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High-throughput sequencing technologies for plant pest diagnosis: challenges and opportunities

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High-throughput sequencing (HTS) technologies have revolutionized plant pest research and are now raising interest for plant pest diagnostics, with plant virus diagnostics at the forefront of development. However, the application of HTS in plant pest diagnostics raises important challenges that plant health regulators will have to address. Adapted infrastructures, technical guidelines and training are pivotal for further use and adoption of the HTS technologies in the phytosanitary framework.

1. Introduction

Early diagnosis and a rapid response are crucial to reduce the risk of entry and spread of plant pests into an area. In 2016, the Commission on Phytosanitary Measures adopted a recommendation recognizing that ‘pest diagnosis is a cross-cutting issue that underpins most International Plant Protection Convention (IPPC) activities. In order to take action against a pest, it must be accurately identified. To enable safe trade, pest diagnosis must further be completed quickly and to a high level of confidence’ (FAO, 2016). National Plant Protection Organizations (NPPOs) routinely perform pest diagnosis to support export certification, import inspections, pest surveillance and

eradication programmes. Plant pests can be managed most effectively when control measures are implemented at an early stage of infestation. Plant pest diagnostics is based on the use of a range of methods underpinned by different biological principles (e.g. bioassay, biochemical, isolation/extraction methods, molecular methods, morphological and morphometric and serological methods), some of them being highly specific and others more generic. The ability to detect plant pests varies with the sensitivity and specificity of the detection tools used. The recent development of high-throughput sequencing (HTS) technologies, also called next-generation sequencing (NGS) or deep sequencing, has revolutionized the research on plant-associated organisms. These techniques are beginning to be

used for pest diagnostics, in particular for viruses. In this paper, the opportunities offered by these new technologies for the diagnosis of plant pests are discussed. The challenges they will pose and the issues that need to be addressed for appropriate use of HTS within phytosanitary frameworks are highlighted, with an emphasis on plant viruses for which the adoption of HTS technology is more advanced.

2. What is HTS?

HTS technologies enable the simultaneous sequencing, and thus detection, of any organism present in a sample (see Fig. 1). These technologies are broad-spectrum and generic in nature and can potentially be used to replace a wide range of conventional methods, especially in the screening stage. For known pests, including regulated ones, such unbiased approaches allow the detection of very distant isolates or strains of the same pest. Moreover, HTS technologies have the ability to reveal in any plant sample the presence of unexpected as well as unknown organisms that might be potential threats.

HTS technologies are widely applied in research, accelerating the discovery of new potential pests from symptomatic (resolving the aetiology of disease) and asymptomatic plants and substrates (soil, water, wood, etc.). In the field of plant virology, HTS is revolutionizing scientists' understanding of the ubiquitous nature of viruses and clearly demonstrates that we are just beginning to touch on a large and previously uncharacterized viral diversity (Simmonds *et al.*, 2017; Zheng *et al.*, 2017; Yutin *et al.*, 2018). However, these discoveries are often only based on the identification of a partial genomic sequence and the biology of such new species is most often very poorly characterized or not characterized at all. Thus, the increased use of HTS technologies for plant pest diagnosis will accelerate the pace of discovery of new plant pests for which challenging and quick decisions will have to be taken by NPPOs on the basis of very limited information and imprecisely evaluated potential phytosanitary risks.

3. Opportunities for the use of HTS in pest diagnosis

HTS technologies open new possibilities and opportunities in routine diagnostics for (a) understanding the status of a pest in a region through surveillance programmes, (b) certifying nuclear stock and plant propagation material, (c) (post-entry) quarantine testing to prevent the introduction of pests into a country or area, and (d) monitoring of imported commodities for new potential risks. HTS offers important benefits for each of these applications (Al Rwahnih *et al.*, 2015; Hadidi *et al.*, 2016; Rott *et al.*, 2017).

Knowledge of pest status in a territory is key to phytosanitary regulation and justification of phytosanitary measures. HTS technologies are powerful tools for both

targeted and non-targeted analysis, and their use in surveillance programmes has a lot of potential. Pre-designed automated bioinformatics workflows will allow an efficient analysis of datasets, providing information on the presence or absence of all targeted pest(s) in the selected crop(s). Moreover, national large-scale non-targeted analysis of viruses, for example virome scanning for viruses present in cultivated and wild plants of a botanical family or genus, can be performed with these technologies. Countries that know the potential pests already present in their territory will have an advantage in managing the associated phytosanitary risks.

The certification of nuclear stocks and propagation materials also requires screening of specific targeted pests. As such, HTS analysis can be applied in a similar way as described for surveillance programmes. HTS analyses have the potential to eventually replace lengthy and tedious resource-intensive biological indexing for regulated pests, shortening and streamlining indexing cycles; increasing the pace of flow of novel varieties to growers is an additional advantage. In this respect, in several field applications, HTS has been found to be superior to standard bioassays for the detection of low-titre viruses (Al Rwahnih *et al.*, 2015).

Post-entry quarantine testing, as required, for example, for imports of potato germplasm, prescribes specific testing for a number of pests and a broad screen for other pests by visual inspection and bioassays. For screening of specific pests, targeted HTS analysis can be applied. However, HTS will also allow a non-targeted analysis of the generated datasets expanding the post-entry testing to unexpected and unknown pests present in imported plant material.

Other advantages of HTS technologies are related to an increase in the amount of genomic data obtained compared with conventional molecular techniques. This can improve researchers' understanding of the genomic diversity within a species (or at a lower taxonomic level), resulting in a better taxonomic assignment for unambiguously identified pests, a better appraisal of the impact of a pest's genetic diversity and better design of targeted molecular tests.

HTS offers the possibility to store the generated sequencing libraries and datasets for future analyses. As databases expand with information on new pests and bioinformatics tools improve, historical datasets will become available for re-analysis, enabling NPPOs to perform post hoc bioinformatic surveys. This is a clear benefit compared with conventional molecular methods which require new laboratory analyses on properly stored original samples or isolated nucleic acids.

4. Challenges of the application of HTS within phytosanitary frameworks

Adopting HTS technologies for routine diagnostic purposes raises challenges that may have significant implications within a phytosanitary framework.

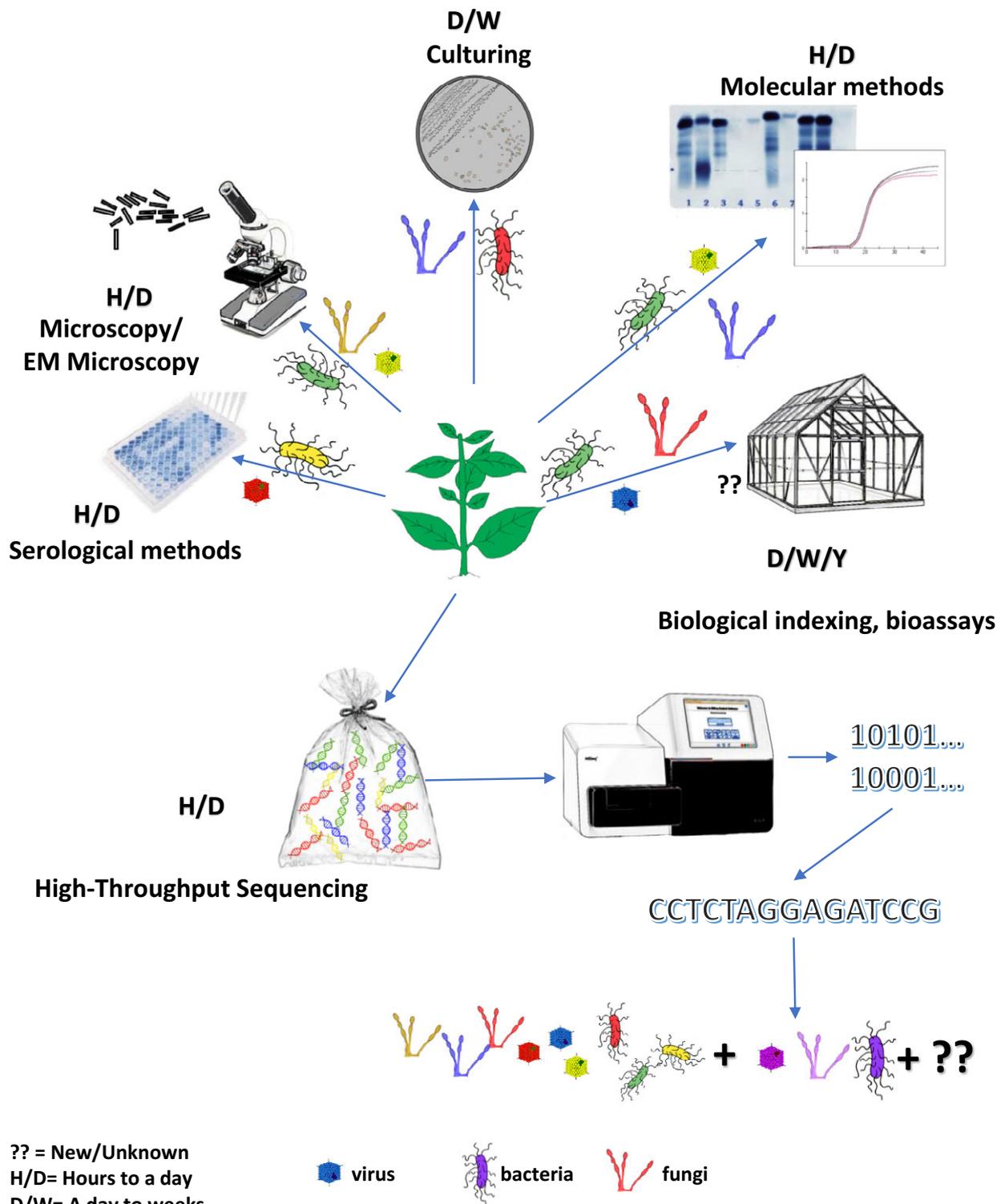


Fig. 1 High throughput sequencing technologies and traditional detection and identification methods in plant pest diagnosis. Examples shown in this figure are for viruses, fungi and bacteria but HTS can be used for other pests such as arthropods and nematodes.

4.1. Laboratory and bioinformatics challenges

As for conventional methods, interpretation of the test results derived from sequence analysis requires a high level of expertise in plant pathology and taxonomy. For laboratories, validation and routine application of HTS technologies demand new bioinformatics skills not previously required in a routine testing laboratory and investment in infrastructure to enable rapid analysis and data storage. Whilst similar principles of validation are required (analytical sensitivity, analytical specificity, repeatability and reproducibility), HTS experiments produce data on a large scale (essentially data for many tests performed all at the same time), making analysis more complex and necessitating novel skills in manipulation and storage of the large numbers of sequence data thus generated.

4.2. Interpretation of results, biological significance of new findings and pest risk analyses

The use of HTS-based diagnosis by regulators will be a major challenge due to the increased number of new pests being discovered for which a biological context may be missing. For plant viruses, Massart *et al.* (2017) proposed a framework within which relevant information can be generated for new findings. This framework will be useful for any novel organism detected by HTS, describing the characterization that should take place when starting from the sole genomic sequence. It is essential that when making regulatory decisions consideration continues to be given to the potential risks associated with the discovery of a new organism.

The complexity of disease aetiology in field conditions may pose additional challenges, for example in the case of mixed viral infections. In fact, plants are commonly infected with more than one virus species, especially in woody, vegetatively propagated plants such as grapevine (Jooste *et al.*, 2015). A possible synergism between co-infecting viral species might lead to unpredictable variations in symptoms, infectivity, accumulation and/or vector transmissibility. In fact, previously undescribed viruses not associated with deleterious symptoms found in some hosts may provide benefits to the host and may have been positively selected for during cultivation (Roossinck, 2015).

The characterization of the biological properties of any organism identified through HTS technologies will need to be performed within a short timeframe in order to provide the basis for assessing the risk it poses. An additional challenge is the allocation of appropriate funding to perform these biological characterizations and risk assessments (Massart *et al.*, 2017). Moreover, it means that pest risk analysis (PRA) processes – from initiation, through to the pest risk assessment and finally the pest risk management – will need to follow the same pace as discovery of new potential pests via HTS technologies.

4.3. Data sharing

Whilst it is standard practice among academics to share HTS datasets generated for research purposes, the sharing of such ‘raw’ datasets is less likely in a regulatory context. There could be benefits in sharing these datasets to facilitate movement of plants (e.g. by sharing information on the health status of propagative material). However, it is recognized that release of such data may pose a risk of the misuse of new findings to hamper the international movement of plants and plant products. Therefore, HTS data management and accessibility is another important decision to be taken by plant health regulators, for which a cost–benefit evaluation will be required. There may be additional complexities for regulators with datasets generated for research purposes that concern endemic pests or even healthy hosts explored by HTS technologies. These datasets may be publicly accessible and inadvertently reveal the presence of regulated pests in previously unknown locations or hosts.

5. What are the main issues to address for plant health regulators in the HTS era?

Implementing HTS technologies for plant pest diagnosis will raise some questions that need to be addressed:

5.1. Technical guidelines for routine analysis by laboratories

HTS technologies, as is the case for other techniques, face similar challenges with regard to proper sampling procedures, nucleic acid template preparation, appropriate controls, contamination and validation issues. HTS also requires confirmation of the results by additional techniques. Contamination is more problematic than with other methods, especially when high-titre infections are encountered and despite additional measures being taken (e.g. rolling programmes of tag use, increased instrument decontamination). Well-defined controls/reference materials and confirmatory testing will solve this problem and help to unambiguously identify infested samples. Thus, technical and bioinformatics guidelines for the use of HTS in plant pest diagnostics are needed to help address these issues. Guidelines will also be needed for reporting the results and for documenting and safeguarding the HTS data and the results of the bioinformatic analyses.

5.2. Infrastructure to analyse, host and share HTS data

Implementation of HTS in plant health will require significant investments in information technology and bioinformatics. Large files up to a few gigabytes per sample are generated and need to be stored and properly backed-up on servers for long periods of time. Transferring files from servers to data analysis computers requires a fast network,

with machines that need high computational power to handle large data files.

5.3. Education/training to adapt to the new paradigm

Expertise in taxonomy of pests, classical diagnostics and epidemiology will continue to be essential. Additionally, analysts will require a more in-depth knowledge on bioinformatics for the appropriate interpretation of the data. Risk assessors, risk managers and plant health regulators should be trained on the background of HTS-based diagnosis to enable them to make fast and appropriate decisions.

5.4. A framework for biological characterization of micro-organisms to support decision-making by the regulators

A framework for the evaluation of biosecurity, commercial, regulatory and scientific impacts of plant viruses and viroids identified by HTS technologies has been proposed by a European consortium of researchers (Massart *et al.*, 2017). It provides useful guidance for strategies for generating the biological and other relevant data needed for PRA. Appropriate communication channels between regulatory authorities (such as NPPOs) and diagnostic laboratories need to be enhanced.

5.5. Reinforcing baseline surveys

The current application of HTS technologies in research and their future large-scale application in pest diagnostics will identify poorly characterized or new potential pests. Ultimately for a NPPO, the key question will be whether this novel finding from HTS is truly a new incursion rather than a pest that has been present in an area or imported commodity but previously unreported due to limitations in existing diagnostic technologies. This will put a far greater emphasis on baseline survey activity to demonstrate what pests are currently present in an area/imported commodity. For example, some countries (such as Belgium) have already launched national-based surveys using HTS to detect any virus present in cultivated and wild plants of selected botanical families or genera within their respective territories.

6. To summarize

In summary, HTS technologies are powerful tools that are rapidly evolving and open up unprecedented possibilities. By overcoming the current challenges, they have the potential to transform the field of plant diagnostics by enabling quick, efficient and broad-spectrum testing using generic procedures applied in a range of diverse disciplines, thus providing a major contribution to the progress of the management of plant pest control programmes.

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Technologies de séquençage génétique à haut débit pour le diagnostic des organismes nuisibles aux végétaux: défis et perspectives

Les technologies de séquençage génétique à haut débit ont révolutionné la recherche des organismes nuisibles aux végétaux et suscitent maintenant de l'intérêt dans le contexte de leur diagnostic, avec au premier plan celui des virus des végétaux. Cependant, l'application de ces technologies au diagnostic des organismes nuisibles aux végétaux fait émerger de nombreux challenges auxquels les personnes développant la réglementation en santé des végétaux devront faire face. Des infrastructures adaptées, des lignes directrices techniques ainsi que la formation sont des éléments clés pour l'utilisation future et l'adoption de ces technologies dans le contexte phytosanitaire.

Применение технологий высокопроизводительного секвенирования для диагностики вредных для растений организмов: существующие проблемы и открывающиеся возможности

Технологии пиросеквенирования (HTS) подняли на качественно новый уровень исследования вредных для растений организмов, и в настоящее время они повышают интерес к вопросам диагностики вредных организмов. При этом диагностика вирусов растений является одной из приоритетных задач. Однако применение HTS для диагностики вредных для растений организмов сопряжено с целым рядом важных проблем, на которые должно быть обращено внимание нормативных органов фитосанитарии. Вот почему для дальнейшего использования и принятия на вооружение технологий пиросеквенирования (HTS) в фитосанитарных структурах необходима их адаптация, согласованные руководящие принципы и тренинг.

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