a large number of undescribed taxa are present as sequences in molecular databases, which may provide unexpected matches with OTUs from sentinel plantings and useful information on previous detection.

During arthropod studies in sentinel nurseries (Roques et al. 2015), systematic sequencing of the “morphospecies” (defined as a group of individuals that are recognized as probably belonging to a same species based on morphological characteristics) of immature stages and adults was achieved using the “barcode” COI gene to compare potentially, newly recognized species with sequence data already present in global genetic databases. However, only a limited number of the organisms found, essentially lepidopteran larvae, could be identified to the species level. Therefore, arthropod DNA barcoding does not replace the classical approach of morphology-based species identification (Hebert and Gregory 2005; Pires and Marinoni 2010). The combination of both techniques has proven successful in numerous cases (Pires and Marinoni 2010; Okiwelu and Noutcha 2014; Kirichenko et al. 2015) and should be applied also in sentinel nurseries and plantations (Roques et al. 2015).

### **Sentinel arboretum**

A sentinel arboretum (Fig. 3) comprises a broad range of both native and non-native tree species from diverse regions around the world, which can allow testing of various ecological hypotheses on biological invasions, as possible host-shifts, one of the main barriers to establishment of alien plant pests and pathogens, can be examined (Kirichenko et al. 2013; Kirichenko and Kenis 2016; Morales-Rodríguez et al. 2018). Non-native species are exposed to inoculum of native, potentially pathogenic organisms harboured by native trees species growing in the same or nearby environment. An expanded assumption here is that all native and non-native tree species planted in





**Figure 3.** Schematic representation of the sentinel arboretum (botanical garden) concept. The exotic and native tree species cultivated in the same area/environment are cross-exposed to inoculum harbored by each of the species. The identification of causal agents of different symptomatology provides a list of new pests or pathogens potentially harmful to those plants in their native environments.

the same area are cross-exposed to inoculum harboured by each of the tree species in a latent native-to-native interaction.

Protocols used in sentinel arboreta should aim to characterize damage morphotypes, followed by isolation or collection, and species level identification of the organisms causing these symptoms. The non-native trees might harbour endophytic microflora since the time of their introduction into arboreta as propagation material (e.g. seeds, seedlings, cuttings). HTS can be useful in detecting non-symptomatic native host endophytic species or latent infections, contributing to characterization of the donor host microbiome and to the description of a novel host-shift event. Recently, using HTS and traditional isolation methods, several novel host-interactions between *Quercus* species and fungal pathogens were described in the Ataturk arboretum in Turkey by Morales-Rodríguez et al. (2018). Differing from sentinel plantations, sentinel arboreta may also allow surveys of the recruitment of insects by mature trees, and especially of particular groups, such as xylophagous pests (Roques et al. 2015).

For the three cases of sentinel plantings presented above, confirmation of pathogenicity on the host plant is an essential step for determining the causal agent of disease (Koch's postulates). Thus, collection and isolation of the organism from symptomatic plants is crucial for establishing the causative relationship between a microbe and the disease or symptoms it produces. This procedure, however, is limited to mainly non-biotrophic organisms which can be cultured onto nutrient media. Once the causal agent is known, additional inoculation trials can be designed and car-

ried out to evaluate its potential host range. Colonizing insects observed on sentinel plants must not be incidental, but clearly capable of completing the entire life cycle on the given host, especially when non-native plants are used in sentinel plantings. This process is difficult to ascertain because rearing possibilities on non-native plants could be limited when such plants are only growing within a sentinel plot. One way to distinguish between incidental species and potential pest could be to consider the number of successive colonization events attained over a number of years by an insect species on the same non-native tree. Roques et al. (2015) considered two groups of insects, a first one (38 species) which had shown five colonization events per year, at least on European trees in China, and a second one (7 species) that has been more frequently observed (more than 15 colonization events per year) and probably more capable of switching to European trees. Hence, repeatability and reliability in the observations are critical to drawing sound conclusions on the potential risks to plant health that are needed for PRA and CRA.

### **Sampling methods used in sentinel plantings**

A first step towards the identification of causal agents of damage is usually the observation and recording of symptoms and signs of infections in the field. In the framework of the COST Action FP1401 Global Warning (a global network of nurseries as early warning system against alien tree pests; [www.ibles.pl/en/web/cost/globalwarning](http://www.ibles.pl/en/web/cost/globalwarning)), an open-access field guide for the identification of damage on woody sentinel plants was published, providing schemes for rough assignment of damage symptoms to relatively broad groups of organisms (Roques et al. 2017).

### **General considerations for sampling**

The successful detection of potentially harmful pests and pathogens in sentinel plantings relies on several conceptual, methodological and organizational factors. Among these, experimental design (i.e. how sentinel plantings are organized, e.g. how many replicates of each tree species), and sampling design (i.e. how, when and what should be sampled) are critical to making sampling as efficient and reliable as possible (Eschen et al. in prep). Similar-looking symptoms might have different causes, and for this reason, the diagnostic procedure can be challenging. Although sentinel plants might be colonized and/or damaged by a broad range of organisms, some general principles about sample collection and preservation apply to all organisms (Kirichenko and Csóka 2017; Prospero et al. 2017). Among these principles, one should consider the following:

1. As different organisms can affect a single plant, the whole plant should be carefully checked for different damage morphotypes (hereinafter referred to as damage characteristic of a certain pest or pathogen) (Tables A1, A2) and the presence of

damaging organisms (Moreira et al. 2017). Samples should be taken from a range of representative symptomatic organs (Nelson and Bushe 2006).

2. Before collecting symptomatic plant material, high-resolution photographs of the whole plant, of the damaged organ(s), and, if present and visible, possible damaging agent(s) should be taken. Categorization of damage morphotypes (Tables A1, A2) might give some hints about the potential causal agents.
3. Cross-contamination from sampling instruments (e.g. secateurs, pruning saw, forceps) should be avoided; this is of particular importance when sampling for pathogens.
4. The best period for sampling varies according to the affected tissues and the suspected causal agents. If possible, at least three samplings per year (spring, summer and fall) should be conducted.
5. Samples should also be taken from apparently healthy tissue to know what healthy plant tissue looks like during normal growth, to potentially detect differences in microbial community composition between healthy and symptomatic tissues, and to study latent infection or endophytes.
6. Proper labelling of sampled material is an essential step without which biological specimens lose their scientific value (Krogmann and Holstein 2010). The minimal data recorded should include locality, GPS coordinates, host plant, date of collection, collector name, and unique identifying number.
7. The stringency of sample disinfection before processing represents an additional variable, especially for biological detection of culturable microorganisms. However, the adoption or not of surface sterilization of samples also represents a conceptual decision. Specifically, in the case of sentinel nurseries, superficial contamination of plants might represent an additional pathway of introduction of alien microorganisms that deserves further attention (Vettraiño et al. 2017).

Apart from these general principles, which apply to all groups of damaging agents, there are approaches for sample collection that are specific to the affected plant tissues and causal agent groups (Table A3).

### **Sampling for detection of pathogens**

Pathogens can affect all plant tissues and cause a broad range of symptoms, which could affect the whole plant (e.g. general dieback) or be more localized (e.g. wilting of individual branches). Based on the tissue affected and the type of damage induced (i.e. damage morphotype, Table A1), it may be possible to recognize which group(s) of causal agent(s) is(are) involved. The strategy for sampling symptomatic material varies according to which tissue is damaged (Table A1). It is important to collect not only the symptomatic parts, to optimize the chances of isolating and identify the causal agent(s). To optimize the chances of isolating the causal agent of the symptoms and not a secondary pathogen, samples should include the region where healthy tissue borders infected tissue (Prospero et al. 2017). Evidence of insect attack (holes in the

bark, galleries under the bark, sawdust, resin flows) may also be helpful for detecting the presence of pathogens, as insects can act as vectors of other damaging organisms (Weintraub 2007; Zhao et al. 2007; Akbulut and Stamps 2012; Drenkhan et al. 2017).

### **Sampling for detection invertebrates**

Similar to pathogens, sampling of invertebrates varies depending on the affected plant tissue (Table A2) (Kirichenko and Csóka 2017). Invertebrate pests are generally sampled while feeding on plant tissue (to exclude collecting occasional agents that might be on the plant by chance) and preserved for identification. When sampled as immature stages, some arthropods, particularly insects, can be reared to adults in the laboratory as it is the preferred stage for species diagnostics (Gillott 2005). Additionally, plant material with typical arthropod damage can be collected and stored in herbarium collections and used for defining feeding guilds that have added value for identification (Roques et al. 2017). To collect pests, various tools might be used, including nets, umbrellas, collecting trays, aspirators, beating sheets, hand lenses, forceps, and sticky and pheromone traps (Gibb et al. 2006).

### **Diagnostic approaches to species identification**

Information on pests and pathogens are needed for pest- and commodity risk analysis including the organism's identification to the species level and its associated hosts. A variety of traditional, inexpensive techniques and advanced molecular methods are available for identification purposes. The key problem, upon detection of a living pest or pathogen is its correct and rapid identification. Molecular tools can satisfy both of these criteria and have, to some extent, the advantage of being automated. These characteristics make molecular diagnostics as complementary methods to classical morphology-based identification (Rao et al. 2006).

### **Pathogen identification**

#### **Classical techniques**

Conventional detection of pathogens involves macroscopic and microscopic examination of symptomatic plant material and isolation of the causal agent. Often, specific isolation protocols, based on optimal requirements for types of pathogens are available, potentially increasing isolation success. However, when working with sentinel plants, there is a risk that causal agents are unknown to science. For this reason, sampled material should be analyzed using a variety of isolation methods, different culture media and temperatures.

Once isolated in pure culture, macroscopic traits, including colony shape, texture and color, and microscopic characteristics of vegetative and reproductive structures are useful criteria for characterization and identification of isolates (Beales 2012).

One problem with the identification of pathogens is the impossibility to grow some organisms on artificial/synthetic media. Obligate parasites such as rust fungi, powdery mildews, viruses and mollicutes require a living host to grow and reproduce. For these organisms vegetative and/or reproductive structure characteristics must be observed on specimens directly from the living host using optical microscopy, or electron microscopy for viruses and mollicutes. Apart from the EPPO protocols, many useful taxonomic manuals, such as Ellis and Ellis (1997), Brenner et al. (2005), Braun and Cook (2012) or Ristaino (2012) can be consulted for morphological identification of fungal, oomycete and bacterial organisms.

### Serological tests

Commercially designed kits, such as enzyme-linked immunosorbent assays (ELISA) and lateral flow devices (LFDs) (Lane et al. 2007) are available for detecting and identifying common and known plant pathogens such as the bacterial pathogens *Ralstonia solanacearum* (Smith) Yabuuchi and *R. pseudosolanacearum* Safni (EPPO 2018), and viral pathogens like tomato yellow leaf curl begomovirus and tomato mottlebegomovirus (EPPO 2005). With sentinel systems, species-specific serological tests are however unlikely to prove useful, since many of the target microorganisms could be unknown. Thus, only genus-specific LFDs are useful for rapid in situ screening of samples and the selection of appropriate isolation methods for further laboratory testing. For example, for suspected *Phytophthora* infections, commercial LFDs can give a positive signal enabling the isolation protocol to be oriented towards the use of *Phytophthora* selective media in the laboratory (Lane et al. 2007).

### Molecular barcoding

Molecular-based techniques using polymerase chain reaction (PCR) and Loop-mediated isothermal amplification (LAMP) assays are generally more specific and much faster than conventional techniques and can be applied to non-culturable microorganisms. Plant protection organisations routinely rely on diagnostic methods based on PCR assays, e.g. EPPO Standards ([https://www.eppo.int/RESOURCES/eppo\\_standards](https://www.eppo.int/RESOURCES/eppo_standards)). The most commonly used markers for molecular identification of fungal pathogens are the ribosomal DNA transcribed spacers, particularly the internal transcribed spacer (ITS) regions ITS1 and ITS2 (Schoch et al. 2012; Romanelli et al. 2014). Although ITS regions perform generally well as barcoding markers for many fungal taxa, this region is less useful for some genera, such as *Fusarium* or *Penicillium*, as these taxa have narrow or no barcode gaps in the ITS regions (Raja et al. 2017). Thus, additional regions must be sequenced. Commonly used regions include the two largest subunits of RNA polymerase II (RPB1, RPB2),  $\beta$ -tubulin regions or translation elongation factor 1  $\alpha$  (TEF1 $\alpha$ ),

which can resolve identification of individual species within the various groups (Schoch et al. 2012). These gene regions are routinely used, depending on the organism (Romanelli et al. 2014). The 16S ribosomal RNA gene and chaperonin-60 (cpn60) are used as bacterial barcode marker genes and to study bacterial phylogeny (Chakraborty et al. 2014). Detection and identification of phytoplasma and spiroplasma are primarily based on 16S rRNA (16Sr) amplification followed by restriction fragment length polymorphism analysis (Bertaccini et al. 2019). When genetic information is available, PCR and reverse transcription PCR are used to detect plant viruses (Jeong et al. 2014).

Rapidly evolving high-throughput sequencing (HTS) technologies enable simultaneous identification of thousands of organism species from numerous and complex samples, with protocols available for viruses, bacteria, fungi, oomycetes and animal pests (Abdelfattah et al. 2018; Tedersoo et al. 2018). The available HTS platforms and details for analysis steps are outlined in Tedersoo et al. (2018). Selecting molecular markers of enough resolution, primers of high affinity to templates, negative and positive control samples and reliable reference sequence databases are the most important factors for HTS-based pest and pathogen identification (Tedersoo et al. 2018). Correct reference data are critical in the precise identification of plant pathogens and, at present, not all publically available databases are sufficiently accurate to enable accurate identification (Jayasiri et al. 2015). Thus, it is crucially important to improve and correct pest and pathogen sequences in publicly databases (Nilsson et al. 2014)

Third-generation sequencing technologies such as PacBio ([www.pacificbiosciences.com](http://www.pacificbiosciences.com)) and Oxford Nanopore ([www.nanoporetech.com](http://www.nanoporetech.com)) present the possibility to sequence long reads. These technologies have not yet been used in sentinel systems. The benefits arising from amplifying other regions (with sequences longer than ITS1 or ITS2), that could give better identification at the species level, are countered by the absence of adequate reference databases to blast the result obtained. Moreover, these sequencing technologies currently have higher error rates compared with Illumina (Weirather et al. 2017). Despite this problem, it is necessary to emphasize that the new HTS system, such as the MinION device from Oxford Nanopore has great promise as a useful tool in field applications since its portability allows for in situ (on-site) analysis and real-time data generation, thus making the workflow fully versatile.

The use of HTS platforms for biosecurity purposes such as identifying latent or potentially opportunistic pathogens in asymptomatic host tissues requires some consideration of the technological limitations, including the quality of data output (e.g. Illumina MiSeq). While bioinformatics processing can provide useful data output for biodiversity studies (e.g. metacommunity analysis), blast searching of filtered sequence data against custom or public databases generally results in a limited number of identified species, but with many OTUs assigned to higher taxonomic levels. This problem arises due to following reasons: 1) the low power of single-marker short sequences in differentiating taxa, 2) the low taxonomic coverage of databases, and 3) sequencing errors accumulated in the output reads (the sum of amplification and HTS errors). The result is a limited number of OTUs assigned at the species level which may give some value to biodiversity studies but not for biosecurity purposes.



## **Invertebrate identification**

### **Classical techniques**

The observation and evaluation of damage on plants is the first step towards a diagnosis of damaging arthropod and nematode pests. Damage morphotypes can be effectively utilized in sentinel planting surveys as an identifier to assign phytophagous pests to certain feeding guilds, prior to species identification using morphology-based taxonomy (Roques et al. 2017). Classical taxonomy based on morphological characteristics is undoubtedly a powerful tool for arthropod and nematode identification, but some limitations exist, mainly due to the immense diversity and existing gaps in taxonomic knowledge. In most cases, keys are useful only for certain geographic regions and are often based on the identification in the adult stage (Gillot 2005). Furthermore, morphology-based taxonomy may not be helpful for discrimination of closely related species (e.g. sibling or cryptic species) (Bickford et al. 2007). Moreover, disagreements between taxonomists on defining morphological characters, redefining and synonymizing the species may complicate species identification procedures (Okiewelu and Noutcha 2014). Developments in visualizing tools (electron, fluorescent and scanning microscopy) have led to immense improvements in classical taxonomy and continue to contribute to the precision of morphological observations of arthropods and their documentation, which greatly increased the accuracy of species identification (Klaus and Schawaroch 2006; Lee et al. 2009). Some biometric parameters of arthropod body characters could provide added value for distinguishing species (Su et al. 2015). The nematode species can be identified based on the morphological features of the sexual organs of adult male nematodes (Seesao et al. 2016). Knowledge of species biology (life cycle, phenology) and ecology (range, habitat, ecological niche, host plant association) may provide important additional data when identifying taxa (Panizzi and Parra 2012).

The rapid development of computer vision technologies has led to applications in highly promising automatized arthropod identification platforms based on multivariate biometric features of the taxon. This novel approach, based fully on classical taxonomy and computer algorithms, allows species identification procedures to be performed even by non-taxonomists, with a high degree of reliability (Watson et al. 2003; Hassan et al. 2014; Yang et al. 2015; Favret and Sieracki 2016; Wang et al. 2017). Despite being highly attractive, automated species identification suffers from a number of limitations, the most significant being the limited applicability of automated platforms which have for now been created only for a few groups of insects (e.g. individual families of Lepidoptera or Diptera) (Watson et al. 2003; Yang et al. 2015; Favret and Sieracki 2016; Wang et al. 2017), whereas other large groups of important arthropod pests remain far outside the scope of these systems. The process preceding the automated species identification can be tedious, including specimen preparation for scanning and precise positioning for digitizing and recognition by the software. In addition, the computer algorithms may not always be perfect and identification ac-

curacy may not be satisfactory. Despite these and other disadvantages, this developing technology and its possible utilization in mobile devices and other digital instruments in user-friendly mode, would be in high demand for modern forestry and agriculture (Wang et al. 2017) and could also be highly applicable to the identification of potential arthropod pests in sentinel nurseries and plantations.

### **Molecular barcoding**

DNA barcoding is a well-known molecular approach to species identification (Hebert et al. 2003), applicable to any life stage of arthropods, including immature stages (egg, larva, pupa) most often be identified reliably to species level by morphological characteristics (Hebert and Gregory 2005). The method can be highly useful in sentinel plantings, where the pests are usually found in immature stages (Roques et al. 2015).

For arthropods, DNA barcoding uses a short genetic marker – a fragment of mitochondrial DNA (mtDNA) of the cytochrome oxidase I gene (COI; barcoding fragment 658 bp) (Hebert et al. 2003). However, this gene might not always be enough to delineate arthropod sibling species robustly and other molecular methods are required, including nuclear sequencing and/or amplified fragment length polymorphism genotyping (Dasmahapatra et al. 2010; Kirichenko et al. 2015).

As for pathogens, one of the limitations of DNA barcoding is the lack of appropriate reference databases, which would cover all formally described arthropods. To date, comprehensive databases have been accumulated mainly for certain insect taxa (e.g. Lepidoptera and Coleoptera on <http://www.boldsystems.org/>; Ratmasingham and Hebert 2007), whereas other groups of arthropods remain underrepresented. In the existing databases, inaccuracies may also appear which can lead to misidentification. The quality and accuracy of the sequences stored in the genetic databases might not always be satisfactory, especially considering that any user can access and add sequences (Hebert and Gregory 2005). In a recent survey of insects that colonized a sentinel plantation in China, DNA barcoding enabled to reliably identify only one quarter of sample insect species (Roques et al. 2015)

For nematodes, several genes are targeted for identification such as the mitochondrial cytochrome b locus (mtDNAcytb) (Mattiucci et al. 2003), the gene encoding the mitochondrial cytochrome oxidase 2 (COX2) (Valentini et al. 2006) and the mitochondrial cytochrome oxidase 1 (COXI) (Blouin, 2002), the ribosomal RNA of the small (ssrRNA) and large subunit (lsrRNA) (Hu et al. 2001). Other nuclear genes were also selected such as the internal transcribed spacer 1 (ITS1) of rDNA to identify Strongylidae and Anisakidae (Roeber et al. 2013). NEMBASE (<http://www.nematodes.org/nembase4>), a publicly available database, provides access to sequences and associated meta-data on parasitic nematode expressed sequence tags (Elsworth et al. 2011). WormBase is an international consortium of biologists and computer scientists dedicated to the research community and providing accurate, current, accessible information concerning the genetics, genomics, and biology of *Caenorhabditis elegans* Maupas and related nematodes (<http://www.wormbase.org>).

## Conclusions

Invasive pests and pathogens are major threats to the health of plants and forests. Key to controlling these invasions are preventative measures that will allow for early detection of potentially damaging organisms preferably before they are introduced to a new region. Sentinel plants can have a fundamental role in this early detection and help predict associated risks to plants in the importing country. The three sentinel plantings described offer different possibilities to provide information useful for PRA (sentinel plantations), for CRA (sentinel nurseries), or for studying host-shift events and novel pest/pathogen interactions (sentinel arboreta).

The protocols and diagnostic approaches to follow will therefore vary amongst these systems. For sentinel plantations, the main focus is on symptoms found on the plants and the identification of the causal agent(s) for which classical identification methods are the key. In contrast, the focus for sentinel nurseries and sentinel arboreta should be on identifying a large number of taxa associated with the host irrespective of whether they are causing damage.

HTS technologies are and will continue to play a pivotal role in the study of biological invasions. In sentinel systems, HTS can help filter information on pest or pathogen taxa so as to focus the sampling efforts and identification only on target species. DNA barcoding and metabarcoding are powerful tools that can give an early warning and confirmation of potential causal agents of damage and can permit the study of the microbial community associated with woody hosts to ascertain the origin and functional role of individuals in different environments. However, reliance on HTS data must be weighed against the accuracy of bioinformatics analysis and depth of the sequence database; and be cognizant on what constitutes a positive or negative result (Martin et al. 2016). Inevitably, the combined use of the different identification techniques – morphology-based, classical and molecular approaches – in sentinel systems may prove beneficial in increasing knowledge of potentially harmful pests and pathogens and potential host shifts if introduced to a new region outside their natural range. The information generated can be highly valuable to plant protection agencies in helping to prioritise organisms for PRA and CRA and contributing to the development of preventative phytosanitary measures, ultimately safeguarding forest and tree resources and their native biodiversity.

The following recommendations can be given to promote the use of data collected through sentinel plantings: 1) better communication between scientists and NPPOs at national and international levels, in particular when potentially damaging pests and pathogens are detected, achieved through increased networking and joint training activities; 2) support from scientists for NPPOs by providing updated pest records and a prioritization strategy of detected organisms; 3) clear communication from NPPOs to scientists about data needs and usage for PRA; and 4) recognition of sentinel plantings as a useful tool by NPPOs, for example through the development of a Standard for Phytosanitary Treatments in sentinel plantings.

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## Appendix I

**Table AI.** Diagnostic approach for the identification of plant pathogens.

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach
Foliage (leaves and needles)			
Discolouration and necrosis	Necrotic spots or patches of different shapes and colours, ring- or net-shaped lines, bands, reduced leaf size; possible presence of reproductive structures on necrotic area	Fungi, oomycetes, mollicutes, viruses, bacteria	1. Isolation from symptomatic tissue 2. Molecular barcoding from cultures 3. Serological test from symptomatic tissue 4. Morphological description of signs (OM <sup>1</sup> )
Mould	Soot-like or powdery deposit on the surface; mycelial mats, reproductive structures	Fungi	1. Isolation from symptomatic tissue 2. Molecular barcoding from cultures
Rust	Blisters and/or pustules on the surface (fruiting bodies)	Fungi (biotrophic)	1. Morphological description of signs (OM <sup>1</sup> ) 2. Molecular barcoding from symptomatic tissue/signs
Mildew	White powdery mycelium and reproductive structures (including fruiting bodies) on the surface	Fungi (biotrophic), oomycetes	1. Morphological description of signs (OM <sup>1</sup> ) 2. Molecular barcoding from symptomatic tissue/signs
Reproductive structures (flower, catkins, cones, fruits, seeds)			
Discolouration and necrosis	Discolorations, necrotic spots; reproductive structures (fruiting bodies)	Fungi, bacteria	1. Morphological description of signs (OM <sup>1</sup> ) 2. Molecular barcoding from symptomatic tissue/signs
Rust	Blisters and/or pustules on the surface (fruiting bodies)	Fungi (biotrophic)	1. Morphological description of signs (OM <sup>1</sup> ) 2. Molecular barcoding from symptomatic tissue/signs

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach
Mould	Soot-like or powdery deposit on the surface; mycelial mats, reproductive structures	Fungi	1. Isolation from the symptomatic tissue 2. Molecular barcoding from cultures
Mildew	White powdery mycelium and reproductive structures (including fruiting bodies) on the surface	Fungi (biotrophic)	1. Morphological description of signs (OM <sup>1</sup> ) 2. Molecular barcoding from symptomatic tissue/signs
Fruit rot (mummification)	Entire or partial discolourations, chalky or sponge-like appearance, necrotic spots; fungal mycelium and reproductive structures	Fungi	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures
Stems, branches and twigs			
Butt and stem rot	Bark lesions, eventually with exudates; fruiting bodies	Fungi, oomycetes, bacteria	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures
Bark necrosis (canker)	Localised necrotic lesions, swollen or sunken, eventually with exudates; reproductive structures (fruiting bodies)	Fungi, oomycetes, bacteria	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures
Witches' broom	Concentration of young shoots, which are thicker and shorter than normal ones; reproductive structures (fruiting bodies)	Fungi, bacteria, viruses, mollicutes, hemiparasitic plants	1. Direct symptom observation 2. Isolation from symptomatic tissue 3. Molecular barcoding from cultures or symptomatic tissues (e. g mollicutes)
Epicormic shoots/fasciation	Sprouts growing from dormant buds, flattened, elongated shoots and flower heads	Fungi, bacteria	1. Direct symptom observation 2. Isolation from symptomatic tissue 3. Molecular barcoding from cultures or symptomatic tissues (e. g mollicutes)
Shoot blight or dieback	Discolorations, wilting or crooking from the tip of the shoots, eventually exudates	Fungi, oomycetes, bacteria, mollicutes	1. Direct symptom observation 2. Isolation from symptomatic tissue 3. Molecular barcoding from cultures or symptomatic tissues (e. g mollicutes)
Roots			
Root rot	Wood decay and eventually staining, root exudates; fruiting bodies	Fungi, oomycetes	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures

<sup>1</sup> Optical Microscopy



**Table A2.** Diagnostic approach for the identification of invertebrate plant pests.

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach <sup>1</sup>
Foliage (leaves and needles)			
Discolouration	Spots, galleries of different shapes, size and colours, mosaic-like discoloration	Insects (leaf-mining, sucking), mites	1. Collecting damaged leaves for presence of damaging agent 2. Sampling insects from mines, or on leaf surface; herbarizing leaves with typical damage 3. Rearing larvae to adults 4. Morphological identification and/or DNA barcoding (MI & DNA <sup>2</sup> )
	Chlorosis, yellowing or browning. External symptoms reflect infestation of wood or roots	Nematodes	See the sections “Stems, branches and twigs” and “Roots”
Lack of surface/tissue parts	Skeletisation, perforation, holes, cut-outs, rough eating	Insects, snails and slugs	1. Identifying damage type 2. Sampling feeding larvae and adults directly from leaves or by beating branches. 3. MI & DNA
Other coating/covering	Foth, wax, spittle, webbing	Insects, mites	1. Sampling damaging agent by removing the coating or opening the construction (nests)
Construction	Nests		2. MI & DNA
Deformation	Rolling, curling, twisting, reduced size	Insects, mites	1. Collecting damaged leaves for damage type identification 2. Sampling arthropods by opening the rolls and deformed tissues; herbarizing leaves with typical damage
Outgrowth of plant tissue	Galls		3. MI & DNA
Reproductive structures (flower, catkins, cones, fruits, and seeds)			
Discolouration	Entire or partial (spotted) discolouration, necrotic spots	Insects, mites	1. Sampling mites or insect larvae by opening the affected organ 2. MI & DNA
Other coating/covering	Presence of resin flow, white dusting, shield or felt-like covering, etc.	Insects (sap-feeders) or mites	1. Sampling mites, sucking aphids, etc. from the affected organ 2. MI & DNA
Internal damage: tunnels, holes	Damage invisible at the beginning; later detected as tissue deformation, presence of openings and insect frass on the surface	Insects	1. Sampling larvae/adults from damaged organs/tissue 2. At early-stage, X-ray seeds for the presence of the damaging agent inside 3. Rearing larvae in damaged organs to adults 4. MI & DNA
External injuries	Gnawing, rough eating (lack of tissues parts)	Insects	1. Sampling feeding larvae (nymphs) or adults directly from damaged organs 2. MI & DNA (any development stage)
Deformation	Distorted or shrivelled organs/tissues (especially flowers, conelets)	Insects, mites	1. Sampling by opening damaged organs/tissues
Outgrowth of plant tissue or abnormal growth	Swollen organs, gall formations		2. MI & DNA (any development stage)

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach <sup>1</sup>
Apparently sound seeds	Apparently sound	Insects	X-raying to reveal presence of larvae
Stems, branches, and twigs			
Coating/covering	Presence of white dust shield or felt-like covering, etc.	Insects (sap-feeders)	1. Sampling insect from damaged surface 2. MI & DNA
Internal damage: galleries	Damage invisible at the beginning; later detected through the presence of holes on the bark, insect frass on the surface	Insects	1. Sampling by opening bark with holes or insect frass on the surface 2. Collecting fragments of bark or wood with typical galleries for damage morphotype identification 3. MI & DNA
Internal damage: embolism of xylem tissue	Disruption of water transport in the tissues (timber) accompanied by external symptoms: plant stunting, wilting and foliage discoloration	Nematodes	1. Remove bark and inspect sapwood 2. Collect nematodes 3. MI & DNA
External injuries	Scars on bark, debarking/bark stripped (girdling or pruning)	Insects	1. Sampling the damaging agent feeding on the bark or by opening swollen plant tissue
Outgrowth of plant tissue	Swollen tissues, gall formations		2. MI & DNA
Roots			
Deformations, root knot or galls, necrosis, atrophy	Thickenings in a variety of shapes, stunting, appearance of necrotic spots, dying-off roots. Accompanied by plant stunting, wilting and foliage discoloration.	Insects, nematodes	1. Sample externally feeding larvae 2. Collect affected fragments of roots, examine externally and dissect knots and galls to find insect larvae or nematodes (using magnification) 3. MI & DNA
Injuries (internal and/or external)	Debarking/bark stripped, tunnels, holes and/or frass at root collar	Insects	1. Sampling damaging agent
Coating/covering	Wax, dust		2. MI & DNA (any development stage)

<sup>1</sup>As a rule, morphological identification of damaging agent is applicable to adult stage solely, whereas for DNA-barcoding any development stage can be used; <sup>2</sup>MI & DNA: Morphological identification and/or DNA barcoding.

**Table A3.** Sampling methods used in sentinel plantings.

Sampling pathogens		
Tissue	Collection	Preservation
Foliage	<ul style="list-style-type: none"> <li>• Whole leaves/needles should be collected, not only symptomatic parts</li> <li>• If symptoms occur on foliage at different stages all developmental stages should be collected</li> <li>• If symptoms concern whole shoots (e.g. wilting), it is likely that the causal agent has infected the twig/branch and not the foliage, which should also be checked</li> </ul>	<ul style="list-style-type: none"> <li>• Leaves/needles should be collected dry and rapidly processed, avoiding long storage</li> <li>• Leaves with diagnostic damage type should be stored in herbarium collection</li> </ul>
Reproductive structures <sup>1</sup>	<ul style="list-style-type: none"> <li>• Whole reproductive structures should be collected</li> <li>• If symptoms occur on foliage at different stages all developmental stages should be collected</li> </ul>	<ul style="list-style-type: none"> <li>• Apart from cones, seeds and some fruits are better kept dry</li> </ul>

Shoots, twigs, branches, stems	<ul style="list-style-type: none"> <li>• Samples should include the region where healthy tissue borders infected tissue. If symptoms occur on a small branch or sprout, the entire symptomatic section of the branch or shoot should be collected</li> <li>• For vascular diseases and to a lesser extent butt and stem rots, symptoms are often only seen when the bark is removed, and the wood exposed</li> </ul>	<ul style="list-style-type: none"> <li>• Wood tissues should be kept in humid conditions and stored cold (5–8 °C)</li> </ul>
Roots	<ul style="list-style-type: none"> <li>• Carefully remove the soil to expose the main superficial roots. Samples should include the region where healthy tissue borders infected tissue</li> <li>• Since roots are generally infected by soil-borne organisms, soil samples should be collected from the rhizosphere of trees with symptomatic roots</li> </ul>	<ul style="list-style-type: none"> <li>• Roots tissues should be kept in humid conditions and stored cold (5–8 °C)</li> </ul>
Visible signs of pathogen damage <sup>2</sup>	<ul style="list-style-type: none"> <li>• Fruiting bodies and mycelial fans (below the bark) are reliable indicators of pathogen presence and should be sampled either alone or with the substrate on which they grow</li> </ul>	<ul style="list-style-type: none"> <li>• Samples should be stored cold (5–8 °C) and processed rapidly to avoid long storage</li> </ul>

## Sampling invertebrates

Tissue	Collection	Preservation
Foliage	<ul style="list-style-type: none"> <li>• Leaves with typical damage caused by endophagous arthropods (mines and galls), which are often host plant specific, should be preserved as herbarium specimens as they might provide essential information for taxon identification at a later stage</li> </ul>	
Reproductive structures	<ul style="list-style-type: none"> <li>• Organs with visible damage symptoms should be collected, with immature individuals present inside</li> <li>• The fruits, cones or seeds can be collected from the ground under a tree or by beating branches over sheets or netting</li> <li>• Seeds can be extracted from fruits or cones and a subset of seeds with no visible signs of damage must be X-rayed to assess the possible presence of larvae inside. Collected seed can also be kept in the laboratory until adult emergence</li> </ul>	<ul style="list-style-type: none"> <li>• Preserve arthropods in ethanol, either at 70% for morphological identification or 96% for molecular identification</li> </ul>
Shoots, twigs, branches and stems	<ul style="list-style-type: none"> <li>• Pests feeding on plant tissues can be sampled directly from the surface or by debarking</li> <li>• Immature insect stages hidden in plant tissues can be sampled together with a healthy plant fragment and reared in the laboratory</li> <li>• For assessing the presence of wood nematodes, wood discs, chips or sawdust should be collected from the sapwood of symptomatic trees, if possible at different stem heights for further diagnostics</li> <li>• Stem sections with dark staining in the sapwood often indicating the presence of blue stain fungi, or signs (holes, galleries) of xylophagous insects should also be sampled</li> </ul>	<ul style="list-style-type: none"> <li>• Slugs and snails can be stored in water in sealed containers</li> <li>• Mites should be preserved in a mixture of ethanol and lactic acid</li> <li>• Plant tissues can be preserved until their processing as described above.</li> </ul>
Roots	<ul style="list-style-type: none"> <li>• The base of the trunk and the roots should be first inspected for the presence of holes and sawdust (frass) and dissected to find pests</li> <li>• Fine feeder roots showing disease symptoms should also be sampled</li> <li>• Litter and soil around the damaged roots should be inspected</li> <li>• For diagnostics of root-knot nematodes fine roots and soil must first be collected</li> </ul>	

<sup>1</sup> i.e. flowers, fruits, catkins, cones and seeds; <sup>2</sup> The term 'visible' means everything observable in the field to the naked eye, or with simple, portable magnifying instruments