Deliverable Summary D3.4: Guidelines for new detection methods (LAMP, RTqPCR, VOCs, Single Cells) of forest PnPs

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PU Public	**PU**
CI Classified, as referred to Commission Decision 2001/844/EC	
CO Confidential, only for members of the consortium (including the Commission Services)	

HOMED

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°771271

1. Summary

Objectives:

Alien pathogens are exponentially increasing, challenging the sustainability of agriculture and forestry crops and natural ecosystems. The major pathway of non-native plant pathogen introduction is the international trade of plants, mainly ornamental (Santini et al. 2013).

We posit that one of the most rational approaches to limit the introduction of new pathogens within EU borders was to set up a harmonized pool of novel rapid, sensitive, specific and user-friendly tools for effective phytosanitary inspection and interception. For this aim, we optimised four Real-time LAMP protocols, three qPCR assays and one VOC detection system.

Teams involved:

CNR, MENDELU, UNIPD.

2. Introduction

Alien pathogens are exponentially increasing, challenging the sustainability of agriculture and forestry crops and natural ecosystems. The major pathway of non-native plant pathogen introduction is the international trade of plants, mainly ornamental (Santini et al. 2013).

The overall aim of a plant health policy is to safeguard and improve the health and quality of commercially produced plants and plant products. A key element of such policy is to prevent the introduction and spread of harmful, non-native organisms and to take action through regulation of such organisms if they do become established. In Europe, based on specific pest risk analyses (PRA), about 250 plant pests and pathogens not present, or with a limited extent in the EU, are regulated (Anonymous 2016). Regulations are applied to these lists of pests or pathogens, while all the other consignments not included in the list can be introduced without any limitation. In this context, the control measures depend on the proper identification of diseases and the causal agents. Without proper identification of the disease and the disease-causing agent, disease control measures can be a waste of time and money leading to further plant losses. Proper disease diagnosis is therefore vital.

Objective

The objective was to set up a harmonized pool of novel rapid, sensitive, specific and user-friendly tools for effective phytosanitary inspection and interception.

3. Results

Table 1. Detection methods developed in the frame of HOMED (X) and already reported in literature (+)

Pathogen	**Detection method**				**Literature supported by HOMED**
	Real-Time PCR (qPCR)	**LAMP**	**VOC**	**Single Cell sorting**	
Ceratocystis platani	+	X	X	X*	Aglietti et al., 2019; Brilli et al., 2020; Luchi, pers. comm.

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Xylella fastidiosa	X	X			Aglietti et al., 2019
Phytophthora ramorum / P. lateralis	+	X			Aglietti et al., 2019
Fusarium circinatum	+	X			Stehlikova et al., 2020
Caliciopsis moriondi	X				Migliorini et al., 2020
Cryptostroma corticale	X				Dvorak, pers. com.
Dothistroma sp.	+	X			Aglietti et al., 2021
Lecanostica acicola	+	X			Aglietti et al., 2021

3.1. Results on qPCR and LAMP techniques

Real-Time quantitative *PCR (qPCR)*

Real-time quantitative PCR (qPCR) assays, allows for rapid and specific detection of fungal pathogens from environmental samples. The advent of this molecular technique has enabled faster and more sensitive diagnostic tools for the identification and quantification of disease-causing agents. The accuracy and reliability of qPCR may also enable the detection of latent fungal infections before symptoms occur and the detection of fungal pathogens that are difficult to culture. This technique is very precise and sensible, allowing the detection and quantitation of very small amounts of pathogen's DNA and is hence a powerful tool for early surveillance and detection of pathogens in healthy plant tissue, before symptom expression in the host, as well accurate epidemiological studies of the spread of pathogens and their presence on different vectors and hosts.

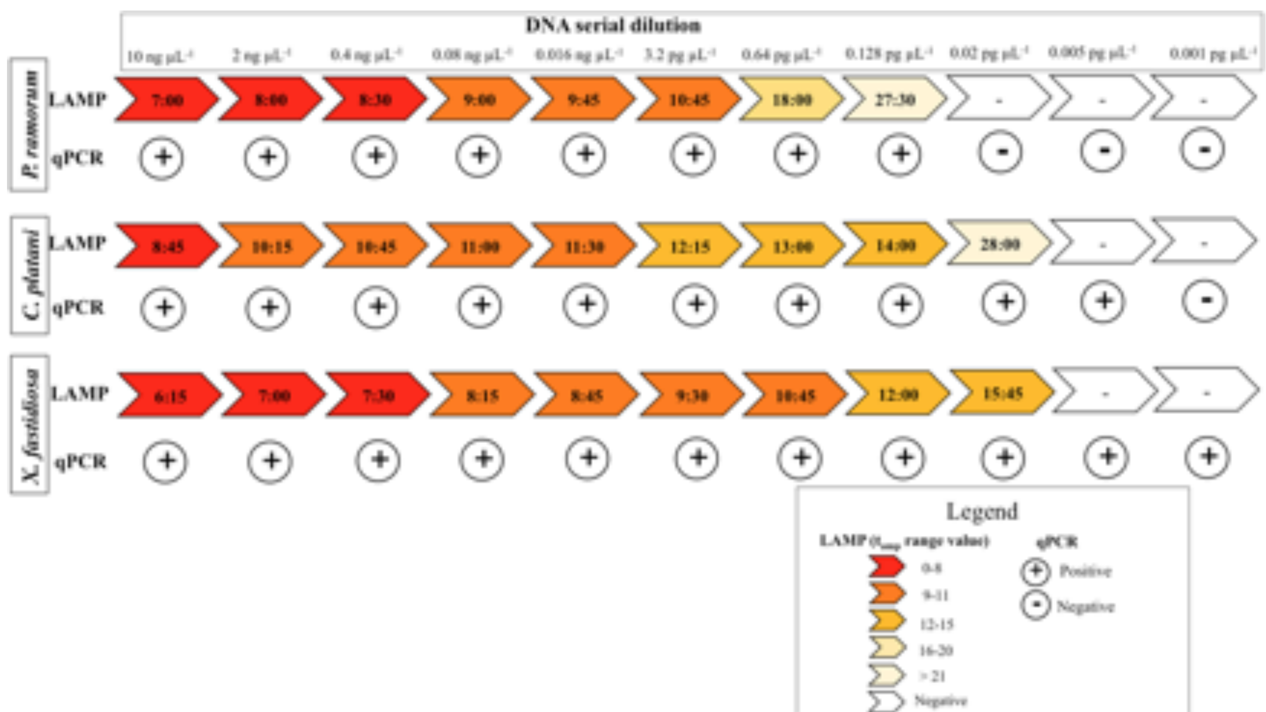

Figure 1. Comparison of sensitivity of some assays set up in the frame of HOMED.

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Real-time quantitative PCR assay for Cryptostroma corticale

This assay has been developed for the purpose of a very sensitive and specific detection of the causal agent of the Sooty bark disease of maples and the Maple bark disease of human. Due to its increasing importance, it has been chosen by the consortium of HOMED project as one of the target species. Its occurrence was molecularly detected in microscopic slides obtained from national pollen information services.

C. corticale has been introduced to different parts of Europe during the twentieth century, but especially in the last decade it is becoming an emerging pathogen. Especially in central Europe it is currently causing significant damages to native species of maples (*Acer pseudoplatanus, A. platanoides*).

Figure 2. Symptoms due to *C. corticale* infection

C. corticale specific assay has been developed. Primers and hydrolysis probes were designed, tested on reference strains and their specificity was proved on other endophytic and parasitic fungi commonly isolated from maple branches. This very sensitive assay was successfully validated on samples obtained from the pollen information service of four countries, giving positive results in three of them.

Successful validation of this assay for the abovementioned purpose means, that it can be probably used for detection protocols with much lower risk of insufficiency of pathogen's DNA. Apart of qPCR identification of pure cultures it can serve for a detection from plant tissues after appropriate modification of the extraction protocol as well as for all kinds of aerobiological studies.

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LAMP diagnostic assays

In order to provide sensitive, specific, but also simple and fast tools to be used for pathogens interception at points of entry four LAMP (Loop mediated isothermal amplification) assays were set up in the frame of the project.

The opportunity to have an accurate and rapid detection of the quarantine pathogens directly in the field by a portable instrument, represents a great advantage to preventing introductions and for applying control measures. Most of the LAMP based assays recently developed for plant pathogens, including the one developed for *P. ramorum* by Tomlinson et al. (2007) and for *X. fastidiosa* by Harper et al. (2010), are based on laborious and time-consuming isothermal amplification reactions. On the contrary, the main parameters used to assess the positivity of a sample in a LAMP real-time assay, as those developed in the frame of Homed (Aglietti et al., 2019; Stehlikovà et al., 2020; Aglietti et al., 2021), are amplification time and annealing temperature resulting by fluorescence analysis results provided by the instrument.

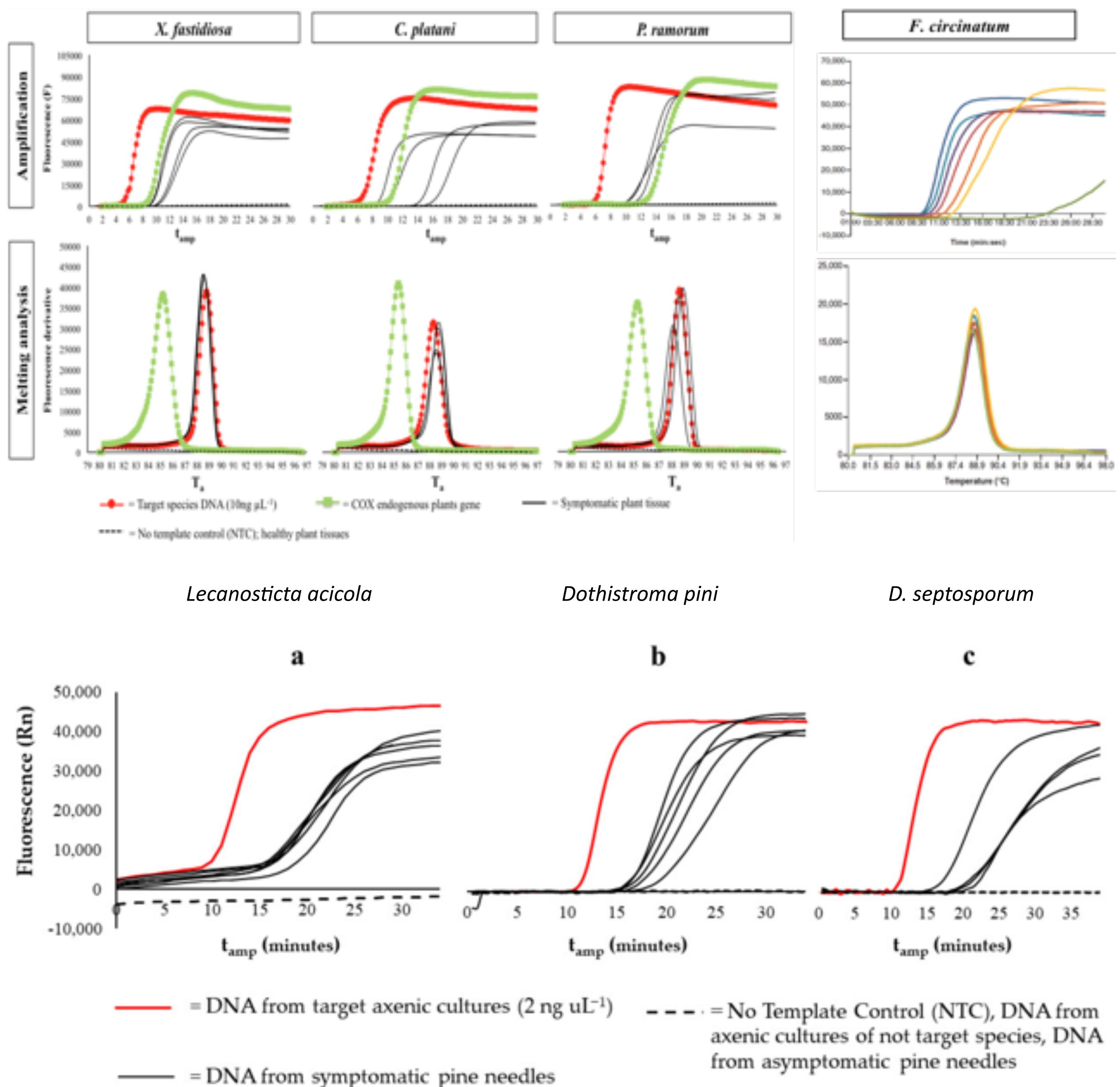

Figure 3. Examples of kinetics of LAMP assays developed in the frame of HOMED

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The use of rapid, specific and sensitive point-of-care methods like the LAMP assays developed in this study could enable phytosanitary services to make immediate management decisions, helping in containing environmental and economic losses. The application of such a portable diagnostic tool, requiring minimum equipment and a few, if any, specific scientific skills could be profitably used to check the health status of live plants or plant parts at the points of entry or in field, thus reducing time of analyses and allowing a prompt management of disease.

3.2. Result on VOCs detection techniques

Since phytosanitary services have to deal with a large and increasing number of plants to be inspected at ports of entry, spending one hour to run a single analysis still remains a time constraint. Moreover, these molecular tools can sample just a few plants among an entire consignment. Therefore, finding a technique that allows to screen large plant consignment in a short period of time may effectively help to speed up the inspection work, thus avoiding the introduction of new plant pathogens.

A peculiarity of *Ceratocystis* spp. is the emission of volatile organic compound(s) (VOC), which resemble the fruity aroma of bananas. In the frame of HOMED it was investigated whether is possible to differentiate *C. platani* among other fungal species commonly associated with hosts plants growing in the same ecosystems as plane trees through the emission of specific VOC; then, it was verified whether these "targeted" VOC could be used to detect plane trees infected by *C. platani*.

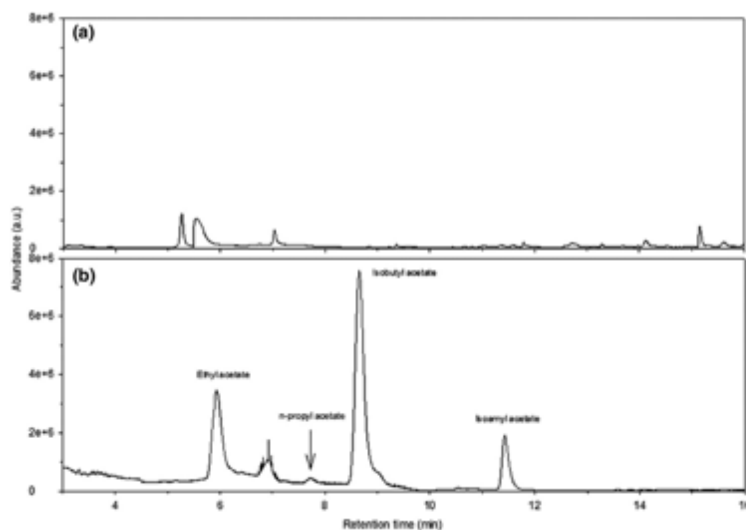

Figure 4. Chromatograms showing the VOC emitted in the headspace of the vials containing: (a) only PDA medium; (b) pure cultures of *Ceratocystis platani* grown on PDA medium.

Specific VOC emitted in vitro by pure culture of C. platani (i.e. isobutyl acetate, isobutyl alcohol and isoamyl acetate) are also released in vivo from the bark of infected asymptomatic plane trees already 4 dpi (days post inoculation), and continued to be detectable even after 32 days (Table 2).

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Days post-inoculation (dpi)	Presence of symptoms on inoculated plants	ethyl acetate (%)	n-propyl acetate (%)	isobutyl acetate (%)	isobutyl alcohol (%)	isoamyl acetate (%)	isoamyl alcohol (%)
1	No visible symptoms	-	-	-	-	-	-
4	No visible symptoms	-	-	92.5 ± 1.4	6.5 ± 1.3	1.0 ± 0.1	-
8	No visible symptoms	-	-	92.2 ± 1.4	7.7 ± 1.4	-	-
10	Wilting of apical leaves	-	-	89.8 ± 2.9	10.2 ± 2.9	-	-
17	Wilting and chlorosis of leaves; necrosis on bark	-	-	100.0 ± 0.0	-	-	-
32	Extensive chlorosis of the leaves and longitudinal necrosis on stem; plant death	100.0 ± 0.0	-	-	-	-	-

Table 2. Percentages of single VOC out of the total sum of the six targeted VOC measured over time in the plane trees stem where *C. platani* has been inoculated (values are means of three replicated measurements ± percentage uncertainty). Symptoms were detected in all the investigated seedlings at the same dpi.

Ceratocystis platani emits the same blend of VOC both when grown on media and on living tissues of plane trees. Thus, this blend of VOC could be potentially exploited as a non-invasive early diagnostic marker of *C. platani* infection, even before symptoms are visible.

In the frame of HOMED it was reported for the first time the use of a VOC blend for the detection of a quarantine plant pathogen.

VOC fingerprinting could represent an innovative method to help phytosanitary services in rapid surveying of plant consignments at ports of entry by sampling the air within a container where plants have been stored.

3.3. Result on Single-Cells analysis

Agents of tracheomycosis can start their infective process with a few spores circulating in the host vessels. In the frame of HOMED it has challenged the potential of DEPArray™ technology, a microchip-based digital sorter, which combines precise microfluidic and microelectronic enabling precise, image-based isolation of single spores, which can then be analyzed by other molecular detection methods.

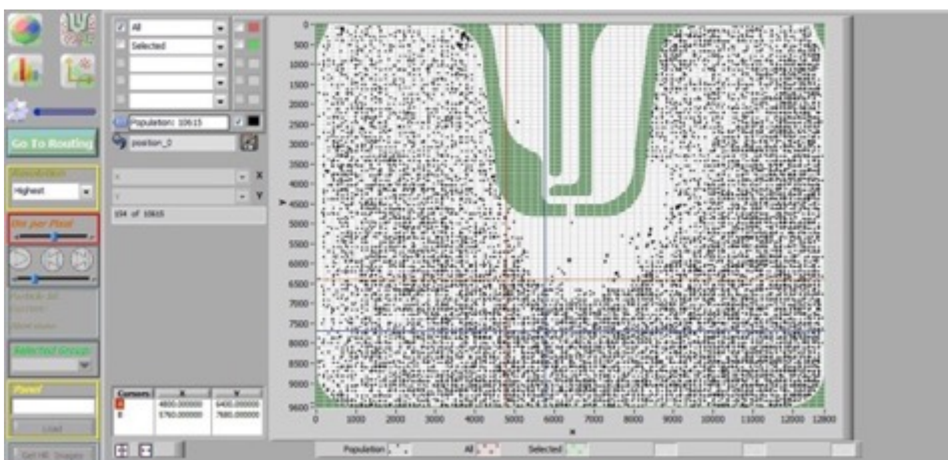

Figure 5. *C. platani* conidia in DEPArray cartridge.

In vitro DEPArray™ method resulted effective to detect single conidia of the pathogen.

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