UNIVERSITÀ DEGLI STUDI FIRENZE

PROT. N° 199965 POS. III/6.62
DATA 04-11-2019

# DOTTORATO DI RICERCA IN SCIENZE AGRARIE E AMBIENTALI

CICLO XXXII

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# Development of diagnostic techniques for studying quarantine plant pathogens

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Chiara Aglietti 20 Marzo 2020

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Chiara Aglietti 20th of March 2020

# ABSTRACT

The aim of this thesis was to optimize and improving reliable, fast, sensitive and specific field-deployable tools for the early detection of quarantine plant pathogens. In the first part of the thesis the work was concentrated in developing a field-applicable LAMP-based assay for the detection of *Xylella fastidiosa*, *Phytophthora ramorum* and *Ceratocystis platani*. Each assay, optimized on the portable instrument Genie II ® (Optigene, UK), was based on the conventional LAMP reaction and showed the capability to detect *X. fastidiosa*, *C. platani* and *P. ramorum* with high specificity and sensitivity in only 30 minutes also on plant samples for which a rapid kit method for in field-DNA extraction was also utilized. However, the assay targeting *C. platani* and *P. ramorum* were able to detect also *C. fimbriata* and *P. lateralis*, having also many cross reactions with other *Phythophtora* species. Even if the specificity was assessed by results obtained from melting analyses, that gave different temperature between target and non-target species, improving the specificity of a LAMP assay was needed. The second part of the thesis was hence concentrated in improving the chemistry and the specificity of a LAMP assay. The use of sequence-specific LAMP probes was analyzed by the development of a conventional and FRET-assimilating probe-based LAMP method targeting *Fusarium circinatum*, a pine pathogen for which specificity is a very important requirement concerning diagnostics. The capability of increasing the specificity using this novel LAMP chemistry was assessed by comparing LAMP results of conventional and probe-based LAMP reaction developed for *F. circinatum*: with conventional reaction many cross reactions were obtained with phylogenetically closest Fusaria while with the probe-based method only *F. temperatum* was amplified as cross reaction. Due to positive results obtained applying the probe-based method on wood samples DNA extracted with the field method the suitability for using it into the field was also assessed. The same probe-based LAMP chemistry was then implemented for multiplex application concerning pine needles *pathogens Dothistroma septosporum, Dothistroma pini and Lecanosticta acicola*, obtaining as preliminary results that of having a multiplex specific reaction directly in the field in about 10 minutes. Concerning this third part of this work, the possibility to apply the described method on crude samples was investigated concerning pine needles for which preliminary test to optimize a field suitable crude extraction method were carried out with promising results. As in the last part of this work was assessed that in Italy the distribution of *Dothistroma septosporum* is widespread by applying a TaqMan-based qPCR method while *L. acicola* was reported only in restricted places and *D. pini* was never reported, the developed LAMP method could be useful to prevent and monitoring their spread and introduction.

# INDEX

**INTRODUCTION** 1
1 Invasive pathogens: risks and regulation 1
*1.1 Xylella fastidiosa* 5
*1.2 Ceratocystis platani* 7
*1.3 Phytophthora ramorum* 8
*1.4 Fusarium circinatum* 9
*1.5* Needle blights (*Dothistroma septosporum, D. pini, Lecanosticta acicola*) 11
2 Diagnostic methods for plant pathogens: evolution and importance 15
3 Point-of-care methods: transferring diagnostics into the field 22
*3.1 Loop-mediated isothermal AMPlification (LAMP)* 26
*3.2 Improving LAMP reaction: probes and multiplexing* 30
**AIM** 33
**MATERIALS AND METHODS** 34
**Development and optimization of Loop-mediated isothermal AMPlification (LAMP)-based assays** 34
*Conventional LAMP reaction optimization: the case of Xylella fastidiosa, Ceratocystis platani and Phytophthora ramorum* **(Paper I)** 34
*Improving the specificity of a LAMP assay: the case of F. circinatum* **(Paper II)** 35
*Application of LAMP probes for multiplexing: the case of Dothistroma septosporum, D. pini and Lecanosticta acicola* **(Paper III)** 36
**Transferring diagnosis into the field: rapid and simple DNA extraction methods** 38
*Application of a rapid and simple DNA extraction kit to be used into the field* **(Paper I)** 38
*Optimization of a field deployable rapid DNA extraction method from crude pine needles samples* **(Paper III)** 38
**Application of molecular tools to study plant pathogens** 39
*Assessing the presence of quarantine pathogens: the case of Dothistroma septosporum, D. pini and Lecanosticta acicola in Italy* **(Paper IV)** 39
**RESULTS AND DISCUSSIONS** 40
**Early detection and pests control** 40
*Conventional LAMP reaction optimization: the case of Xylella fastidiosa, Ceratocystis platani and Phytophthora ramorum* **(Paper I)** 40
*Improving the specificity of a LAMP assay: increasing specificity* **(Paper II)** *and multiplexing* **(Paper III)** 43
**Transferring diagnosis into the field: rapid and simple DNA**

**extraction methods** 46
**Using molecular tools to study plant pathogens** 48
*Assessing the distribution of quarantine pathogens: the case of Dothistroma septosporum, D. pini and Lecanosticta acicola in Italy* **(Paper IV)** 48
**FUTURE PERSPECTIVES** 50
**REFERENCES** 53
**RINGRAZIAMENTI** 68

## APPENDIX

## Papers I-IV

The present thesis is based on the following papers and manuscripts, which will be referred to by their Roman numbers:

**I.** Aglietti C., Luchi N., Pepori A. L., Bartolini P., Pecori F., Raio A., Capretti P., Santini, A. (2019). Real-time loop-mediated isothermal amplification: an early-warning tool for quarantine plant pathogen detection. *AMB Express*, *9*(1), 50.

**II.** Stehlíková D., Luchi N., Aglietti C., Pepori A. L., Diez Casero J. J., Santini A.: Real-time loop mediated isothermal amplification assay for a rapid detection of *Fusarium circinatum,* Manuscript submitted to BioTechniques, The international Journal of Life Science Methods

**III.** Aglietti C., Villari C., Ghelardini L., Marchi G., Barnes I., Capretti P.: Development and optimization of sequence-specific LAMP assays to target *Dothistroma pini*, *D. septosporum* and *Lecanosticta acicola* needle blights, Manuscript

**IV.** Ghelardini L., Aglietti C., Capretti P., Cerboneschi M., Gionni A., Goti E., Loria F., Maresi G., Moricca S., Marchi G.: Molecular detection of *Dothistroma* Needle Blight in protected pine forests in Italy. Manuscript submitted to Management of Biological Invasions

# INTRODUCTION

## 1) Invasive pathogens: risks and regulation

Plant diseases are increasingly recognized as a worldwide threat to forestry, agriculture and biodiversity conservation (Fisher *et al.,* 2012; Ghelardini *et al.,* 2017). In the last few decades, in Italy as in other countries, plant diseases were mainly related to the introduction of invasive alien species that have caused serious phytosanitary emergencies. This is not a new problem and among all kind of plant pathogens, fungi have long been known to cause epidemics and constitute a widespread threat to plant species. Historically, plants, and pathogens evolved in unique regional assemblages, largely isolated from other assemblages by geographical barriers (Santini *et al.,* 2018). When barriers are broken, non-indigenous pathogenic organisms are introduced into new environments, potentially finding suitable hosts lacking resistance genes and conditions favoring pathogenic behavior; this process may result in epidemics of newly emerging diseases (Santini *et al.,* 2018). Biological invasions are tightly linked to human activities and have been a constant feature throughout human history (Santini *et al.,* 2018). As an example, in the nineteenth century, late blight led to starvation, economic ruin and the downfall of the English government during the Irish potato famine as a consequence of *Phytophthora infestans* introduction from South America. Or in the twentieth century, Dutch elm disease caused by the fungus *Ophiostoma novo-ulmi* subsp. *americana* that was introduced with elm logs from North America to Europe (Hubbes, 1999) likewise, the chestnut blight caused by the importation of *Cryphonectria parasitica*-infected Asian chestnut trees to the east coast of the United States, which led to the destruction of the North American chestnuts (Fisher *et al.,* 2012). The threat of plant disease has not abated, but it is heightened also due to microbial adaptation to new ecosystems, brought about by trade and transportation, and by climate fluctuations (Fisher *et al.,* 2012). As explained by Garbelotto *et al.,* (2008), the main factors that can influence a disease development are: 1) pathogens have to arrive in the new area where 2) ecological conditions must be favorable for their growth and

3) hosts in the new environment must to be susceptible. At the origin of an invasion there is, therefore, movement of pathogens, which recently has increased due to commercial trade in association with food and products transportation (Liebhold *et al.,* 2012; Santini *et al.,* 2013; Smith *et al.,* 2007). The most common pathway for the unintentional introduction of plant pests across borders was demonstrated to be the international trade of woody plants, especially live plants for planting (Migliorini *et al.,* 2015). Non-native organisms have a higher risk of establishment when they are carried with their hosts. Indeed, pathogens can survive, and possibly grow or multiply, on the host's tissues or in the soil during transit (Migliorini *et al.,* 2015). As an example, one-gram soil samples from airplane passenger footwear contained eight genera of plant pathogenic fungi regulated by the New Zealand Ministry of Agriculture and Forestry, demonstrating just how easily living pathogens can be accidentally transported across borders globally (McNeill *et al.,* 2011). However, human activities are not only associated with the dispersal of pathogenic fungi, they also interact with key fungal characteristics, such as habitat flexibility, environmental persistence and multiple reproductive modes, to cause the emergence of disease. Many fungi are adaptable in their ability to undergo genetic recombination, hybridization or horizontal gene transfer, causing the clonal emergence of pathogenic lineages but also allowing the formation of novel genetic diversity leading to the genesis of new pathogens (Fraser *et al.,* 2005). Reproductive barriers in fungi are known to evolve more rapidly between sympatric lineages that are in the nascent stages of divergence than between geographically separated allopatric lineages, in a process known as reinforcement. As a consequence, anthropogenic mixing of previously allopatric fungal lineages that still retain the potential for genetic exchange can drive rapid macroevolutionary change. Although the formation of many hybrids is prevented by genome incompatibilities, large phenotypic leaps can be achieved when the hybridization occur, leading to host jumps and increased virulence (Fisher *et al.,* 2012). This is to further be considered in a new environment where the invasive organism do not have natural

enemies that can counteract its action. Indeed, contrary to the classic "invasion paradox'' in which native organisms are presumed to be better adapted to their environment than invasive ones, invasive pathogens are often predicted to have higher pathogenicity and infectivity than native pathogens which have undergone long-term coevolution with the same host, leading to the inability of native hosts to react to non-native pathogen (Garbelotto *et al.,* 2010). Emergent diseases may also be facilitated by ecological factors such as high transmission rates in favorable environments or the ecology of disease vectors. In some cases, the epidemic emergence of native or introduced pathogens was caused by the establishment of novel associations with introduced or native arthropod vectors (Wingfield *et al.,* 2010). However, also climate fluctuations can be a potent cofactor in forcing changing patterns of plant phenology and they are known to govern emerging fungal diseases of plants. Models of climate change for the coming decades predict increases in global temperature, atmospheric CO2, Ozone and changes in humidity, rainfall and severe weather (Fisher *et al.,* 2012). This might have an influence on 1) the physiological and spatial changes that plants may undergo in response to the various components of climate change and 2) pathogen's physiology and dispersal external to their hosts plants. These events could negatively affect the economy of both sectors related to trees, i.e. classical forestry and urban forestry and of course plant nurseries.

In the last 50 years, the world's cultivated area has grown by 12% and, as a result of the significant increase in the yield of major crops, agricultural production has grown between 2.5 and 3 times (Donoso *et al.,* 2018). Agriculture contributes 3.9% of the global gross domestic product and provides employment to nearly 1.3 billion people worldwide (Donoso *et al.,* 2018). However, even though every year new technologies, research, and products help agriculture to maintain integrated management and farming practices, pathogens (mainly viruses, fungi and bacteria) have reduced crop productivity since its dawn, causing losses of at least 10% of global food production (Donoso *et al.,* 2018). Quantifying the damage by invasive pathogens is complex and

only a few studies have calculated the cost of multiple alien diseases either at regional or global scale. It was estimated that invasive diseases and pathogens caused a worldwide loss of US$ 426 billion in 1998 (Ghelardini *et al.,* 2017). In the US, the losses and control costs due to plant diseases introduced from abroad annually reach about US$ 21 billion (Brownlie *et al.,* 2006). With regard to forest trees, the few available quantitative estimates indicate large economic impact by alien pathogens reporting that approximately US$ 2.1 billion in forest products are lost each year due to alien forest pathogens in the US (Pimentel *et al.,* 2005). In Canada, past introductions of harmful invasive plant pests on agricultural crops and forestry cost US$5.7 billion per year (Environment Canada, 2004). Apart from production loss, the full economic costs of invasions include negative side effects on trade of forest products and plants, control expenses due to inspections, monitoring, prevention and response, and ecological and environmental impacts on ecosystems (Ghelardini *et al.,* 2017). In the last decades, the European Union together with other international organizations (IUCN, International Union for Conservation of Nature; WCN, World Conservation Union; EPPO, European Plant Protection Organization) have promoted initiatives aimed at identifying and monitoring damaging species recently introduced or at risk of introduction, among which can be found the Commission implementing regulation (EU 2018/2019, 18$^{th}$ December 2018) that established a provisional list of high risk plants and EPPO lists (A1: species at risk of introduction, A2: introduced damaging species with a limited distribution) that are recognized by more than 50 countries. With the compilation of lists speaking about "Quarantine and health measures", plant material importation was regulated together with commercial way (2002/89/CE). While in several countries such as Australia, New Zealand, Canada and USA there is a strict biosecurity regulation on plant pests (e.g the importation of soil, on its own or as a growing medium around plant roots is forbidden), Europe permits the importation of plants rooted in soil from outside the EU when they are officially declared free from harmful organisms (phytosanitary certificate or plant passport within the EU) and show no

sign of infestation or disease (Annex IV in EU 2000). However, the number of EU-regulated pests is small compared with the much longer lists of quarantine organisms that EPPO recommends to member governments (EPPO 2013). Inspections are usually concentrated on well-known pests and pests that are supposed to affect economically important plants. Where instances are considered low risk, inspections are reduced. Moreover, the available time for the inspection of individual consignments often limits the ability to find pests (Liebhold *et al.,* 2012). Concerning live plants, inspections are usually limited to visual examination of the aerial parts of plants; destructive sampling is practiced only in exceptional cases. Ordinary inspections may fail to detect regulated and non-regulated/unknown pests and pathogens, especially if these are asymptomatic, or if incipient symptoms are limited to the roots (Migliorini *et al.,* 2015). For these reasons, even careful inspection coupled with classical diagnostics methods will not completely prevent the introduction of new pathogens, the development of more efficient tools to detect plant diseases, based on cutting-edge molecular technologies, and the establishment of a more effective interaction with decision bodies, may help to efficiently deploy the necessary responses and safeguard systems (Lau and Botella, 2016). Such tools could be applied for fastening inspection at borders and ports of entry. Among invasive plant pathogens that are regulated at international levels, *Xylella fastidiosa*, *Phytophthora ramorum*, *Fusarium circinatum* and species causing needle blights (*D. septosporum*, *D. pini*, *L. acicola*) are acquiring great importance due to many new outbreaks (Santana *et al.*, 2016;Welsh *et al.,* 2014) causing social economic damages and to the necessity of their management and control.

## 1.1) *Xylella fastidiosa*

*Xylella fastidiosa* is a quarantine gram-negative bacterium that causes considerable economic damage by occluding the xylem of over 350 different hosts (Denancè *et al.,* 2017), mainly causing economical damage to grape vine (*Vitis vinifera, V. labrusca, V. riparia*), citrus (*Citrus spp.,*

*Fortunella*), Almond (*Prunus dulcis*), Peach (*P. persica*) and Coffee (*Coffea spp*.) but it was also found on Oleander (*Nerium oleander*), Blueberries (*Vaccinium corymbosum, Vaccinium virgatum*) and Avocado (*Persea americana*), or on some tree species such as *Ulmus americana*, *Liquidambar styraciflua*, *Platanus occidentalis, Quercus spp.* and *Acer rubrum* (Loconsole *et al.,* 2014). It was assessed that the bacterium can be carried by many wild plants such as shrubs and herbaceous species (i.e. *Cynodon dactylon*, *Calendula arvensis* and *Malva sylvestris*) without highlighting symptoms that usually manifest as chlorosis and necrosis of parts or of the entire crown. The large array of hosts that this bacterium can affect is related to two main factors: 1) it is a genetically diverse species subdivided into six subspecies, each one being more or less specific to a particular host range and a native zone in the Americas (Denancè *et al.,* 2017) 2) it is naturally dispersed over short distances by a large range of sap-feeding insects (Denancè *et al.,* 2017). The disease was considered to be confined to America until 2013, when it was reported for the first time in Europe (Italy, Apulia), well beyond the limit of natural dispersal, causing Olive Quick Decline (OQD) syndrome (Saponari *et al.,* 2013) and later confirmed also on Oleander (*Nerium oleander*) and other species. Then in 2013, the pathogen was reported on Grapevine and almond trees in Iran (Amanifar *et al.,* 2014); in 2015 in France mainland, in the region of Provence-Alpes-Côte d'Azur, mainly on *Polygala myrtifolia* (Denancè *et al.,* 2017); in 2016 in Mallorca (Spain) on three sweet cherry trees, growing in a nursery (Olmo *et al.,* 2017); in Germany where oleander and rosemary plants in a nursery were found to be contaminated (Denancè *et al.,* 2017) and Portougal (Pereira, 2015). Recently, *X. fastidiosa* subsp. *multiplex* was also detected on many Mediterranean maquis species located in the central part of Italy (Argentario, Tuscany) (Marchi *et al.,* 2018). It is to be considered that importation of coffee plants from the suspected area of origin of the agent of Pierce's disease has been linked to the first known outbreak of Pierce's disease in the USA. Similarly, plum leaf scald is supposed to have been introduced in the 1930s in Brazil by contaminated plant material, assessing that long-

distance dispersal depends predominantly on the human-mediated movement of infected planting and propagating material (Denancè *et al.,* 2017). Furthermore, it is documented that *X. fastidiosa* have high power of recombination and that several cases of intersubspecific recombination events are associated with host shifts (Denancè *et al.,* 2017). Thus, the risk associated with the mixing of isolated strains eventually present before spread and/or introduction should be avoided as this could result in novel genetic combinations with new host ranges. One of the methods to avoid spread and new introductions is improving early detection. There is a wide range of diagnostic DNA-based methods nowadays developed to detect *Xylella fastidiosa* in which are comprised methods adopted by EPPO for its official detection (Francis *et al.,* 2006; Harper *et al.,* 2010; Minsavage *et al*., 1994). Although many of these methods have been used routinely in the laboratory, most of them are not transferable for field inspection. Improving detection is hence needed.

### 1.2) *Ceratocystis platani*

*Ceratocystis platani* (J. M. Walter) Engelbr & T. C. Harr. (*Ceratocystis fimbriata* Ellis & Halsted f. sp. platani Walter), known as the causal agent of plane tree (*Platanus spp*.) canker stain (CSD), is a facultative wound parasite ascomycete that colonizes xylematic tissues, causing the death of trees within few years from infection. In the north-eastern USA, where the fungus is thought to be native, it was first (1930) reported affecting *Platanus x acerifolia* (Aiton) Willd (London plane) then (1960) *Platanus orientalis* L. (Oriental plane). Due to the clonal characteristics of the European *C. platani* population, the fungus is thought to be introduced in France and Italy probably on wood associated with military supplies during World War II. Indeed, its first report in Europe was in Tuscany, Italy (1972) but later it was also found in Armenia, France, Switzerland, Greece, Albania and Turkey where it is causing widespread serious losses in natural *P. orientalis* populations (Lehtijärvi *et al.,* 2017; Tsopelas *et al.,* 2017). As *C. platani* is now established in the western range of *P. orientalis*, the pathogen is likely to continue spreading

eastwards, following the natural distribution of this plane species and causing epidemics and extensive degradation in natural stands (Lehtijärvi *et al.,* 2017). It is also to be considered that *C. platani* is naturally transmitted via root anastomosis, infected water and ambrosia beetles that are considered as the most important means of dispersal, especially in urban areas, together with contaminated sawdust and equipment used for sanitation felling (Luchi *et al.,* 2013). As a quarantine species due to its heavy impact on plane trees and rapidity of spread, specific and sensitive diagnostic tools are necessary to facilitate effective measures for *C. platani* control and eradication, containing the environmental and economic damage that the pathogen may cause both in forests and urban environments. Nowadays, several methods for *C. platani* diagnosis were developed, ranging from classical analyses of symptoms, isolation and culturing to qPCR-based methods (Luchi *et al.,* 2013; Pilotti *et al.,* 2012) that result to date as the most sensitive and specific.

## 1.3) *Phytophthora ramorum*

The genus *Phytophthora* contains a range of many and different species that have been frequently found to cause economically important diseases mainly in greenhouse systems, being capable to damage a huge number of plants species. Recent epidemicological studies have demonstrated that nursery stands across Europe are almost ubiquitously infested by a large array of *Phytophthora* species, identifying nurseries as the major pathway of *Phytophthora* diseases into forests and semi-natural ecosystems within and between continents (Jung *et al.,* 2016). During the last decades, considerable attention has been focused on *Phytophthora ramorum* an invasive pathogen, reported as the causal agent of sudden oak death (SOD), a lethal disease affecting, since the 1990s tanoak (*Lithocarpus densiflorus*) and several oak (*Quercus spp*.) species along the pacific coast of the United States (Rizzo *et al.,* 2002; Tomlinson *et al.,* 2007). Simultaneously, the pathogen has been found in European nurseries causing serious blight of ornamental plants (*Rhododendron, Camelia* and *Viburnum*) (Tomlinson *et al.,* 2007; Werres *et al.,* 2001). Later, the

pathogen has been found for the first time in natural ecosystem on Japanese larch (*Larix kaempferi*) in Ireland and Britain and has been recognized as a serious threat to forestry, causing considerable damages to Japanese larch woods (Brasier and Webber, 2010; King *et al.,* 2015). To prevent the spread of this pathogen across borders and to contain outbreaks at their source, efficient and rapid detection methods are needed. The detection and identification of *P. ramorum*, as well as other *Phytophthora* species, is difficult and require expertise. Even if many DNA-based methods were nowadays developed for this pathogen (Bilodeau *et al.,* 2007; Chandelier *et al.,* 2006; Rollins *et al.*, 2016), improving detection for this species is highly needed.

### 1.4) *Fusarium circinatum*

The genus *Fusarium* is one of the largest fungal genera including many members that can damage many plants of great economic importance, ranging from horticultural to forestry species. Among plant diseases caused by *Fusaria*, are included destructive blights of cereal crops in major producing countries of the world (Ploetz *et al.,* 2006), wilts and root rots of some of the most important field and vegetable crops, such as tomato, pea, melon (Oumouloud *et al.,* 2013), potato (McClure, 1951) cotton and flax, and disorders of many ornamental plants (Armengol *et al.*, 2005) such as aster, carnation, bulbous and woody plants (Viljoen *et al.,* 1994). Among these, *Fusarium circinatum* Nirenberg & O'Donnell (syn. *Gibberella circinata*) is a highly virulent pathogen of pine trees listed as a quarantine organism in Europe (A2 EPPO) and subjected to provisional emergency measures (Vainio *et al.,* 2019). It causes cankers and resinous bleeding on trunk and branches of adult Pines trees, but it can also affect pine seedlings in nurseries (Storer *et al.,* 1998) causing damping off and collar necrosis. It was first described in 1946 in the southeastern USA (Hepting and Roth, 1946), where it occasionally caused damage to Southern pines (*Pinus virginiana*, *Pinus echinata*, *Pinus rigida*). In the mid-1980s, the disease reached the coast of California (Gordon *et al.,* 2001), where it caused extensive dieback of *Pinus radiata* D. Don and other pine species. In Europe, the pathogen was introduced in the early 2000s and is

now established in Spain (Landeras *et al.,* 2005) and Portugal (Bragança *et al.,* 2009) in *P. radiata* plantations, one of the most widely used species in plantation forestry all over the world. In Italy, the pathogen has been reported in 2005 on ornamental *Pinus pinea* L. and *Pinus halepensis* Mill. trees (Carlucci *et al.,* 2007). In France, two different introductions have been reported in 2005 and in 2008 on different exotic pine species and on *Pseudotsuga menziesii* Mirb. In both Italy and France, the disease foci were eradicated. Pitch canker is also present in South Africa (Wingfield *et al.,* 1998), Chile (Wingfield *et al.,* 2002), Haiti, Mexico (Guerra-Santos, 1999), and Japan (Muramoto and Dwinell, 1990) mainly in association with *P. radiata* stands. Further spread of *F. circinatum* is of great concern to many other countries, where highly susceptible pines (i.e., *P. radiata*) are extensively grown in plantations. However, *Fusarium*, the most important genus among those including toxigenic fungi (Geiser *et al.,* 2004), is well known to mycologists and plant pathologists for being one of the genera in which it is most difficult to distinguish species from each other. A number of factors have conspired to create taxonomic systems that poorly reflect species diversity, resulting in the misapplication and inconsistent application of species names to toxigenic and pathogenic isolates (Geiser *et al.*, 2004). Among all, the major cause of the difficulties in classifications is the capacity of its members to vary widely in those morphologic and physiologic characters, including virulence, which are normally used in taxonomy (Snyder and Hansen, 1940) leading to species concepts that are too broad together with a lack of clear morphological characters that can be used for separating species (Geiser *et al.,* 2004). Much of the motivation for the streamlined morphology-based *Fusarium* taxonomic systems of the mid- to late-20th century came from a desire to make identification simple and reliable, and in hindsight at the cost of over-simplification (Geiser *et al.,* 2004) together with the need to distinguish one species from the other for studying and describing these fungi, their host plants, and their correlated diseases (Snyder and Hansen, 1940). With the advent of DNA-based identification, multilocus phylogenetic methods, which allow for the objective identification of

species boundaries in the Fungi, were developed, making it possible to infer relationships among well-defined *Fusarium* species that showed a great deal of species diversity that was vastly under-estimated by all previous morphological treatments (Geiser *et al.,* 2004). While two or more gene genealogies are usually required for this method of classification, in many instances species may be identified accurately using a single DNA sequence marker, thereby validating its diagnostic utility (Geiser *et al.,* 2004). Even if there is not an universally accepted DNA barcode for Fungi (Schoch *et al.,* 2012), the markers of choice for species-level phylogenetics and detection in fungi are usually intron-rich portions of proteincoding genes (Geiser *et al.,* 2004). Among all, the nuclear rRNA cistron has been used for fungal diagnostics and phylogenetics for more than 20 years, referring mainly to the internal transcribed spacer (ITS) region that resulted suitable also for *Oomycota* identification (Schoch *et al.,* 2012). However, many Fusaria within the *Gibberella* clade possess non-orthologous copies of the ITS2, which can lead to both incorrect phylogenetic inferences and identifications (Geiser *et al.,* 2004). Several molecular methods based on these gene regions have been nowadays developed for differentiating *F. circinatum* from other species (Ioos *et al.,* 2019) but few can target *F. circinatum* without having cross reaction with nearest species. For these reasons, studying and improving *F. circinatum* molecular diagnosis is highly required.

## 1.5) Needle blights (*Dothistroma septosporum, Dothistroma pini, Lecanosticta acicola*)

Needle blights are among the most serious needle fungal diseases affecting pine species, occurring in almost every country where susceptible hosts and suitable conditions are found, a range that includes climates from tropical to subarctic (Drenkhan *et al.,* 2016). Among the different causal agents, the ascomycetes fungi *Dothistroma pini*, *D. septosporum* (Fig. 2G) and *Lecanosticta acicola* (Fig. 2H) are of particular concern, causing respectively *Dothistrom*a needle

blight (DNB) and brown spot needle blight (BSNB). Both diseases, that are considered as quarantine plant diseases in many countries, causes premature defoliation (Fig. 2A, 2B), which results in growth reduction and, in extreme cases, mortality (Möykkynen *et al.,* 2016). Although 82 pine species were reported as the most susceptible host species belonging to *Pinus* genus, a growing number of new and non-pine species in the *Pinaceae* have been recorded as hosts of *Dothistroma* and *Lecanosticta* species (Adamnson *et al.,* 2018) showing that the exact number of hosts that could be affected by each of the pathogen species and theirs variations in susceptibility is still unknown (Drenkhan *et al.,* 2019; Möykkynen *et al.,* 2016). As they produce similar symptoms on their hosts (Fig. 2A, 2B, 2C, 2D) and it is very hard to discriminate one from the other (Fig. 2E, 2F) based on morphological characteristics (Drenkhan *et al.,* 2016), up until 2004 DNB was considered to be caused by one pathogen species with occasional variety designations (Drenkhan *et al.,* 2016). It was interchangeably referred to in the literature as either *Dothistroma septospora* (septosporum), *Dothistroma pini*, *Mycosphaerella pini* or *Scirrhia pini* (Drenkhan *et al.,* 2016). Similarly, *L. acicola* was initially included in *Scirrhia* genus and up until to 2012 it was identified based only on morphological characteristics (Van Der Nest *et al.,* 2019), making it very hard to distinguish *L. acicola* from nearest *Lecanosticta* species. As a consequence of the high number of taxonomic changes, it is hard for researchers to know which species was being studied or referred to before 2004 (Drenkhan *et al.,* 2016), making unclear the origin and real distribution of each species. Until the 1990s, *Dothistroma* needle blight was mainly known for its devastating effects on pine health in plantation forests in the Southern Hemisphere, especially in New Zealand where it caused an estimated loss of NZD $19.8 million per year during the 2000s (Bulman *et al.,* 2016), Australia and Africa (Bradshaw *et al.,* 2019) while *L. acicola* was confined in the USA and Mexico until 1940s when it was reported in Spain (Janousek *et al.,* 2016). However, severe disease epidemics mainly due to *Dothistroma septosporum* together with the many new outbreaks of *L. acicola* (Van Der Nest *et al.,* 2019) are now a global phenomenon,

with widespread death of native and plantation pines, particularly in Canada and Europe (Bradshaw *et al.,* 2019), causing concern for the commercial and environmental importance of *Pinus spp* hosts. Regarding Italy the only published report on DNB dates back to 1977 on *Pinus radiata* plantations in San Pietro di Caridà, Aspromonte Massif, Calabria (Magnani 1977) and according to EPPO its distribution is restricted to this host into the southern part of the country (Calabria). Similarly, *L. acicola* was reported in Italy only in a botanical garden on the western side on the Lake Garda (Lombardia) affecting *P. mugo* (La Porta and Capretti, 2000). But, as assessed by Möykkynen *et al.,* 2016 that modelled the probability of DNB spread in Europe based on climate conditions and on distances of spores dispersal, there are many other places in Italy with suitable conditions for the spread of these pathogens, including also locations suited in the North and Central part of the country (Fig. 1). Also, considering that conditions suitable for DNB establishment are quite similar to that required by BSNB, new outbreaks could be highly possible. DNA-based diagnostics is at date very limited for these three species, rely mainly on few classical PCR and qPCR protocols (Ioos *et al.,* 2010), that are based on laborious and time-consuming reactions needing a lab for being applied.

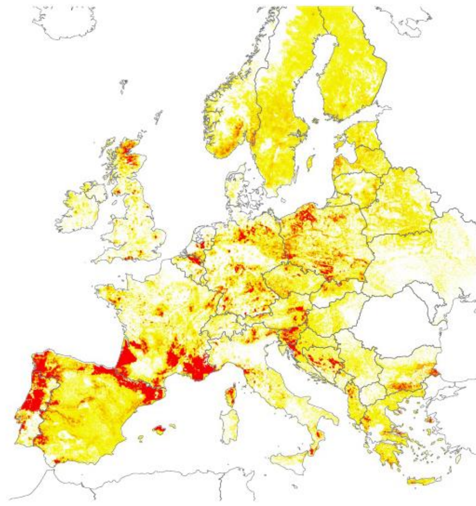


**Figure 1:** Probability of spread of *Dothistroma septosporum* by 2015. Red tone: 60%–100%; yellow tone 1%–10%. Credits: Möykkynen *et al.,* 2016

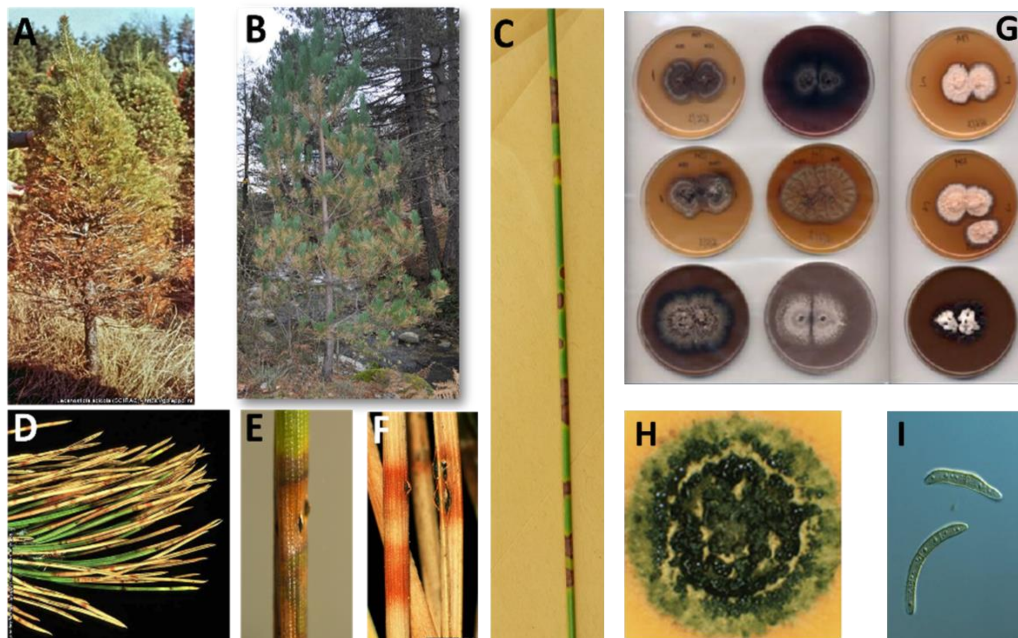



**Figure 2:** Symptoms on full trees (A,B) and needles (C,D), and fruiting bodies (E,F) of *Lecanosticta acicola* (A,C, E) and *Dothistroma spp*. (B,D,F), respectively. Cultures on MEA of *L. acicola* (H) and *Dothistroma* (G), conidia of *L. acicola* (I). Photo credits: A, D, F, H http://gd.eppo.int; B,E, C. Aglietti; C, C. Villari, G, I Mullett and Barnes 2012.

## 2) Diagnostic methods for plant pathogens: evolution and importance

Performing efficient diagnostics of any kind is part of a decision-making process (eradication and containment measures), which in the case of plant pests and diseases is usually done to prevent or limit pathogens' spread that can have severe economic and social consequences (Boonham, 2014; Tomlinson *et al.,* 2010). Concerning notifiable pests, measures are usually needed to prevent their incursion into a new environment often resulting in the destruction of infested consignments. On the contrary, for non-notifiable pests, actions are more often targeted to ameliorating their impact (Boonham, 2014). However, in each case the faster the pathogen is identified the faster decisions on its management can be taken allowing to apply more effective actions for pest control. Diagnostic methods can be applied to study many aspects of plant pathology, for instance infection dynamics or disease epidemiology, as described in detail below. For this reason, the development of such methods is important also for research purposes. There is a wide range of methods that can be used to recognize and detect plant pathogens. The earliest **conventional methods** used symptom observation, involving field inspections to identify disease symptoms as well as laboratory tests such as pathogen culture on selective media (e.g PDA, MEA) followed by physiological, biochemical and pathogenicity tests (Lau and Botella, 2017). Although conventional diagnostic is still in many cases the most reliable method, still pests can go unnoticed at low levels of infestation or in the case of pathogens at pre-symptomatic infection stages. Indeed, the first symptoms of a disease can occur after a long latent post-infection phase and they may be non-specific, making it difficult to visually observe the problem and leading to unchecked spread of the disease up to such a high level that can be easily seen (Boonham, 2014). Furthermore, culturing the sample on specific media could take days or even weeks depending on the pathogen and could be difficult when it comes to biotrophic pathogens being time consuming, requiring specialized laboratories, and expert operators. As an example, the bacterium *X. fastidiosa* is very difficult to isolate and grow in axenic culture, requiring specific

media and long times to grow (1, 1.5mm of size after 3 weeks of incubation) (EPPO PM 7/24). Also, closely related organisms may be difficult to discriminate based on morphological characters, requiring often advanced knowledge in taxonomy and specialized microbiological expertise which often takes many years to be acquired. In addition, results are usually not conclusive requiring further analysis to investigate the presence of pathogens that, due to the low sensitivity of these methods, can not be recognized when occurring in low quantities. The use of more generic techniques that can be taught quickly and easily to relatively unskilled staff is highly needed. An improvement in diagnostics occur with **antibody technology** that has been used in plant diagnostics since the 1980s becoming popular and powerful tools because of their speed, specificity and inexpensive nature (Lau and Botella, 2017). Antibodies are molecules, produced by mammalian immune systems, that are used to help identify target organisms or substances. There are two traditional methods that can be used for the production of antibodies. The first is based on the injection of the pathogen extraction into an animal. The animal can react to the pathogen by creating antibodies that can be found in its blood. Blood of infected animals is extracted and let clot. Serum is collected and antibodies can be tested with or without purification. Antibodies obtained with this method are called Polyclonal antibodies (PAbs) (Ward *et al.,* 2004). Although this kind of antibodies have been used successfully for detecting plant pathogens, especially for viruses, it is not always sufficiently specific and usually generated in limited amounts. Furthermore, their specificity can vary from batch to batch leading in some cases to cross-reactivity with unrelated pathogenic species (Lau and Botella, 2017; Werres and Steffens, 1994). The second method is made by fusing antibody-producing cells (lymphocytes) from the spleens of an inoculated animal (usually mice or rats) with cultured myeloma cells. This generates many hybrid cell lines (hybridomas), each producing a different single (monoclonal) antibody. These individual cell lines are propagated and single monoclonal antibodies are harvested from the culture medium (Dewey and Marshall, 1996). With the development of monoclonal

antibodies (Mabs), specificity was improved since they target a single epitope in a pathogen protein providing an unlimited supply of standardized reagents with homogeneous binding behavior (Lau and Botella, 2017; Ward *et al.,* 2004). However, monoclonal antibodies are generally slow to produce, expensive to both produce and maintain and occasionally cell lines may die or stop producing the required antibody (Ward *et al.,* 2004). Also, it has been reported that closely related species may share common epitopes and cause Mabs to react positively (Lau and Botella, 2017). In each case, the final aim of an immunodiagnostic assay is to detect or quantify the binding of the diagnostic antibody with the target antigen. It can be done using different methods of detecting antibody/antigen binding, but often it involves coupling the antibody to an enzyme that can be used to generate a color change when a substrate is added (Ward *et al.,* 2004). The most common method is the enzyme linked immunosorbent assay (ELISA) (Engvall *et al.,* 1972) but many antibody-based diagnostic tools such as immunoblot, immunofluorescent test, and lateral flow devices (LFD) (Danks *et al.,* 2000) have been developed and widely used to identify plant pathogens (Lau and Botella, 2017). However, even if developing antibodies for plant viruses has usually been very successful, the immune-based approach is less suitable for more complex organisms such as fungi (McCartney *et al.,* 2003). As a consequence, immunoassays may not be sufficiently sensitive or sufficiently specific to identify the pathogen to the required taxonomic level, as often a quarantine species requires (Tomlinson *et al.,* 2010).

The advent of the polymerase chain reaction (PCR) (Mullis *et al.,* 1989) (Fig. 3) in the 1980s allowed scientists to start exploring **DNA-based approaches** for the detection of unique DNA or RNA sequences carried by the selected organism that allow to differentiate it from others. PCR is a procedure by which DNA can be copied and amplified. It exploits DNA polymerases to amplify specific pieces of DNA using short, sequence-specific oligonucleotides added to the reaction to act as primers. The first and most commonly used of these enzymes is Taq DNA polymerase (from *Thermus aquaticus*), whereas Pfu DNA polymerase (from *Pyrococcus*

*furiosus*) is used widely because of its higher fidelity when copying DNA. Although these enzymes are subtly different, they both have two basic capabilities that make them useful for PCR: 1) they can generate new strands of DNA using a DNA template and primers, and 2) they are heat resistant. The latter attribute is necessary because after each round of DNA copying, the resulting double-stranded DNA (dsDNA) must be "melted" into single strands by high temperatures within the reaction tube (95°C). The reaction is then cooled to allow the oligonucleotide primers to anneal to the now single-stranded template DNA and direct the DNA polymerase enzyme to initiate elongation by adding single complementary nucleotides to create a new complete strand of DNA. Thus, dsDNA is created. This new dsDNA must then be melted apart before the next cycle of copying can occur. Therefore, if the reaction works with perfect efficiency, there will be twice as much specific dsDNA after each cycle of PCR. Amplification reactions do not maintain perfect efficiency because reactants within the PCR are consumed after many cycles, and the reaction will reach a plateau. In addition, self-annealing of the accumulating product may also contribute to the "plateau effect". Because the reaction is able to efficiently amplify DNA only up to a certain quantity before the plateau effect, there is no way to reliably calculate the amount of starting DNA by quantifying the amount of product at the completion of the PCR. That is to say, no matter how much of a specific target DNA sequence is present before PCR, there can be similar amounts of amplified DNA after PCR, and any distinct correlation between starting and finishing quantities is lost (Valasek and Repa, 2005). An improvement was given by the developed of the real-time quantitative PCR (Heid *et al.,* 1996) (Fig.3). It is a recent modification to the polymerase chain reaction that is based on the detection of a fluorescent signal produced proportionally during the amplification of a DNA target, allowing precise quantification of specific nucleic acids in a complex mixture by fluorescent detection of labeled PCR products (Bustin, 2005; Fraga *et al.,* 2014). Rather than having to look at the amount of DNA target accumulated after a fixed number of cycles, real-time PCR assay showed cycle

number when amplification signal of a PCR product is first detected. This is determined by identifying the cycle number at which the reporter dye emission intensity rises above background noise. That cycle number is referred to as the threshold cycle (Ct). The Ct is determined in the exponential phase of the PCR reaction and is inversely proportional to the copy number of the target. Therefore, the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed, and the lower Ct value is observed. Real-time PCR assays are highly reproducible and can easily discriminate between twofold differences in target numbers. At its simplest, real-time PCR can be used as a qualitative assay. However, as fluorescence output is linear to sample concentration over a very broad range, this linear correlation between PCR product and fluorescence intensity can be used to calculate the amount of template present in the exponential phase of the reaction by using a standard curve (Bustin, 2005). Detection can be accomplished using general nonspecific DNA-binding fluorophores (e.g., SYBR Green), fluorophore-labeled primers (e.g., LUX, FRET), or sequence-specific probes (e.g., Scorpions, TaqMan). Several types of probes are available, such as hydrolysis probes (5'-nuclease probes), hybridization probes, molecular beacons, sunrise and scorpion primers, and peptide nucleic acid (PNA) light-up probes (Valasek and Repa, 2005). Each type of probe has its own unique characteristics, but all rely on very similar functioning. They must link a change in fluorescence to amplification of DNA (Valasek and Repa, 2005). However, the most common are nowadays SYBR green I and hydrolysis probes. SYBR green I binds to the minor groove of dsDNA, emitting 1,000-fold greater fluorescence than when it is free in solution. Therefore, the greater the amount of dsDNA present in the reaction tube, the greater the amount of DNA binding and fluorescent signal from SYBR green I. Thus any amplification of DNA in the reaction tube is measured, making specificity the primary concern in its use (Valasek and Repa, 2005). On the contrary, hydrolysis probes (also called 5'-nuclease probes because the 5'-exonuclease activity of DNA polymerase cleaves the probe) offer an alternative approach to the problem of specificity.

These are likely the most widely used fluorogenic probe format and are exemplified by TaqMan probes. In terms of structure, hydrolysis probes are sequence-specific dually fluorophore-labeled DNA oligonucleotides. One fluorophore is termed the quencher and the other is the reporter. When the quencher and reporter are in close proximity, that is, they are both attached to the same short oligonucleotide, the quencher absorbs the signal from the reporter. This is an example of fluorescence resonance energy transfer in which energy is transferred from a “donor” (the reporter) to an “acceptor” (the quencher) fluorophore (Valasek and Repa, 2005). During amplification, the oligonucleotide is broken apart by the action of DNA polymerase (5’-nuclease activity) and the reporter and quencher separate, allowing the reporter’s energy and fluorescent signal to be liberated. Thus, destruction or hydrolysis of the oligonucleotide results in an increase of reporter signal and corresponds with the specific amplification of DNA (Valasek and Repa, 2005). Hydrolysis probes afford similar precision as SYBR green I, but they give greater insurance regarding the specificity because only sequence-specific amplification is measured. From qPCR technique implementation, there has been an improvement of protocols, instruments, and chemistries, which on the one hand is evidence for the popularity and ubiquity of the assay, but also highlights the need to be aware of problems associated with the use of non-standardized assays for diagnostic assays (Bustin, 2005). The greatest advantage these DNA-based techniques have over conventional diagnostic methods is the potential to be highly specific and highly sensitive (McCartney *et al.,* 2003). As they can distinguish between different fungal target species, and within a single species allowing to detect very low amounts of the pathogen’s DNA (eg from a single fungal spore (Williams *et al.,* 2001)), they are usually used when greater sensitivity or more control over specificity is required. Furthermore, they are potentially more reliable than the identification of visual symptoms, as they do not rely on the skills needed to distinguish subtle differences in disease symptoms (McCartney *et al.,* 2003). Even if they can not assess if a pathogen is viable in a sample, targeting genetics regions of selected pathogens, DNA-

based diagnostics can be used to determine particular genetic properties of the pathogen. As an example, they can be used to determine whether the pathogen is resistant or sensitive to particular fungicides and to determine its virulence characteristics, to study the biology of plant pathogenic fungi, pathogen population structure and dynamics, host/pathogen interactions, gene flow in pathogen populations and inoculum movement (McCartney *et al.,* 2003). For these reasons, research in subdisciplines upon which applied plant pathologists depend—etiology, epidemiology, and diagnosis—heavily utilizes nucleic acid (NA)-based detection techniques, with their use greatly outnumbering the use of immunoassays in recent research publications (Vincelli and Tisserat, 2008). Nowadays, the high specificity and sensitivity of molecular DNA-based technologies allows detection of plant pathogens in the early stages of infection, when they are present at low DNA concentrations (Zhou *et al.*, 2000). Many PCR-based assays have been optimize also coupling the PCR reaction with immune-enzymatic methods (e.g PCR-ELISA, immunocapture-PCR) to increase their specificity (Lau and Botella, 2017). However, in order for samples to be subjected to PCR-based testing, they need to be sent to a laboratory with the necessary facilities. Significant advantages for controlling and managing plant pathogens spread could be gained from moving testing closer to the site of sampling. In this way, the time between sampling and identifying the pathogen could be reduced, reducing also the delay for management strategies (Tomlinson *et al.,* 2010).

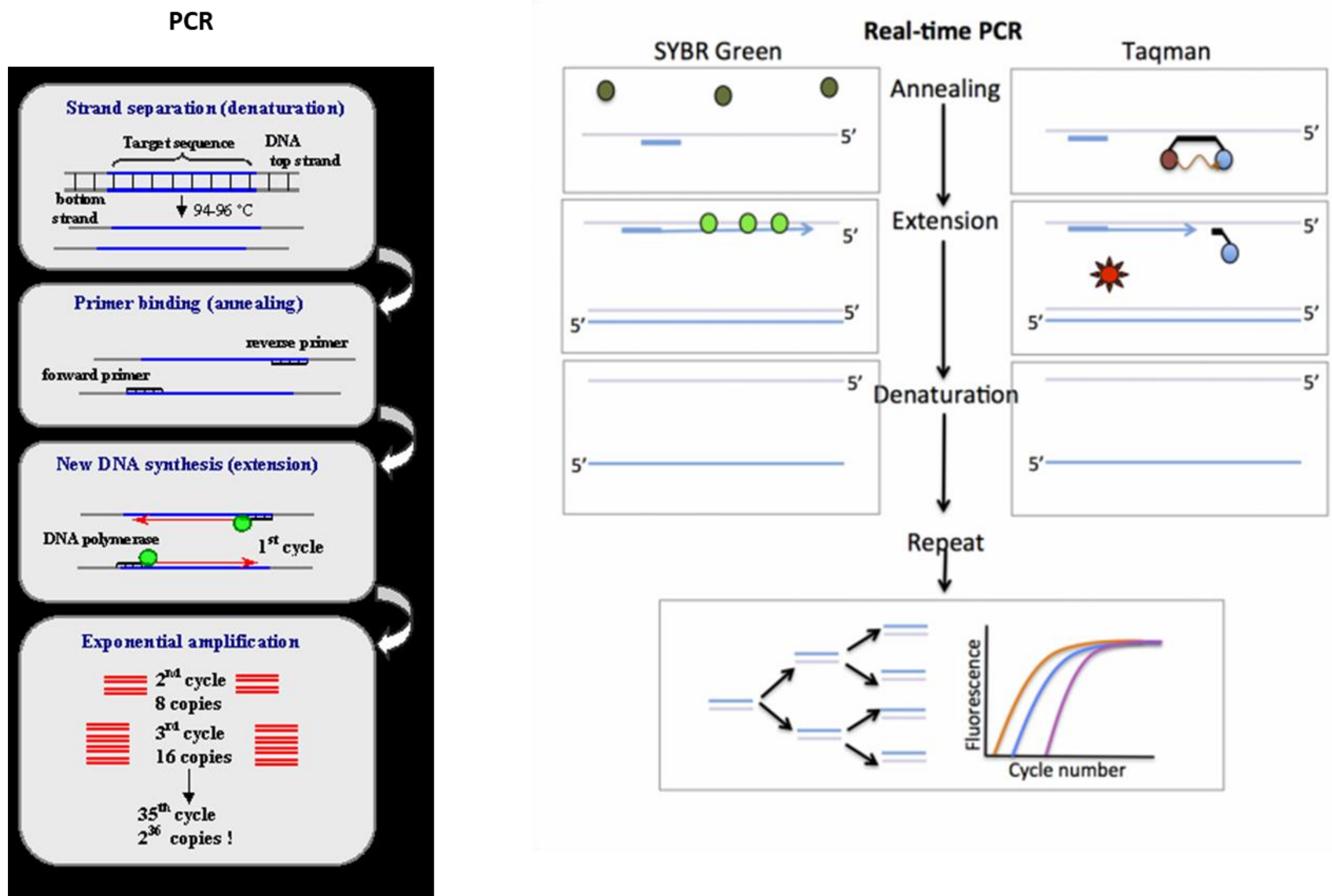



**Figure 3:** Description of the functioning of PCR and real-time PCR reactions. Credits: https://www.ncbi.nlm.nih.gov/ and https://www.ebi.ac.uk/

## 3) Point-of-care methods: transferring diagnostics into the field

Point-of-care (POC) diagnostic assays which do not require sophisticated equipment and can be rapidly and cheaply performed on site are in high demand (Lau and Botella, 2017). Transferring diagnostics directly into the field is a high challenge for controlling and limiting the spread of pathogens, allowing fast responses to threats. It is not useful just to inspection services who benefit from these techniques, they can be deployed throughout farmers, agri-production chain by seed producers, growers, processors, pack-houses etc. to limit losses caused by pathogens and pests (Boonham, 2014). However, a POC diagnostic assay technology integrating the entire process from sample preparation to visualization of results is still elusive (Lau and Botella, 2017).

To properly fit the field-deployable use, a POC diagnostic method should be suitable to be applied in extreme field condition as resource poor endemic areas represents, maintaining high levels of sensitivity and specificity together with being robust, rapid and having user-friendly equipment (simple to perform and interpret in a few steps with minimal training). Lack of effective point of care diagnostic tests is a critical barrier to effective treatment and control of diseases that is acutely demonstrated in neglected infectious diseases, where access to reliable diagnostic testing is severely limited and misdiagnosis commonly occurs (Njiru, 2012). Regarding plant pathogens, a similar situation could be represented by nurseries and greenhouses. Almost every major group of disease-causing microorganisms have been found in irrigation water used in commercial plant nurseries and greenhouses that usually can be sourced from surface water supplies such as ponds, lakes, rivers, and reservoirs (Stewart-Wade, 2011). In addition to this contamination of the initial source, plant pathogens may get into the water at various points of the irrigation network, especially if the water comes into contact with infected plant debris or soil or as often happens it is recycled (Stewart-Wade, 2011). Consequently, infected plants may harbor and liberate large numbers of infective propagules of pathogens into leachate water, which are then delivered to the holding pond and, when the water is recycled for irrigation, are subsequently redistributed to susceptible crops causing symptoms and plant death (Stewart-Wade, 2011). In this way, irrigation water acts as both a primary inoculum source and an effective inoculum dispersal mechanism in many plant-pathosystems, correlating to plant disease (Stewart-Wade, 2011). The availability of early detection methods directly usable at point of care could help also in applying management and treatment in this kind of systems. Developing tools that can be applied by those who work in the field is not a new approach, methods based on latex agglutination (Fig. 4a) have been performed for plant diseases since the early 1980s (Boonham, 2014). Since then more refined ELISA-based methods have been developed for field application, exploiting pathogen and in some cases pest specific antibodies to enable rapid identification (Okong'o-Odera *et al.,* 1993).

However, these methods required a large number of temperature labile reagents, had multiple steps in which reagents have to be added sequentially and the interpretation of the result was often subjective, requiring a fair amount of training and experience to reproduce effectively (Boonham, 2014). The most significant innovation from a field diagnostic purpose, came in the late 1990s with the application of homogeneous test kit formats developed in the bio-medical arena and exploited most notably for hormone detection in pregnancy testing applications (Boonham, 2014). The Lateral Flow Device (LFD) format was exploited initially for plant disease diagnostics for the detection of potato viruses for use in seed certification systems and proved to be a considerable improvement over previous formats (Boonham, 2014). On this kind of field-deployable LFD-based test (Fig.4b), the agglutination is accumulated at a specific location by the presence of a line of target specific antibody, which immobilises the agglutinated latex whilst allowing the background reagents to be washed away by continued flow along the membrane (Boonham, 2014). When results are positive, a colored line appears on the kit providing a non-subjective and clear read out of a positive result against a low background (Boonham, 2014). In addition, the sequential rehydration of reagents as the sample flows along the membrane effectively removes the need for multiple steps to be performed by the user (Boonham, 2014). The most significant drawbacks to LFD approaches to field detection are the availability of reagents with a specificity appropriate for the application and the inherent lack of amplification that limits sensitivity (Boonham, 2014). From then, methods based on DNA and RNA amplification have become tools-of-trade, favored for their great sensitivity and specificity (Boonham, 2014). The implementation of these methods on-site has been investigated for some time, using portable laboratory equipment (Madi *et al.*, 2012). Although a number of companies have produced real-time PCR and PCR equipment for in field detection (Fig.4c), there are significant drawbacks to its field suitability (Boonham, 2014). First, for all PCR-based methods, DNA extraction usually requires complex protocols for the purification of nucleic acids such as

to avoid the co-purification of compounds which could inhibit the enzymes involved in the correct functioning of the amplification reaction (Moré *et al*., 1994). Then, even if in some cases portable appliances and equipped with a battery were available, they were too expensive. The main problem is due to the PCR-based reaction functioning, that required temperature cycles and instruments that can support it, together with controller and sensors that can record the minimal variations of fluorescence given during the amplification (Boonham, *et al.,* 2016). Methods for pathogens detection in the field, as well as being sufficiently sensitive and specific, should also be simple and fast, with results easy to interpret and should demand minimal equipment and facilities (Tomlinson *et al.,* 2010). To solve both of these problems subsequent research has been focused on evaluation of isothermal amplification chemistries (Fig.4d). A number of alternative isothermal techniques (e.g. Nucleic Acid Sequence Based Amplification (NASBA), Recombinase polymerase amplification (RPA) (Piepenburg *et al.*, 2006), Helicase dependent amplification (HDA) (Vincent *et al.,* 2004), Strand Invasion Based Amplification (SIBA) (Hoser *et al.,* 2014) are now available to amplify DNA and RNA, obviating the need for a thermal cycler and giving many advantages from a field point of view. As isothermal reactions they avoid the use of thermal-cycling equipment, allowing reactions to be incubated in a water bath or simple heated block (Tomlinson *et al.,* 2010). Furthermore, working with enzymes more resistant and durable than those used in PCR-based methods they allow to perform analyses from an unpurified DNA, copying very large amounts of DNA efficiently as well as being robust and able to withstand the effects of inhibitors (Boonham *et al.,* 2016). Although a published systematic comparison of a large number of chemistries does not exist, methods based on the Loop mediated AMPlification (LAMP) (Notomi *et al.,* 2000) reaction seems to be the most field-suitable at date developed.

**Figure 4**: field diagnostics tools based on a) Latex agglutination b) Lateral flow devices kit c) Portable PCR d) Isothermal AMPlification reaction test. Credits: Boonham *et al.,* 2014

## 3.1) Loop-mediated isothermal AMPlification (LAMP)

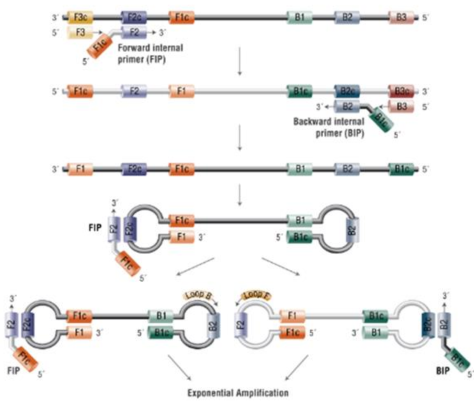
LAMP (Loop mediated isothermal AMPlification) is a recently developed reaction (Notomi *et al.,* 2000) that can amplify a few copies of DNA to $10^9$ in less than an hour under isothermal conditions. It relies on auto-cycling strand displacement DNA synthesis that is performed by a DNA polymerase with high strand displacement activity. Usually, the method makes use of the large fragment of the *Bst* DNA polymerase obtained by *Geobacillus stearothermophilus* (Niessen *et al.,* 2015) that is fused to the maltose binding protein (MBP) of *E. coli*. MBP is used for purification and removed by cleavage of the fused proteins while the large fragment of the *Bst* DNA polymerase, containing the 5′–>3′ polymerase activity but lacking the 5′–>3′ exonuclease activity, is used in the reaction to amplify and displace DNA (Niessen *et al.,* 2015). A set of two specially designed inner and two outer primers, that can hybridize six different regions of the target DNA, are strictly necessary for LAMP reaction (Notomi *et al.,* 2000). The six regions in

which primers will anneal are named as follow: the sequences inside both ends of the target region are designated F2c and B2, two inner sequences 40nt from the ends of F2c and B2 are designated F1c and B1and two sequences outside the ends of F2c and B2 are designated F3c and B3 (Fig.5). The inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), respectively, and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi *et al.,* 2000). FIP is indeed composed by F1c and the sequence (F2) complementary to F2c while BIP contains the sequence (B1c) complementary to B1 and B2. The two outer primers consist of B3 and the sequence (F3) complementary to F3c, respectively (Notomi *et al.,* 2000). The mechanism of LAMP reaction is briefly illustrated in Fig. 5. Thus, in LAMP the target sequence is amplified 3-fold every half cycle (Notomi *et al.,* 2000). The unremitting cycling reaction accumulates products with repeated sequences of target DNA of different sizes (Lau and Botella, 2017). In order to further accelerate the reaction, a third pair of primers (loop primers), hybridizing to the stem-loops except for the loops that had been hybridized by the inner primers, can be added optionally to the reaction (Nagamine *et al.,* 2002). Since *Bst* DNA polymerase has a very high activity, vast amounts of high molecular weight DNA are produced within short time (Niessen *et al.,* 2015). LAMP products can be visualized by using both indirect and direct methods. In the first category are included all the methods, included gel electrophoresis (Fig.7D), that require post-amplification steps often together with the opening of the reaction tubes. Among these, methods based on turbidity (Fig.7A), on hydroxynaphthol blue (HBN) or calcein (Fig.7B,7C) are the most commonly used. The first is based on incorporating deoxynucleotide triphosphates (dNTPs) into the DNA strand during polymerization allowing the formation of pyrophosphate in high amount that, forming stable complexes with bivalent metal ions such as magnesium, calcium, or manganese, precipitate as pellet (Mori *et al*., 2001). A positive reaction can be assessed by measuring the turbidity or observing the sample with the

naked eye. Alternatively, colored dye as hydroxynaphthol blue (HBN), can be added to the reaction. This cause a decrease in the concentration of magnesium due to its binding to pyrophosphate resulting in a changing from violet to sky blue of positive samples (Goto *et al*., 2009). Similarly, Calcein can be used in combination with free manganese ions that have a quenching effect on the chelating dye. As manganese binds strongly to newly formed pyrophosphate, calcein is released to complex free magnesium, resulting in bright green fluorescence (Fischbach *et al.,* 2015). The use of a simple color change method to assess the positive result of LAMP-tested samples is particularly suited for use in the field but opening the tube after the reaction has finished to add the colorimetric dye makes the method extremely vulnerable to carryover contamination due to the very large amount of product generated by LAMP reaction (Tomlinson *et al.,* 2007). Furthermore, some colorimetric dyes reagents can completely inhibit the LAMP reaction at the concentration needed to produce a color change visible with the naked eye (Tomlinson *et al.,* 2007). In addition, even though they might be possible to observe in a laboratory environment, they are difficult to detect in the field due to the different light conditions at different times of the day (Lau and Botella, 2017), leading to false negative results or to losses in detection sensitivity. In addition, the interpretation of positive results as color changing in colorimetric dyes is very subjective, requiring very experienced staff. An alternative to these methods, is the direct detection of isothermally amplified DNA by intercalating dyes (e.g. SYBR Green I, EvaGreen), allowing the detection of amplified DNA (Fig. 7E, 7F) for both end point and real-time analysis (Fischbach *et al.,* 2015). These methods are the most suitable for use in field diagnostics, not requiring post-amplification steps and allowing to further simplify the interpretation of results, usually given in real-time on a monitor. Every intercalating dye emit a weak fluorescence signal in the presence of single-stranded DNA (ssDNA) but higher fluorescence is emitted upon a binding to double-stranded DNA (dsDNA) (Fischbach *et al.,* 2015), meaning that every time somewhat is amplified a positive result is

obtained. Even if the LAMP reaction is highly specific binding on 6 different regions of the target DNA, it is to be considered that it works with large amplicons and hence the fluorescence from intercalating dyes not always enables to distinguish species that differs for few nucleotides. However, a new improvement to LAMP reaction seems to give a solution to this problem, such as the use of fluorescent probes.

**I. Starting material producing step**




**II. Cycling amplification step**

**III. Elongation and recycling step**

**Exponential amplification**

**Figure 5:** Loop mediated AMPlification (LAMP) reaction. Credits: www.neb.com. LAMP reaction starts with inner primer FIP that hybridizes to the target DNA and initiates complementary strand synthesis. Outer primer F3, hybridizes to F3c in the target DNA and initiates strand displacement This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis, leading to the production of a dumb-bell form DNA, which is quickly converted to a stem–loop DNA by self-primed DNA synthesis. This stem–loop DNA then serves as the starting material for LAMP cycling, the second stage of the LAMP reaction. Both of products produced in the second phase then serve as template for a BIP-primed strand displacement reaction used for starting the third phase of the LAMP reaction (Notomi *et al.,* 2000).

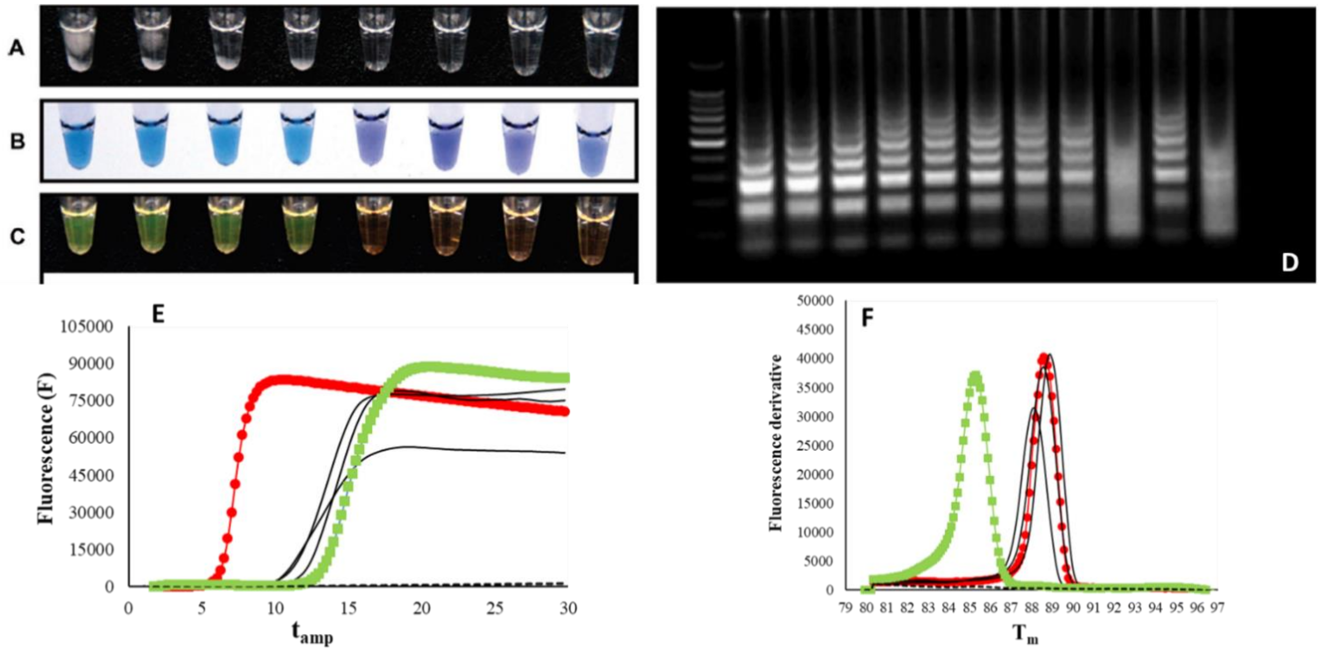



**Figure 6:** Methods for visualization of LAMP products by 1) post amplification analyses observing A) turbidity B,C) color changing by dyes addiction (hydroxynaphthol blue (HBN); calcein) D) amplification products on gel electrophoresis and 2) real-time monitoring of E) products amplification and F) melting curve analysis. Credits: A;B;C Fischbach *et al.,* 2015, D Techathuvanan *et al.,* 2010, E;F Aglietti *et al.,* 2019.

## 3.2) Improving LAMP reaction: probes and multiplexing

Most of the LAMP-based assays nowadays developed, especially in plant pathology (Harper *et al*., 2010; Tomlinson *et al.*, 2007) are based on methods for products visualization that have many drawbacks. Even if, the methods for LAMP real-time monitoring are still relatively undeveloped compared to PCR (Kubota *et al.,* 2011), they are considered as the best option for point-of-care application allowing to work with closed-tubes avoiding the risk of contamination and giving easily interpretable results. The main limitation is that the majority of them are based on nonspecific quenching, mainly based on intercalating dyes (Tanner *et al.,* 2012), consequently increasing the rates of false positive and limiting the discrimination capability of the assay together with its utility. The lack of target specificity and the unique wavelength fluorescence emitted during amplification by intercalating dyes (e.g. SYBRgreen) further means that the above described visualization techniques cannot be used for multiplex technology (Ball *et al.,* 2016),

that is a DNA-based variant detection tool in which two or more target sequences can be amplified at the same time by including more than one set of primers in the same reaction. Considerable time and effort could be saved by simultaneously amplifying multiple sequences in a single reaction, further reducing time of analyses, being more cost effective than singleplex analyses and allowing to work with minimal amounts of samples (Lau and Botella, 2017). Since it was first described in 1988 (Church and Kieffer-Higgins, 1988), multiplexing has been successfully applied in many areas of DNA testing using PCR-based reactions in which the availability of different fluorescent dyes with different wavelengths allows for the designing of a specific probe targeting each chosen pathogen. Similar technologies were recently improved also for LAMP application, allowing for sequence-specific LAMP amplification and multiplexing. The most of them are based on the inclusion in the LAMP reaction of a dye-labeled primer that is incorporated into a target-specific amplicon (Ball *et al.,* 2016). As an example, probes described in Kubota *et al.,* 2011 are composed by a fluorescent labeled strand that contains at the 3' end one of the Loop primers and that is quenched by a complementary strand having at the 5' end a dark quencher (e.g. Black hole). Once the chosen Loop primer is amplified the two strands separate resulting in a destruction of the probe that allows the emission of fluorescence by the incorporated dye (Fig.7). As assimilating probes they require first the correct functioning of each primer on the target region and then the correct amplification of the specific Loop primer by which fluorescence is dependent (Kubota *et al.,* 2011), highly increasing the specificity of the assay and making possible to distinguish differences in very few DNA bases. The possibility of marking this kind of LAMP-applicable probes with every kind of fluorescent dye used in qPCR for TaqMan probes allows to perform multiplex analyses (Kubota *et al.,* 2015). However, other multiplexing techniques for LAMP or other isothermal strand displacement techniques have been described: displacement of a bound quencher (Yi *et al.,* 2006), fluorescence resonance energy transfer (FRET) (Kubota *et al.,* 2011), a combination of labeled primers and intercalating dyes (Kouguchi

*et al.,* 2010), or strand displacement of a quencher bound to a probe targeting the loop region of the amplicon (DARQ) (Tanner *et al.,* 2012), quenching of unincorporated amplification signal reporters (QUASR) (Ball *et al.,* 2016). The majority of these sequence-specific LAMP techniques were nowadays applied for viruses and bacteria (e.g *Ralstonia solanacearum*, *Salmonella enterica*), few targeting fungal plant pathogens (e.g *Magnaporthe oryzae*) were nowadays developed.

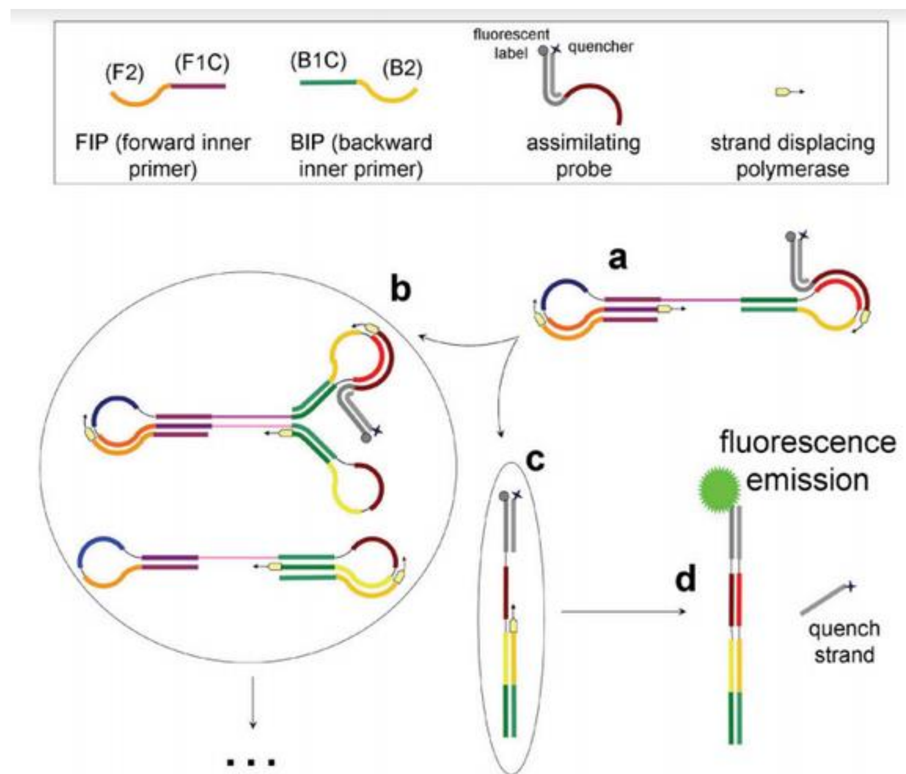



**Figure 7:** LAMP reaction functioning with probes. Credits: Kubota *et al.,* 2011

# AIM

The **main aim** of this thesis was to optimize and improving reliable, fast, sensitive and specific field-deployable tools for the early detection of quarantine plant pathogens. In the **first part of the thesis** (**Paper I**) the work was concentrated in developing a field-applicable LAMP-based assay for *Xylella fastidiosa*, *Phytophthora ramorum* and *Ceratocystis platani*. The assay, optimized on the portable instrument Genie II ® (Optigene, UK), was based on the conventional LAMP reaction and a simple and rapid kit method for in field-DNA extraction was utilized for wood and plant tissues samples. The **second part of the thesis** was concentrated in improving the chemistry and the quality of a LAMP assay. The use of sequence-specific LAMP probes was analyzed by the development of a conventional and FRET-assimilating probe-based LAMP method targeting *Fusarium circinatum* (**Paper II**), a pine pathogen for which specificity is a very important requirement concerning diagnostics. The capability of increasing specificity with this chemistry and its quantitative performance was analyzed from a field-application point of view. The same probe-based LAMP chemistry was then implemented for multiplex application concerning pine needles pathogens *Dothistroma septosporum*, *Dothistroma pini* and *Lecanosticta acicola* (**Paper III** preliminary results). Regarding pine needles pathogens, an extraction method that can be applied in the field directly on crude samples was optimized.

As it may be difficult or too long to isolate some pathogen species using traditional techniques, molecular methods might be used also for identifying and studying some features regarding plant pathogens. In this thesis, a TaqMan-based qPCR assay was applied to investigate the presence and the distribution of *Dothistroma septosporum*, *D. pini* and *Lecanosticta acicola* in Italy (**Paper IV**).

# MATERIALS AND METHODS

## Development and optimization of Loop-mediated isothermal AMPlification (LAMP)-based assays

*Conventional LAMP reaction optimization: the case of Xylella fastidiosa, Ceratocystis platani and Phytophthora ramorum (**Paper I**)*

Each real-time LAMP assay targeting *X. fastidiosa*, *C. platani* and *P. ramorum*, was optimized on the portable field-deployable real-time fluorometer Genie® II (OptiGene Limited, Horsham, UK). As required by LAMP reaction (Notomi *et al.,* 2000) six specific primers were designed using the software LAMP Designer (OptiGene Limited, Horsham, UK) on the basis of specific genetic regions previous selected by reason of sequences alignments results: the consensus sequences of the ribosomal RNA gene (ITS1-5.8 S-ITS2) was chosen for *P. ramorum* (Genebank Acc. No. KC473522) and *C. platani* (Genebank Acc. No. EU426554.1), while for *X. fastidiosa* the ribosome maturation factor (RimM) gene belonging to Co.Di. Ro strain was selected (JUJW01000001). The specificity of each assay was assessed by testing the DNA extracted from a total of 84 pure fungal and bacterial cultures in which were included: different strains of each target species, fungal and bacterial species phylogenetically related to target pathogens, as well as out-group species and common host colonizers. The limit of detection (LOD) of each LAMP assay was tested by using an 11-fold 1:5 serial dilution (ranging from 10 ng $\mu L^{-1}$ to 0.001 pg $\mu L^{-1}$) of each standard genomic DNA template (*X. fastidiosa*; *C. platani*; *P. ramorum*). To assess the capability of each assay to diagnose each pathogen in plant tissues samples, plant samples DNA were analyzed from naturally infected hosts including: (i) Two symptomatic plants of each of the following Mediterranean maquis species: *Rhamnus alaternus, Calicotome spinosa, Cistus incanus, Spartium junceum, Prunus dulcis*, affected by *X. fastidiosa subsp. multiplex* (recently detected by Tuscany Regional Phytosanitary Service—EPPO 2019); (ii) 10 *Platanus* × *acerifolia* symptomatic trees infected by *C. platani* (Florence, Italy), (iii) 10 DNA samples extracted from

symptomatic *Olea europaea* leaves with *X. fastidiosa subsp. pauca* infections, kindly provided by M. Saponari (IPSPCNR,Bari); (iv) 10 DNA samples from *Viburnum tinus* leaves having symptoms similar to *P. ramorum*. As negative control, DNA from fresh tissue collected from 10 healthy plant of each tested species (*Olea europaea, Rhamnus alaternus, Calicotome spinosa, Cistus incanus, S. junceum, Prunus dulcis, Platanus × acerifolia and Viburnum tinus*) was included. Each DNA sample was tested on LAMP both using developed primers for *X. fastidiosa*, *C. platani* and *P. ramorum* and COX (cythocrome oxidase) LAMP primers optimized by Tomlinson *et al.,* 2010 that can amplify plant DNA. Results (sensitivity and specificity) were compared to that obtained by applying on the same samples the qPCR protocol developed by Luchi *et al.,* 2013, Migliorini *et al.,* 2019 respectively for *C. platani* and *P. ramorum*. A new qPCR assay targeting *X. fastidiosa* was developed and used for comparison.

*Improving the specificity of a LAMP assay: the case of F. circinatum (**Paper II**)*

Two different LAMP-based assays targeting *F. circinatum* were performed and optimized on the portable instrument Genie® II (OptiGene Limited, Horsham, UK). The first, was based on conventional LAMP (cLAMP) reaction for which only LAMP primers (Notomi *et al.,* 2000) are needed. cLAMP primers for targeting *F. circinatum*, were designed based on the specific genetic region selected by Luchi *et al.,* 2018 as target for developing a qPCR TaqMan-based assay for this species. The methodology used for primers design was the same reported in the previous work. In the second assay, a FRET-based fluorogenic probe was added to the reaction following Kubota *et al.,* 2011, for evaluating its capability of increasing the specificity of the assay and of quantifying the target DNA. Using this method, the probe is composed by a fluorescent strand, built on a selected Loop primer, and a quencher strand, both incorporated into the LAMP amplicon. The fluorescence is produced by the amplification of the selected specific Loop primer, on which the probe is built, increasing the specificity of the assay (Kubota *et al.,* 2011). Due to

its 100% homology only with *F. circinatum* obtained on BLAST® (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.,* 1990) confirming high specificity, the backward loop primer (BLP) was selected and used to design the fluorescent strand of the assimilating probe that was marked with FAM (6-carboxyfluorescein) dye at the 5' end. The specificity and the sensitivity of the developed LAMP assay were tested both with cLAMP and qLAMP assay, maintaining DNA concentrations and using the methodology described for the previous work of LAMP optimization. DNA extracted from symptomatic pine tissues (infected bark) and symptomatic seedlings samples were tested by using cLAMP and qLAMP targeting *F. circinatum.* Results were compared. A test with LAMP COX primers (Tomlinson *et al.,* 2010) was included to further validate results obtaining by processing plant tissues. All the LAMP results were then compared with that obtained by testing the same DNA samples with the qPCR method targeting *F. circinatum* developed by Luchi *et al.,* 2018.

*Application of LAMP probes for multiplexing: the case of Dothistroma septosporum, D. pini and Lecanosticta acicola* **(Paper III,** preliminary results)

Three qLAMP assays respectively targeting *D. septosporum*, *D. pini* and *L. acicola* were developed. Primers and probes were designed using Primer Explorer (V.4, Eiken Chemicals, Tokyo, Japan, http://primerexplorer.jp/e/) based on specific gene regions selected by comparing all the available sequences of elongation factor (EF1- α) and beta-tubulin (β-tub2) genes described in Ioos *et al.,* 2010, Janoušek *et al.,* 2016; Quaedvlieg *et al.,* 2012; Van der Nest *et al.,* 2019. The beta-tubulin (β-tub2) gene was chosen for *D. septosporum* (GenBank Acc. No. FJ467298) and *D. pini* (GenBank Acc. No. FJ467304), the elongation-factor (EF1-α) gene for *L. acicola* (GenBank Acc. No. KJ938441). The assays were optimized using the portable instrument BioRanger (Diagenetix, INC.). Differently to the portable instrument described in the previous works, this has two optical channels (470nm/550nm; 575nm/640nm), allowing to follow two

reactions at one time. To have the possibility of doing multiplex reactions, *L. acicola* and *D. pini* fluorescent strands were marked with the FAM (6-carboxyfluorescein) dye at the 5' end while that targeting *D. septosporum* was marked with TAMRA (carboxytetramethylrhodamine) at the 5' end. Specificity and sensitivity tests were made in singleplex as previous described. Symptomatic and asymptomatic pine needles were collected from symptomatic plants for analyzing the capability of each assay to recognize properly each pathogen in plant tissues, even in a latent phase. Each DNA needle sample was then tested with the developed primers an probes on LAMP. To further validate results isolations were made from positive needles samples using 1.5% MEA and following Adamson *et al.,* 2015. Results were compared with that obtained by applying on the same samples the qPCR assay developed by Ioos *et al.,* 2010, that target *D. septosporum*, *D. pini* and *L. acicola*. Preliminary tests to assess the capability of these assays to work in multiplex were made for **Paper III**. First, multiplex reactions were optimized by including two primer and probe sets (respectively targeting *D. septosporum-D. pini*/*D. septosporum-L. acicola*) in the same tube. Maintaining the same final reaction volume and the same DNA concentration used in singleplex LAMP reactions, several concentrations of probes and primers targeting each species were tested including in the reaction only one target DNA at a time. Due to the best amplification results, one of the multiplex reaction previous tested was chosen and used for assessing the capability of the reaction of amplifying two DNA (*D. septosporum-D. pini*/*D. septosporum-L. acicola*) at one time. To verify the possibility of applying the same multiplex reaction using different LAMP primer sets working with the same probe chemistry, a test was made using the optimized multiplex reaction with primer and probe sets developed and optimized by Villarilab (Warnell School of Forestry & Natural Resources, University of Georgia, USA) to detect the fungus *Raffaelea lauricola* and cytochrome oxidase gene of plants DNA.

**Transferring diagnosis into the field: rapid and simple DNA extraction methods**

*Application of a rapid and simple DNA extraction kit to be used into the field* **(Paper I)**

To have the possibility to apply the developed LAMP assays directly on-site, a simple and rapid DNA extraction method is required. The same plant species used in the optimization of the conventional LAMP for *X. fastidiosa*, *C. platani* and *P. ramorum* were extracted using a field-applicable Plant Material DNA extraction kit (OptiGene), for evaluating its suitability for a field application. Small pieces of plant material (c.a. 80 mg) were placed into a 5 mL bijou with ball bearing and 1 mL lysis buffer. Bijous were shaken vigorously for 1 min to ground the plant material. Plant material solution (10 µL) was transferred into a vial containing 2 mL dilution buffer and mixed. Finally, 3 µL of dilution buffer containing DNA has been used as template for LAMP reaction, both with developed primers and with the COX (cythocrome oxidase) primers (Tomlinson *et al.,* 2010) that target plant DNA. Results were compared to that obtained by extracting DNA from the same samples with a laboratory kit. The same portable extraction field Kit was also used on DNA wood samples infected with *F. circinatum* and were tested with the qLAMP assay targeting *F. circinatum* to evaluate the suitability of the probe-based reaction for field application.

*Optimization of a field deployable rapid DNA extraction method from crude pine needles samples* ***(Paper III)***

Preliminary tests for optimizing a crude extraction method from both mycelium and plant samples that can be applied into the field were made. For mycelium, a small amount of fungal tissue was put into a 1.5 ml contained 100 µl pure water and centrifugated at 14000rpm for 1min. Then, the supernatant was discarded and 100 µl of fresh lysis buffer (50mM sodium phosphate at PH 7.4, 1mM EDTA and 5% glycerol) was added. The mixture was then incubated at 85°C for 20-30min. The same method was adopted for needles, adding a first step in which needles were minced by

using pestle and mortar and using 1ml of lysis buffer instead of 100 µl. A 1:10 dilution was made and the mixture was then incubated as previous described. To verify the extraction of DNA using this method a classical PCR using ITS4 and ITS5 primers was made (Gardes and Bruns, 1993). PCR cycles were set as follow: 5min at 95°C, 45 sec at 95°C, 30 sec at 50°C, 30min at 72°C and 10sec at 72°C to terminate the reaction. PCR products were verified on 1% Agarose gel using 5 µl of the extracted DNA as template.

### Application of molecular tools to study plant pathogens

*Assessing the distribution of quarantine pathogens: the case of Dothistroma septosporum, D. pini and Lecanosticta acicola in Italy (**Paper IV**)*

In June 2017 widespread and locally severe foliar symptoms resembling DNB were observed in La Sila Massif, a mountain plateau at about 12 hundred meters elevation, covered with native forests of Corsican Pine (*Pinus nigra* subsp. *laricio* (Poir.) Maire) in the Parco Nazionale della Sila, a protected area for biodiversity conservation in the southernmost continental Italy. In autumn 2017, sporadical and moderate symptoms of DNB were observed on *Pinus cembra* L. growing in natural growing in natural forests of Paneveggio-Pale di San Martino Nature Park, Region Trentino Alto-Adige, a protected area at the opposite end of Italy, towards the border with Austria. The symptoms became more severe and widespread during 2018 on *P. cembra* and also on *Pinus mugo* Turra subsp. *mugo* naturally growing in the same area. Finally, in late summer 2018 similar symptoms accompanied by heavy defoliation were observed in Val Sarentino, Region Trentino Alto-Adige, on native *P. mugo* and *P. cembra* forests. Symptomatic pine needles with or without visible conidiomata from individual trees (100 samples in total, 3-10 trees per site) growing at several sites in Italy in 2017,2018 and 2019 were analyzed. DNA was extracted from 5-mm-long needle pieces (about 70 mg) with red band symptoms and bearing or not conidiomata. Each extracted DNA was tested by real-time PCR using the TaqMan probe assays

for *D. septosporum* and *D. pini* described in Ioos *et al.,* (2010). To assess the efficiency of DNA extraction from needles and consequently their DNA amplifiability, all the obtained DNA from needles were also tested using the real-time PCR assay targeting 18S rDNA developed by Ioos *et al.,* (2010). Conventional PCR was applied on a subsample of positive needle DNA extracts using primers DStub2-F and DStub2-R as described in Ioos *et al.,* (2010) and recommended by EPPO (PM 7/46(3) 2015). For each sample, 2 µl of PCR product was visualized after electrophoresis in 1% agarose gel (Sigma-Aldrich) in 1 × Tris-acetate-EDTA (TAE) buffer and staining with ethidium bromide (0.5 µg mL-1). Obtained DNA fragments were then purified from the agarose gel by using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sent for Sanger sequencing to StarSEQ® GmbH (Mainz, Germany). Nucleotide sequences obtained from sequencing were analyzed using CHROMAS LITE v. 2.01 (Technelysium, South Brisbane, Australia) and MUSCLE (Edgar 2004) as implemented in MEGA 7 (Kumar et al., 2016). To further confirm results obtained by applying the real-time PCR assay on needles DNA, analyzed DNA sequences were compared with that of the GenBank database (National Center for Biotechnology Information (NCBI), Bethesda, MD) using BLAST® in order to check the correspondence to the expected target.

# RESULTS AND DISCUSSIONS

## Early detection and pests control

### *Conventional LAMP reaction optimization: the case of Xylella fastidiosa, Ceratocystis platani and Phytophthora ramorum (*Paper I)

Results of the first work assessed the capability of each assay to detect *X. fastidiosa*, *C. platani* and *P. ramorum* with high specificity (Table 1 in **Paper I**) and sensitivity (Fig.2 in **Paper I**). The main parameters used to assess the positivity of a sample in a LAMP real-time assay, as the one developed in the present work, are amplification time ($t_{amp}$) and annealing temperature ($T_a$)

resulting by fluorescence analysis and given on the portable instrument monitor as curves of amplification and melting. The complete analysis (amplification and annealing) was obtained in only 30 minutes, starting to have positive results at c.a 7 min (Table 1 in **Paper I**). All the DNA samples of *X. fastidiosa* that include *X. fastidiosa* (Co.Di.Ro), *X. fastidiosa subsp. fastidiosa* (Xff) and *X. fastidiosa subsp. multiplex* (Xfm) were positively amplified by LAMP assay, and the melting curve showed a specific peak ($T_a$) ranged between 88.78 and 88.98 °C. Bacterial DNA extracted from the other strains were not amplified, confirming the theoretical amplicon specificity analyzed on BLAST ® that gave 100% homology only with *Xylella fastidiosa*. On the contrary, the assay targeting *C. platani* and *P. ramorum* were able to detect also *C. fimbriata* and *P. lateralis* that resulted amplified with the same $T_a$=88°C obtained also for the target species. Regarding *P. ramorum* assay, other *Phytophthora* species were amplified but they showed different amplification curves (with different $t_{amp}$) or different melting curves (with different $T_a$). Regarding these results it is to consider that the *P. ramorum* LAMP amplicon showed 99% homology (due to only 2 bases of differences in the ITS region) with *P. lateralis* sequences. Similarly, the *C. platani* LAMP amplicon showed complete homology (100%) with *C. fimbriata* and 99% homology with *C. neglecta*, *C. ecuadoriana* and *C. manginecans*. These species are almost morphologically indistinguishable and phylogenetically very close (Kroon *et al.,* 2012; Martin *et al.,* 2014), but they were reported on very different hosts: *P. lateralis* attacks *Chamaecyparis* spp. and other *Cupressaceae* (Hansen *et al.,* 2000; Robin *et al.,* 2011), and *C. fimbriata* is the agent of sweet potato black rot (Okada *et al.,* 2017), not compromising the utility of the LAMP developed detection tool. Regarding sensitivity (Fig. 2 in **Paper I**), qPCR showed higher sensitivity with respect to LAMP in *X. fastidiosa* and *C. platani* detection, while for *P. ramorum* LOD was the same as that of LAMP (0.128pg $\mu l^{-1}$ for *P. ramorum*, 0.02 pg $\mu l^{-1}$ for *X. fastidiosa* and *C. platani*). All symptomatic host plant samples were amplified successfully with the LAMP assay designed for each target pathogen. Results were further validated by COX gene

amplification, showing a specific melting peak at $T_a$= 85 °C for each analyzed plant sample (both healthy and infected tissues). The opportunity to have an accurate and rapid detection of the three quarantine pathogens considered in this study 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. As an example, the LAMP protocol adopted by EPPO for *X. fastidiosa* detection and developed by Harper *et al.,* (2010), requires ca. 60 min to amplify 1.4 pg $\mu L^{-1}$ pathogen DNA in host (*Vitis vinifera*) DNA. In comparison, the assay developed in the current study requires only ca. 15 min to amplify 0.02 pg $\mu L^{-1}$ of *X. fastidiosa* DNA in dd-water. No previous LAMP assay has been developed for *C. platani* to our best knowledge. The use of a simple method for visualizing amplification products (e.g. Hydroxynaphtal blue dye used in Harper *et al.,* 2010) could be particularly suited for use in the field, but opening the tube in post-amplification makes the method extremely vulnerable to carryover contamination due to the very large amount of product generated by LAMP reaction (Tomlinson *et al.,* 2007). Furthermore, even though they may be possible to observe in a laboratory environment, they are difficult to detect in the field due to the different light conditions at different times of the day (Lau and Botella, 2017), leading to false negative results or to losses in detection sensitivity. To solve this problem, in this work a real-time method of products visualization was improved allowing to simplify the interpretation of results and to avoid post reaction analyses. 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, thus allowing a prompt reaction.

*Improving the specificity of a LAMP assay: increasing specificity* (**Paper II**) *and multiplexing* **(Paper III,** preliminary results**)**

The correct functioning of the assimilating LAMP-probes (Kubota *et al.,* 2011) inserted in the reaction was assessed for each qLAMP developed assay. Each target species DNA (*F. circinatum*, *D. pini*, *D. septosporum* and *L. acicola*) was amplified correctly when also the probe was included in the reaction maintaining high fluorescence and starting to have results at $t_{amp}$=10min for *D. pini*, *D. septosporum* and *L. acicola* (**Paper III**), $t_{amp}$=14min for *F. circinatum* **(Paper II)**. Comparing the cLAMP to the qLAMP both developed for *F. circinatum* **(Paper II)**, a delay in amplification of c.a 4min was observed when also the probe was included. However, whit this improvement it was possible to obtain higher specificity for this assay **(Paper II)**. The BLAST ® search applied on the complete amplicon of *F. circinatum* LAMP assay, showed a complete homology (100%) only with *F. circinatum* sequences available on the GenBank database (NCBI). However, high homology (from 97.14 to 97.89 %) were obtained with other *Fusarium* species (*F. oxysporum*, *F. quttiforme*, *F. begonia*, *F. ananatum*, *F.fujikuroi*, *F. bulbicola*, *F. subglutinans, F. bactridioides, F. anthophilum, F. mexicanum, F. temperatum*), a situation that was very similar to that obtained for *P. ramorum* in the previous described work **(Paper I)** for which many cross reactions were registered. Indeed, using the cLAMP developed for *F. circinatum*, results were very similar to that obtained for *P. ramorum*, having many cross reactions with other *Fusaria* species (**Paper II**) that given a melting temperature ($T_a$=88.53°C) that was very similar to that obtained for *F. circinatum*, the target species of the assay ($T_a$=88.83°C). On the contrary, using the qLAMP in which the assimilating probe was included, only *F. temperatum* resulted as cross reaction. Considering that when the probe is included in the assay it is impossible to obtain a melting temperature, the distinction between *F. circinatum* and *F. temperatum* was based on the maximum fluorescence reached in amplification. Indeed, it was observed that qLAMP amplification fluorescence of *F. circinatum* was always of c.a 25.000 F while *F. temperatum* was

of c.a 5.000 F. A cross-reaction with *F. temperatum* DNA was observed also in the qPCR protocols nowadays developed (Ioos *et al.,* 2019; Luchi *et al.,* 2018), underlining the high difficult in distinguish the two species and validating the improvement obtained in the qLAMP assay. Furthermore, it is to be considered that *F. temperatum* was reported only on *Zea mays* (Scauflaire *et al.,* 2011), not compromising the utility of the assay. The same high specificity was obtained also when the developed probe was used for *D. septosporum*, *D. pini* and *L. acicola* for which no cross reactions were obtained with the tested species (Table 1 in **Paper III**). Considering that cLAMP techniques is not sequence specific but rather it can detect the total amplification occurring in a reaction (Ball *et al.,* 2016), it results highly prone to detection of nonspecific amplification, which can occur with LAMP even in the absence of the specific target (Ball *et al.,* 2016) species, consequently increasing the rates of false positive and limiting the discrimination capability of the assay together with its utility. Results obtained by applying the probe improvement show an advance in technology that can improve in field detection, validating the utility of these assays also in distinguishing between species that have very similar morphological (*D. septosporum, D. pini, L. acicola*) or genetical (*Fusaria*) features. In addition, the use of this improvement has not compromised sensitivity that resulted high (3.2 pg $\mu L^{-1}$ for *D. septosporum*, 0.64 pg $\mu L^{-1}$ for *D. pini*, 0.128 pg $\mu L^{-1}$ for *L. acicola*) and the same of that obtained in cLAMP for *F. circinatum* (0.5 pg $\mu L^{-1}$ for both cLAMP and qLAMP assay). Even if some qPCR assay developed for *F. circinatum* (Ioos *et al.,* 2009; Luchi *et al.,* 2018) resulted higher sensitive than the LAMP developed protocol having a detection limit of respectively 0.06 pg $\mu L^{-1}$ and 0.4 fg $\mu L^{-1}$, conventional PCR and qPCR reported as species specific (Ramsfield *et al.,* 2008; Schweigkofler *et al.,* 2004) reported a lower detection limit of 10 pg $\mu L^{-1}$. Regarding the LAMP assays developed for *D. pini, D. septosporum* and *L. acicola*, for *D. pini* and *L. acicola* the sensitivity was comparable to that obtained in the qPCR protocol (Ioos *et al.,* 2010) used for comparison in which a detection limit of 0.1 pg $\mu L^{-1}$ was assessed. On the contrary the sensitivity,

was lower for *D. septosporum* probably due to the lower efficiency of TAMRA dye that was used for marking the *D. septosporum* LAMP probe. The possibility to screen plant samples in low times with high specificity and sensitivity that are comparable to qPCR protocols was assessed also by positive results given by applying qLAMP developed assay on DNA extracted from plant symptomatic samples (wood, seedlings, pine needles). No reactions inhibitions were registered when processing plant DNA including assimilating probes and $t_{amp}$ were similar (from 10min to 14 min) to that obtained from axenic cultures DNA, further confirming the efficiency of each assay. Furthermore, positive results were obtained also from needles samples, collected from infected plants, with only incipient symptoms, allowing to confirm the utility of each assay to recognize pathogens in different phases of the infection including early stages (Table 2 in **Paper III**). Considerable time and effort could be saved by simultaneously amplifying multiple sequences in a single reaction, further reducing time of analyses, being more cost effective than singleplex analyses and allowing to work with minimal amounts of samples (Lau and Botella 2017). The assimilating-probe LAMP method used in **Paper II** and **Paper III** for *F. circinatum*, *D. pini*, *D. septosporum* and *L. acicola*, was described as capable to support multiplex reaction (Kubota *et al.,* 2011) and was applied on *Salmonella enterica* by Kubota *et al.,* 2015. Preliminary multiplexing tests applied on *D. pini*, *D. septosporum* and *L. acicola* showed the capability of the qLAMP developed assays (**Paper III**) to amplify in the same reaction the DNA of two species at one time (*D. pini*/*D. septosporum*-*L. acicola*/*D. septosporum*). Even if, compared to FAM dye, lower amplification fluorescence was obtained when using the TAMRA dye used for marking the *D. septosporum* probe, DNA of each pathogen was correctly amplified when in the same reaction were included the two primer sets, also using the portable instrument BioRanger (Diagenetix, INC.). Each tested combination of LAMP assay (*D. pini*/*D. septosporum*-*L. acicola*/*D. septosporum*) resulted capable to amplify the DNA of the two target species at one time with time of amplification that were comparable to that obtained in singleplex reaction

($t_{amp}$=13min). The efficiency of the optimized multiplex reaction was also validated by positive amplification results obtained by using the same conditions used in **Paper III** with two different LAMP primer sets working with the same probe chemistry and respectively targeting the fungus *Raffaelea lauricola* and plant DNA. Even if the development and optimization of multiplex LAMP reaction are only at the first steps of research needing to be further investigated, the preliminary results obtained in this study show how an advance in technology can provide more cost-effective efficient tools to prevent the introduction or limit the spread of diseases that can have severe economic, ecological and social consequences.

### Transferring diagnosis into the field: rapid and simple DNA extraction methods

The majority of LAMP-based assays developed so far for plant pathogens are still elusive regarding integrating the entire process from sample preparation to visualization of results (Lau and Botella, 2017), having as the main problem applying DNA extraction in field conditions. DNA extraction kit required usually thermolabile and degradable reagents, expertise and many steps to be correctly applied, and are not suitable for in-field use. In this work first a commercial field-deployable kit was applied and evaluated (**Paper I** and **Paper II**), then preliminary tests for developing a crude extraction method to be applied in the field were made (**Paper III**). Results obtained by applying the field-suitable commercial kit (Optigene) were as follow. All the symptomatic host plant DNA samples (*Olea europaea, Rhamnus alaternus, Calicotome spinosa, Cistus incanus, S. junceum, Prunus dulcis, Platanus × acerifolia*, *Viburnum tinus, Pinus spp.* wood and seedlings) extracted in **Paper I** and **Paper II** using the field-suitable (OptiGene) DNA extraction kit, were amplified successfully with the LAMP assay designed for each target pathogen (*X. fastidiosa, C. platani*, *P. ramorum* and *F. circinatum*). To further confirm the efficiency of the field-suitable kit, all the samples were processed with the COX (cythocrome oxidase) LAMP primers that gave positive results for all the plant samples further confirming the

efficiency of the DNA rapid extraction kit. Results were compared with that obtained by applying the same LAMP primers on the same plant samples extracted with the laboratory commercial kit (Omega Bio-tek), obtaining for each sample the same melting temperature ($T_a$=85°C) and the same amplification temperatures ($t_{amp}$) (Fig. 8) and confirming the reliability and the efficiency of the field suitable kit. Even if the application of this field-suitable Kit could be difficult when dealing with a large quantity of samples, it resulted capable to give the same results of the compared laboratory kit in shorter time (5min for the field kit, 1hour for the laboratory kit) that were registered also in comparison with other field extraction kit applied by Tomlinson *et al.,* 2007 for *P. ramorum* (30-50min) (Table 3 in **Paper I**), allowing the complete application of each LAMP assay directly on the site of sampling. However, a critical disadvantage for a field-suitable diagnostic method, is the absolute requirement for nucleic acid extraction, which makes them difficult to perform in resource limited settings (Mikita *et al.,* 2014). Since Kaneko *et al.,* 2007 found that the tolerance of LAMP reaction to the presence of biological compounds in clinical samples was higher than a PCR-based method resulting in lower amplification inhibition, the DNA extraction step could be omitted using LAMP further reducing the cost of diagnosis application and the time required for a complete analysis. Following Mikita *et al.,* 2014 and Tomlinson *et al.,* 2013 that applied Direct Boil-LAMP and crude extractions respectively on blood and plant samples, the optimization of a crude extraction method to be included in the LAMP protocols developed for *D. pini*, *D. septosporum* and *L. acicola* was evaluated (**Paper III**). Positive preliminary results were obtained by applying the crude extraction method on both fungal tissues and pine needles. Clear bands were obtained on 1% Agarose gel by processing the PCR products of extracted DNA from all the tested mycelia, confirming that DNA was extracted and amplified. Regarding needles DNA, only weak bands were obtained with the 1:10 dilutions (Fig. 9). Even if these needles samples were amplified, very little product was obtained. This might be due to the presence of higher quantities of PCR in pine needles samples. Further work

for the optimization of the method is required.

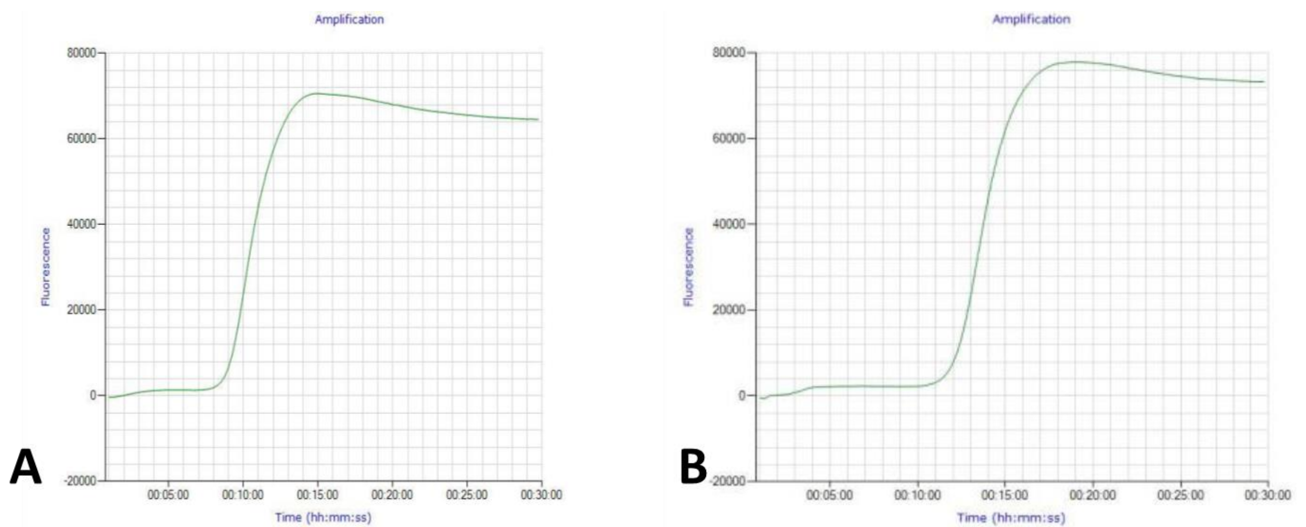



**Figure 8:** LAMP amplification plots of plant DNA amplified using COX LAMP primers. Samples extracted with A) Laboratory suitable Kit E.Z.N.A (OMEGA bio-tek) B) Field suitable Kit (Optigene)

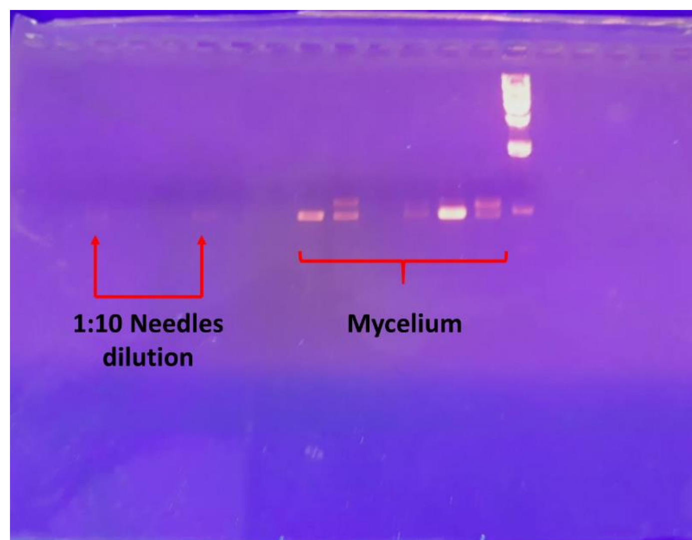



**Figure 9:** Results obtained on 1% agarose gel by processing DNA samples amplified with PCR (ITS1-ITS4) and extracted from mycelium and pine needles using crude extraction method.

## Using molecular tools to study plant pathogens

*Assessing the distribution of quarantine pathogens: the case of Dothistroma septosporum, D. pini and Lecanosticta acicola in Italy* ***(Paper IV)***

Challenges of molecular diagnostics focused around the need to rapidly and accurately identify

the causal agent of plant disease. In this context a rapid diagnosis is crucial to intercept a new pathogen before its spreading in natural ecosystem, but also to correctly manage the disease. In this context the classical methods (based on isolation and immunological assay) are time consuming and showed low sensitive in comparison with molecular approaches, especially regarding *D. pini*, *D. septosporum* and *L. acicola* for which isolations and identification are difficult due to the high morphological similarity of the three species and time consuming, requiring from 7 to 15 days to obtain mycelium grown (Mullet and Barnes, 2012). For these reasons, the qPCR assay developed by Ioos *et al.,* 2010 was applied in this work to study the distribution of these three pathogens in Italy. Positive amplification results (Ct values from 25.9 to 32.7) obtained by applying the real-time PCR on needles samples, confirmed that *D. septosporum* has spread in the Sila Massif (Calabria) on about 40 thousand hectares of natural forests of Corsican Pine, identifying this fungal species as the main causal agent of the high *P. nigra* subsp. *laricio* defoliation. Even if for this area the majority of samples belonged to *Pinus nigra* species, the needles of a few other *Pinaceae* species (i.e. *Pinus Sylvestris* L., *Pinus radiata*, *Pseudotsuga menziesi* (Mirb.) Franco, *Cedrus atlantica* (Endl.) Manetti ex Carrière) were observed as symptomatic and resulted positive to *D. septosporum* real-time assay. The pathogen was not detected at lower altitudes (11 hundred meters) nor in the few other areas of the Calabria Region including two locations some hundred kilometers southward in the Aspromonte Massif, where samples having symptoms similar to DNB were found. Positive amplification results (Ct values from 25.9 to 32.7) were also obtained by applying the *D. septosporum* real-time PCR assay on *P. cembra* and *P. mugo* samples from Colbricon (Paneveggio,Trento), San Martino Reinswald and Valdurna (Val Sarentino, Bolzano), confirming the extensive observed defoliation. All the results obtained by the *D. septosporum* qPCR analyses were further validated by positive results given by the amplification of each needle DNA sample with 18s rDNA internal control. The β-tub2 gene region fragment (231 bp) amplified and sequenced from a subsample of positive needle

DNA extracts following the procedure by Ioos *et al*., (2010) showed 100% identity to D. septosporum reference strain Genbank ID: KX364411.1. The other DNB species *D. pini* was never detected by the application of species-specific real-time PCR assay.

# FUTURE PERSPECTIVES

In last years, a part of research has dealt with developing and ameliorating diagnostics techniques for implementing their quality and efficiency. In particularly, molecular-based techniques have acquired great importance mainly in medical studies where high sensitivity and specificity was required (Lauri and Mariani, 2009; Zaravinos *et al.*, 2009). Taking as an example diagnostics tools for medical application, plant pathologists have started to be interested to diagnostics for applying it as a method of prevention, management and control of plant diseases. With the recent implementation of new simpler molecular reactions (Notomi *et al.,* 2000), research has allowed to study new technologies suitable for a field use, enabling to transfer the analysis directly on the site of sampling and further reducing the delay for having results and applying control measures or treatments. Even if many field-suitable assays were developed to control medical and food safety issues (Mikita *et al*., 2014), in plant pathology there are only the first steps toward the use of field-suitable molecular assays as a disease management decision supporting tool. For these reasons, the aim of this work was to study, developing and improving field suitable diagnostic for plant pathogens referring to Loop mediated isothermal amplification (LAMP). The majority of the assays developed for plant pathogens are based on methods of product visualization that don't allow full application into the field (Harper *et al.,* 2010; Tomlinson *et al.,* 2007). The few LAMP protocols developed for plant pathogens that work using an easy real-time visualization method (Zhang *et al*., 2013), are often based on the insertion of intercalating dyes in the reaction, as used in **Paper I**. Every intercalating dye emit a weak fluorescence signal in the presence of single-stranded DNA (ssDNA) but higher fluorescence is emitted upon a binding to double-

stranded DNA (dsDNA) (Fischbach *et al.,* 2015), meaning that every time somewhat is amplified a positive result is obtained. This was assessed in **Paper I** when dealing with *Phytophthora ramorum* and *Ceratocystis platani*, for which many other *Phytophthora* and *Ceratocystis* species were amplified. However, the distinction with other species was possible on the basis of the melting temperature results that allowed to obtain specific diagnosis. The completely application in the field of a DNA-based method is often limited by the extraction DNA method that usually is difficult to be applied into the field due to thermolabile reagents and long time required. The use of a field-suitable kit (Optigene) resulted to be a positive solution for transferring diagnosis in the field in an easy, rapid, specific and sensitive way. Indeed, it was efficiently used to detect on site *X. fastidiosa* on Mediterranean maquis species (**Paper I**) that recently were assessed as infected in Tuscany (Marchi *et al.,* 2018). In the same way, it was also applied in the city of Florence to detect the infection of *Ceratocystis platani* on urban plane trees (*Platanus spp.*). The application of these assays could be a useful improvement to rapidly detect these species limiting, preventing and monitoring their spread in urban areas (*Ceratocystis platani*) and in natural ecosystems (*X. fastidiosa; P. ramorum*). Even if from a quarantine point of view the identification of the subspecies in *Xylella fastidiosa* species is not important, the possibility of having a field-suitable assay that can distinguish one from the other in an high specific way could be a further improvement to assess the capability of each subspecies to spread in different conditions and to colonize different hosts species. Recently, probe-based technologies for LAMP application that allow to increase the specificity of a diagnostic assay were developed (Ball *et al.*, 2016; Kubota *et al*., 2011; Tanner *et al.*, 2012). Even if in plant pathology they are still little applied (Villari *et al.*, 2017), they were evaluated and used in this work to develop a species-specific field-suitable LAMP assay targeting *F. circinatum* **(Paper II).** Since the technology was able to distinguish between *Fusarium* species that differ for few oligonucleotides without losses in sensitivity, it could also useful to develop a field-suitable LAMP assay capable of distinguish between *Xylella*

subspecies. The same high specificity was assessed for the three probe-based LAMP assays developed for *D. septosporum*, *D. pini* and *L. acicola* (**Paper III**), allowing also multiplex reactions that could further reduce time and costs of each analysis (Kubota *et al.,* 2015; **Paper III**). Since only *D. septosporum* was found as widespread in Italy (**Paper IV**), these protocols could be applied to prevent the introduction of *D. pini* and *L. acicola*. However, little is known about the ecological and epidemiological requirements of these three fungi, especially regarding Italy. Each developed assay, could be applied for further monitoring the presence of *D. septosporum* in Italy, making a correlation between its growth and climate and ecological parameters of each place. Since LAMP probes are reported as capable to quantify the amplificated DNA (Kubota *et al.,* 2011) and some authors have applied it in a quantitative manner (Oscorbin *et al.,* 2016; Villari *et al.,* 2017), its capability of quantifying will be further investigated referring to *D. septosporum*. Future research will build upon this work and will include testing the suitability of the assay in quantifying pathogen inoculum in naturally infected sites with the purpose of analyzing airborne spore concentration by means of the LAMP assay, study the correlation with environmental variables and understand disease epidemiology to develop the best management strategies.

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# RINGRAZIAMENTI

Voglio fare un ringraziamento particolare al Dott. Nicola Luchi e al Prof. Paolo Capretti che mi hanno sempre aiutato, sostenuto, incoraggiato e insegnato e che ho trovato sempre disponibili nel momento del bisogno. Grazie dei consigli, delle ore passate a ragionare e del tempo che mi avete sempre dedicato. Ringrazio inoltre la Dott.ssa Luisa Ghelardini e il Dott. Alberto Santini che mi hanno aiutato nella realizzazione di parti del lavoro e nella stesura e correzione della tesi e la Dott.ssa Caterina Villari che mi ha ospitato nel suo lab. e coinvolto nella realizzazione di un nuovo e interessante progetto, in quel mondo strano e desolato del Sud degli Stati Uniti d'America. Voglio inoltre ringraziare i miei compagni di avventura e amici Alessandra, Giuseppe, Samuele e Francesca per i bei momenti passati durante questo percorso e per aver risollevato il morale di molte giornate non sempre luminose in quel delle Cascine, il Dott. Antonio Esposito con cui ho condiviso l'ufficio e il caos sulla scrivania e tutte le persone che hanno in qualche modo contribuito alla realizzazione di questo progetto.

# PAPER I

Aglietti et al. AMB Expr (2019) 9:50
https://doi.org/10.1186/s13568-019-0774-9

AMB Express

ORIGINAL ARTICLE

Open Access

# Real-time loop-mediated isothermal amplification: an early-warning tool for quarantine plant pathogen detection

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**Abstract**

An effective framework for early warning and rapid response is a crucial element to prevent or mitigate the impact of biological invasions of plant pathogens, especially at ports of entry. Molecular detection of pathogens by using PCR-based methods usually requires a well-equipped laboratory. Rapid detection tools that can be applied as point-of-care diagnostics are highly desirable, especially to intercept quarantine plant pathogens such as *Xylella fastidiosa*, *Ceratocystis platani* and *Phytophthora ramorum*, three of the most devastating pathogens of trees and ornamental plants in Europe and North America. To this aim, in this study we developed three different loop mediated isothermal amplification (LAMP) assays able to detect each target pathogen both in DNA extracted from axenic culture and in infected plant tissues. By using the portable instrument Genie® II, the LAMP assay was able to recognize *X. fastidiosa*, *C. platani* and *P. ramorum* DNA within 30 min of isothermal amplification reaction, with high levels of specificity and sensitivity (up to 0.02 pg $\mu L^{-1}$ of DNA). These new LAMP-based tools, allowing an on-site rapid detection of pathogens, are especially suited for being used at ports of entry, but they can be also profitably used to monitor and prevent the possible spread of invasive pathogens in natural ecosystems.

**Keywords:** Alien pathogens, Canker Stain Disease, Isothermal amplification, LAMP, Olive Quick Decline Syndrome, Portable diagnostics, Sudden Oak Death

## Introduction

Invasive alien species represent a primary threat to biodiversity, economy and human health. International trade, tourism and other human activities break geographical barriers introducing non-native pathogenic organisms into new environments where they eventually find susceptible hosts and environments (Fisher et al. 2012; Migliorini et al. 2015; Santini et al. 2018). In Europe the accidental introduction of three quarantine pathogens, *Xylella fastidiosa*, *Ceratocystis platani* and *Phytophthora ramorum* with infected plants or wood material, has led to epidemics with heavy economic and ecological impacts.

*Xylella fastidiosa* is a bacterium reported on more than 350 different hosts (Denancè et al. 2017) and since 2013 is responsible for Olive Quick Decline Syndrome in Southern Italy (Apulia) (Saponari et al. 2013), more recently it has been found in Tuscany (Central Italy) (EPPO 2019); *Ceratocystis platani* is an ascomycetous fungus reported as the causal agent of Canker Stain Disease (CSD) of plane tree (*Platanus*) in urban and natural ecosystems (Lehtijärvi et al. 2018; Tsopelas et al. 2017). *Phytophthora ramorum* is an oomycete causing Sudden Oak Death (SOD) in the USA (Rizzo et al. 2002) but the pathogen has also been found in European ornamental nurseries (Werres et al. 2001) and in plantations of Japanese larch (*Larix kaempferi*) in Great Britain (Brasier and Webber 2010).

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In the last decades alien plant pathogens are exponentially establishing in Europe (Santini et al. 2013). The European Union (EU) has an open-door phytosanitary system, which means that plants not specifically regulated can enter, therefore, inspections are concentrated on well-known pests and mostly limited to visual examination of aerial parts of plants. Traditional inspection methods are time consuming and labor-intensive, requiring specialized laboratories and expert operators. Furthermore, the first disease symptoms can occur after a long latent phase of the infection and they may be non-specific (e.g. *X. fastidiosa*), hampering detection efforts and, therefore, timely management of potential outbreaks. Serological and immunoassay-based methods are available, but their low sensitivity and specificity make them unreliable for phytosanitary inspections. For these reasons, sensitive and specific tools for effective phytosanitary inspection and interception are required to prevent new pathogen introductions. Nowadays, the high specificity and sensitivity of molecular DNA-based technologies allows detection of pathogens in the early stages of infection, when they are present at low DNA concentrations (Bilodeau et al. 2007; Chandelier et al. 2006; Harper et al. 2010; Luchi et al. 2013; Rollins et al. 2016). Although many of these methods have been used routinely in the laboratory, most of them are not transferable for field inspection, seriously limiting their adequacy for point-of-care application (Lau and Botella 2017). Point-of-care methods, besides being sensitive and specific, should also be simple and fast, producing results that are easy to interpret and demanding minimal equipment and facilities (Tomlinson et al. 2010a). For these purposes, an affordable LAMP (Loop mediated isothermal amplification) technique (Notomi et al. 2000), seems to be the most suitable. Recently several LAMP assays have been developed for both field and lab use especially for human and animal diseases and food safety control (Abdulmawjood et al. 2014; Lucchi et al. 2010). Up to now, even if many LAMP-based assays were developed for plant pathogens (Chen et al. 2013; Dai et al. 2012; Hansen et al. 2016; Harper et al. 2010; Moradi et al. 2014; Peng et al. 2013; Sillo et al. 2018; Tomlinson et al. 2007), only a few tests (Bühlmann et al. 2013; Franco Ortega et al. 2018; Harrison et al. 2017; Tomlinson et al. 2010b, 2013) were optimized and applied on portable instrument for on-site use. The use of portable detection instruments is a major driving force to achieve point-of-use, and real-time monitoring of analysed samples, allowing rapid detection.

The aim of this study was to optimize a reliable, fast and sensitive diagnostic assay using a LAMP portable instrument for early detection of *X. fastidiosa*, *C. platani*, and *P. ramorum.* These new protocols will be available to be used for research aims and for phytosanitary inspection, in order to prevent further introductions and spread of these pathogens.

## Materials and methods

### Microbial strains and DNA extraction

In addition to the targeted pathogens, fungal and bacterial species phylogenetically related to target pathogens, as well as out-group species and common host colonizers were used to optimize the molecular assay (Table 1).

Mycelium of fungal and oomycete isolates (stored at 4 °C in the IPSP-CNR collection) was grown on 300PT cellophane discs (Celsa, Varese, Italy) on potato dextrose agar (PDA; Difco, Detroit, MI, USA) in 90 mm Petri dishes and maintained in the dark at 20–25 °C according to species requirements. After 7–10 days the mycelium was scraped from the cellophane surface and stored in 1.5 mL microfuge tubes at − 20 °C.

Bacterial strains (stored at − 80 °C in the IPSP-CNR collection) were grown on Luria–Bertani (LB) agar for 24 h at 25 ± 2 °C. Single colonies were picked-up and transferred to tubes containing 5 mL of LB that were incubated in an orbital shaker at 25 ± 2 °C and 90 rpm overnight. One millilitre of each suspension was used for DNA extraction. Fungal and oomycete DNA suitable for molecular analysis was extracted from mycelium by using the EZNA Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA), as described by Migliorini et al. (2015). DNA from bacteria was extracted by using EZNA Bacteria DNA Kit (Omega Bio-tek) according to the procedure described by the manufacturer. DNA from the quarantine pathogens *X. fastidiosa*, *E. amylovora*, *P. ramorum* and *P. lateralis* were kindly provided by different collectors (see Table 1). Concentration of extracted DNA was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

### Plant DNA samples

Plant samples were analyzed from naturally infected hosts including: (i) Two symptomatic plants of each of the following Mediterranean maquis species were collected in March 2019: *Rhamnus alaternus*, *Calicotome spinosa*, *Cistus incanus*, *Spartium junceum*, *Prunus dulcis*, affected by *X. fastidiosa* subsp. *multiplex* (recently detected by Tuscany Regional Phytosanitary Service—EPPO 2019); (ii) 10 *Platanus* × *acerifolia* symptomatic trees infected by *C. platani* (Florence, Italy).

About 80 mg (fresh weight) of plant material, i.e. leaves of Mediterranean maquis species and wood of *P.* × *acerifolia* plants, were used for genomic DNA extraction by using two different extraction protocols: (i) on-site by using Plant Material DNA extraction kit (OptiGene), according to manufacturer's instructions. Briefly, small pieces of plant material (c.a. 80 mg) were placed in a 5 mL

**Table 1 List of isolates used in this study**

| Species | Isolate code | Group[b] | Host | Origin | Collector[c] | Molecular assay[d] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | LAMP[e] | | qPCR[f] |
| | | | | | | $t_{amp}$ (min:s) | Ta (°C) | Detection |
| *X. fastidiosa assay* | | | | | | | | |
| *Xylella fastidiosa* subsp. *pauca* | Co.Di.Ro[a] | T | *Olea europaea* | Italy | M. Saponari | 7:15 | 88.98 | + |
| *Xylella fastidiosa* subsp. *fastidiosa* | Xff[a] | T | *Prunus dulcis* | USA | J. Chen | 14:20 | 88.78 | + |
| *Xylella fastidiosa* subsp. *multiplex* | Xfm[a] | T | *Liquidambar styraciflua* | USA | S. Russell | 7:00 | 88.83 | + |
| *Pseudomonas savastanoi* pv. *savastanoi* | ITM05[a] | CHC | *Olea europea* | Italy | G. Marchi | – | – | – |
| *Pantoea agglomerans* | PaFL1[a] | CHC | *Olea europea* | Italy | G. Marchi | – | – | – |
| *Pseudomonas fluorescens* | KL218[a] | CHC | *Actinidia deliciosa* | Italy | G. Marchi | – | – | – |
| *Xanthomonas arboricola* pv. *pruni* | Xap | PR | *Prunus laurocerasus* | Italy | A. Raio | – | – | – |
| *Pseudomonas savastanoi* pv. *nerii* | Ps.sav | CHC | *Nerium oleander* | Italy | A. Raio | – | – | – |
| *Pseudomonas koreensis* | KL217[a] | NP, O | *Actinidia deliciosa* | Italy | G. Marchi | – | – | – |
| *Pseudomonas syringae* | KL34[a] | O | *Actinidia deliciosa* | Italy | G. Marchi | – | – | – |
| *Pseudomonas syringae* | KL32[a] | O | *Actinidia deliciosa* | Italy | G. Marchi | – | – | – |
| *Pseudomonas viridiflava* | KL24[a] | O | *Actinidia deliciosa* | Italy | G. Marchi | – | – | – |
| *Pseudomonas mediterranea* | C5P1rad1[a] | O | *Chrysanthemum* sp. | Italy | M. Fiori | – | – | – |
| *Pseudomonas corrugata* | C2P1 rad[a] | O | *Chrysanthemum* sp. | Italy | M. Fiori | – | – | – |
| *Pseudomonas syringae* pv. *photiniae* | CFBP2899[a] | O | *Photinia glabra* | Japan | CFBP | – | – | – |
| *Pectobacterium carotovorum* | C24[a] | O | *Zantedeschia aethiopica* | Italy | G. Marchi | – | – | – |
| *Pectobacterium carotovorum* | C6[a] | O | *Zantedeschia aethiopica* | Italy | G. Marchi | – | – | – |
| *Pantoea agglomerans* pv. *gypsophilae* | Ehg824-1[a] | O | *Gypsophila paniculata* | Israel | G. Marchi | – | – | – |
| *Pseudomonas syringae* pv. *actinidiae* | KL103[a] | O | *Actinidia deliciosa* | Italy | G. Marchi | – | – | – |
| *Pseudomonas syringae* pv. *tabaci* | GSPB1209[a] | O | *Nicotiana* sp. | Italy | G. Marchi | – | – | – |
| *Pseudomonas syringae* pv. *viburni* | CFBP1702[a] | O | *Viburnum* sp. | USA | CFBP | – | – | – |
| *Sphingomonas* sp. | KVPT7FA[a] | NP, CHC | *Actinidia deliciosa* | Italy | G. Marchi | – | – | – |
| *Stenotrophomonas malthopylia* | St | NP, PR | *Capsicum annum* | Italy | A. Raio | – | – | – |
| *Xanthomonas campestris* pv. *campestris* | Xcc | PR | *Brassica* spp. | Italy | A. Raio | – | – | – |
| *Xanthomonas arboricola* pv. *juglandis* | Xaj | PR | *Juglans regia* | Italy | A. Raio | – | – | – |
| *Erwinia amylovora* | Ea12 | O | *Pyrus communis* | Italy | C. Cainelli | – | – | – |
| *Agrobacterium tumefaciens* | LMG37 | O | *Prunus* spp. | USA | BCCM/LMG | – | – | – |
| *Agrobacterium vitis* | CFBP5523 | O | *Vitis vinifera* | Australia | CFBP | – | – | – |
| *C. platani* assay | | | | | | | | |
| *Ceratocystis platani* | CBS117355 | T | *Platanus* sp. | France | CBS-KNAW | 10:08 | 88.14 | + |
| *Ceratocystis platani* | Cp3 | T | *Platanus* × *acerifolia* | Italy | IPSP-CNR | 12:15 | 88.55 | + |
| *Ceratocystis platani* | Cp6 | T | *Platanus* × *acerifolia* | Italy | IPSP-CNR | 9:08 | 88.30 | + |

**Table 1 (continued)**

| Species | Isolate code | Group[b] | Host | Origin | Collector[c] | Molecular assay[d] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | LAMP[e] | | qPCR[f] |
| | | | | | | $t_{amp}$ (min:s) | Ta (°C) | Detection |
| *Ceratocystis platani* | G160 | T | *Platanus × acerifolia* | Turkey | T. Dogmus | 16:58 | 88.93 | + |
| *Ceratocystis platani* | DB203 | T | *Platanus × acerifolia* | Turkey | T. Dogmus | 14:08 | 88.78 | + |
| *Ceratocystis platani* | CBS115162 | T | *Platanus occidentalis* | USA | CBS-KNAW | 8:00 | 88.83 | + |
| *Ceratocystis platani* | Cp24 | T | *Platanus × acerifolia* | Italy | IPSP-CNR | 8:00 | 88.43 | + |
| *Ceratocystis fimbriata* | CBS 115167 | PR | *Syngonium* sp. | USA | CBS-KNAW | 10:05 | 88.45 | − |
| *Ceratocystis fimbriata* | CBS 118126 | PR | *Syzgium aromaticum* | Sulawesi | CBS-KNAW | 15:10 | 88.70 | − |
| *Ceratocystis fimbriata* | CBS 115175 | PR | *Mangifera indica* | Brazil | CBS-KNAW | 9:30 | 88.20 | − |
| *Ceratocystis fimbriata* | CBS 115174 | PR | *Eucalyptus* sp. | Brazil | CBS-KNAW | 14:00 | 88.50 | − |
| *Ceratocystis fimbriata* | CBS 115171 | PR | *Colocasia esculenta* | Brazil | CBS-KNAW | 10:13 | 88.60 | − |
| *Ceratocystis fimbriata* | CBS 74040 | PR | *Crotolaria juncea* | Brazil | CBS-KNAW | 13:38 | 88.00 | − |
| *Sarcodontia pachyodon* | Sp5 | O, CHC | *Platanus × acerifolia* | Italy | IPSP-CNR | – | – | − |
| *P. ramorum* assay | | | | | | | | |
| *Phytophthora ramorum* | Pram[a] | T | *Rhododendron* sp. | Greece | P. Tsopelas | 7:58 | 88.68 | + |
| *P. ramorum* | LSVM123[a] | T | *Rhododendron* sp. | France | R. Ioos-N. Schenck | 7:07 | 88.48 | + |
| *P. ramorum* | LSVM362[a] | T | *Rhododendron* sp. | France | R. Ioos-N. Schenck | 6:15 | 88.73 | + |
| *P. ramorum* | LSVM386[a] | T | *Rhododendron* sp. | France | R. Ioos-N. Schenck | 6:72 | 88.68 | + |
| *P. ramorum* | LSVM390[a] | T | *Rhododendron* sp. | France | R. Ioos-N. Schenck | 7:00 | 88.53 | + |
| *P. ramorum* | LSVM391[a] | T | *Leucothoe* sp. | France | R. Ioos-N. Schenck | 6:30 | 88.53 | + |
| *P. ramorum* | LSVM401[a] | T | *Rhododendron* sp. | France | R. Ioos-N. Schenck | 7:07 | 88.53 | + |
| *P. ramorum* | LSVM402[a] | T | *Rhododendron* sp. | France | R. Ioos-N. Schenck | 7:00 | 88.53 | + |
| *P. ramorum* | LSVM405[a] | T | *Rhododendron sp.* | France | R. Ioos-N. Schenck | 7:15 | 88.48 | + |
| *P. lateralis* | Plat[a] | PR | *Chamaecyparis lawsoniana* | France | C. Robin | 9:00 | 88.43 | + |
| *P. alni* subsp. *uniformis* | Ph68 | PR | *Alnus cordata* | Italy | G. P. Barzanti | 23:27 | 90.47 | − |
| *P. cactorum* | PCA1[a] | PR | *Aesculus hippocastanum* | Germany | J. Schumacher | – | – | − |
| *P. × cambivora* | Ph21[a] | PR | *Castanea sativa* | Italy | A. Vettraino | 25:22 | 90.67 | − |
| *P. cinnamomi* | 28SA | PR | *Laurus nobilis* | Italy | IPSP-CNR | 16:37 | 89.62 | − |
| *P. cinnamomi* | Ncfc[a] | PR | Unknown | Italy | IPSP-CNR | – | – | − |
| *P. cryptogea* | 13SA | PR | *Prunus laurocerasus* | Italy | IPSP-CNR | 22:07 | 89.78 | − |
| *P. citricola* | 51RC | PR | *Viburnum lucidum* | Italy | IPSP-CNR | – | – | − |
| *P. citricola* | Pcl1[a] | PR | Unknown | Germany | T. Jung | 27:27 | 89.82 | − |
| *P. citrophthora* | 33SB | PR | *Euonymus* spp. | Italy | IPSP-CNR | 20:05 | 89.23 | − |

**Table 1 (continued)**

| Species | Isolate code | Group[b] | Host | Origin | Collector[c] | Molecular assay[d] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | LAMP[e] | | qPCR[f] |
| | | | | | | $t_{amp}$ (min:s) | Ta (°C) | Detection |
| *P. citrophthora* | Ph9[a] | PR | *Convolvulus* sp. | Italy | S. O. Cacciola | 18:15 | 89.58 | − |
| *P. europaea* | PE1[a] | PR | Unknown | Germany | T. Jung | 15:12 | 90.42 | − |
| *P. foliorum* | 2015–1454[a] | PR, CHC | *Rhododendron* | UK | A. Pérez-Sierra | 11:15 | 89.08 | − |
| *P. gonapodyides* | PG7[a] | PR | *Quercus robur* | Germany | S. Leonhard | – | – | − |
| *P. gonapodyides* | IHTM | PR | *Alnus cordata* | Italy | IPSP-CNR | – | – | − |
| *P. megasperma* | Ph78 | PR | *Prunus avium* | Italy | G. P. Barzanti | – | – | − |
| *P. megasperma* | PMI[a] | PR | *Quercus robur* | Germany | S. Leonhard | – | – | − |
| *P. nicotianae* | 1RB | PR | *Myrtus communis* | Italy | IPSP-CNR | – | – | − |
| *P. palmivora* | 44RC | PR | *Prunus laurocerasus* | Italy | IPSP-CNR | – | – | − |
| *P. quercina* | PQ4[a] | PR | *Quercus robur* | Germany | S. Leonhard/J. Schumacher | – | – | − |
| *P. syringae* | Psy2[a] | PR | Unknown | Germany | J. Schumacher | 17:45 | 89.48 | − |
| *Elongisporangium anandrum* | PYA[a] | O | *Quercus robur* | Germany | S. Leonhard | – | – | − |
| *Phytopythium litorale* | 40SB | O | *Prunus laurocerasus* | Italy | IPSP-CNR | – | – | − |
| *Elongisporangium undulatum* | 76SB | O | *Cupressus sempervirens* | Italy | IPSP-CNR | – | – | − |
| *Mortariella* sp. | 26RA | O | *Arbutus unedo* | Italy | IPSP-CNR | – | – | − |
| *Diplodia mutila* | Dm | O | *Quercus* spp. | Italy | IPSP-CNR | – | – | − |
| *D. pinea* | 128 | O | *Pinus resinosa* | USA | M. A. Palmer | – | – | − |
| *D. scrobiculata* | 124 | O | *Pinus resinosa* | USA | M. A. Palmer | – | – | − |
| *D. seriata* | UCD 352 | O | *Vitis vinifera* | USA | J. R. Urbez-Torres | – | – | − |
| *D. seriata* | WP-J10 | O | *Vitis vinifera* | Australia | S. Savocchia | – | – | − |
| *Geosmithia pallida* | IVV7 | O | *Ulmus* spp. | Italy | IPSP-CNR | – | – | − |
| *Ophiostoma novo ulmi* subsp. *americana* | HI72 | O | *Ulmus* spp. | USA | IPSP-CNR | – | – | − |

[a] Samples provided as DNA

[b] For each molecular assay developed in this study different groups of isolates were tested: target species (T); phylogenetically related species (PR), CHC = common host colonizers species (CHC); out-group species (O)

[c] *CBS* Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre—Royal Netherlands Academy of Arts and Sciences (KNAW), Utrecht, The Netherlands; *CFBP* French Collection of Plant Pathogenic Bacteria, INRA, France; *IPSP-CNR* Institute for Sustainable Plant Protection—National Research Council, Firenze, Italy; *BCCM/LMG* Bacteria Collection Laboratorium voor Microbiologie Universiteit Gent Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium

[d] Molecular assays are referred to LAMP and qPCR described in Table 2

[e] $t_{amp}$ amplification time, $T_a$ annealing temperature, – not detected

[f] + positive, − negative

bijou with ball bearing and 1 mL lysis buffer. Bijous were shaken vigorously for 1 min to ground the plant material. Plant material solution (10 μL) was transferred into a vial containing 2 mL dilution buffer and mixed. Finally, 3 μL of dilution buffer containing DNA has been used as template in a LAMP assay;

ii) in laboratory by using EZNA Plant DNA Kit (Omega Bio-tek). Plant material of all the collected samples for DNA extraction was transferred to 2 mL microfuge tubes with two tungsten beads (3 mm) (Qiagen) and 0.4 mL lysis buffer P1 EZNA Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA) then ground with a TissueLyser (Qiagen) (30 oscillations/s for 1 min). DNA was extracted from all samples using the EZNA Plant DNA Kit (Omega Bio-tek) (Migliorini et al. 2015).

In addition to the above samples, the optimization of LAMP assay was conducted by using the following DNA samples stored at −80 °C (IPSP-CNR DNA collection): (i) 10 DNA samples extracted from symptomatic *Olea europaea* leaves with *X. fastidiosa* subsp. *pauca* infections. DNA was kindly provided by M. Saponari (IPSP-CNR, Bari) and extracted in CTAB buffer (Loconsole et al. 2014); (ii) 10 DNA samples from symptomatic *Viburnum tinus* leaves affected by *P. ramorum* extracted by using EZNA Plant DNA Kit (Omega Bio-tek).

As negative control, fresh tissue collected from 10 healthy plant of each tested species (*Olea europaea*, *Rhamnus alaternus*, *Calicotome spinosa*, *Cistus incanus*, *S. junceum*, *Prunus dulcis*, *Platanus × acerifolia* and *Viburnum tinus*) were extracted by using both Plant Material DNA extraction kit (OptiGene) and EZNA Plant DNA Kit (Omega Bio-tek), as previously described.

### LAMP primer design

The six LAMP primers included: two outer primers (forward primer, F3; backward primer, B3) two inner primers (forward inner primer, FIP; backward inner primer, BIP) and two loop primers (forward loop primer, FLP; backward loop primer, BLP), as required by LAMP reaction (Notomi et al. 2000). Primers were designed using LAMP Designer software (OptiGene Limited, Horsham, UK) (Table 2) on the basis of the consensus sequences of the ribosomal RNA gene (ITS1-5.8 S-ITS2) for *P. ramorum* (KC473522) and *C. platani* (EU426554.1), while for *X. fastidiosa* the ribosome maturation factor (RimM) gene belonging to Co.Di.Ro strain was chosen (JUJW01000001). All designed primers were synthesized by MWG Biotech (Ebersberg, Germany) and are reported in Table 2. The specificity of newly designed primers was further tested using nucleotide–nucleotide BLAST® (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990).

### Real-time LAMP assay conditions

Real-time LAMP reactions were performed and optimised on the portable real-time fluorometer Genie® II (OptiGene Limited, Horsham, UK). DNA samples were amplified for 30 min in Genie® Strips (OptiGene Limited, Horsham, UK) with eight 0.2 mL isothermal reaction tubes with a locking cap providing a closed-tube system. Each isothermal amplification reaction was performed in duplicate, in a final volume of 25 μL. The reaction mixture contained 15 μL Isothermal Master Mix (ISO-001) (OptiGene Limited, Horsham, UK), 7 μL LAMP primer mixture (at final concentrations 0.2 μM of each F3 and B3, 0.4 μM of each FLP and BLP and 0.8 μM of each FIP and BIP) and 3 μL of template DNA. For each run two tubes including 3 μL dd-water were tested as No Template Control (NTC). LAMP amplification reactions were run at 65 °C for 30 min, followed by an annealing analysis from 98 to 80 °C with ramping at 0.05 °C per second that allow the generation of derivative melting curves (Abdulmawjood et al. 2014).

The main parameters used by Genie® II system to assess the positivity of a sample are: amplification time ($t_{amp}$) and amplicon annealing temperature ($T_a$). The $t_{amp}$ is the time (expressed in min) where the fluorescence second derivative of the signal reaches its peak above the baseline value, while the $T_a$ is the temperature (expressed in °C) at which double-stranded DNA product dissociates into single strands.

### Specificity and sensitivity of real-time LAMP assays

For each target pathogen (*X. fastidiosa*, *C. platani* and *P. ramorum*) the specificity of the real-time LAMP assay was tested by using genomic DNA extracted from bacterial, fungal or oomycete strains (Table 1), at a final concentration of 10 ng $\mu L^{-1}$. The limit of detection (LOD) of the LAMP assay was tested by using an 11-fold 1:5 serial dilution (ranging from 10 ng $\mu L^{-1}$ to 0.001 pg $\mu L^{-1}$) of each standard DNA template (*X. fastidiosa* - Co.Di.Ro strain; *C. platani* - isolate Cp24; *P. ramorum* - isolate Pram).

### Real-time LAMP assay in naturally infected plants

To check the suitability of extracted plant DNA for downstream analysis the cytochrome oxidase (COX) gene was used as endogenous plant gene according to Tomlinson et al. (2010a) (Table 2).

The effectiveness of the real-time LAMP assay was then tested on DNA extracted from naturally infected hosts (*Olea europaea*, *Rhamnus alaternus*, *Calicotome spinosa*, *Cistus incanus*, *S. junceum*, *Prunus dulcis*, *Platanus × acerifolia* and *Viburnum tinus*) to detect each respective target pathogen (*X. fastidiosa*, *C. platani* and

**Table 2 List of primer set used in this study**

| Target species | Molecular assay | Primer code | Sequence | References |
|---|---|---|---|---|
| *Phytophthora ramorum* | LAMP | Phy-r_F3 | 5′-ACGTTGTTGGTTGTGGAG-3′ | This study |
| | | Phy-r_B3 | 5′-CCAATTGAGATGCCAGCA-3′ | |
| | | Phy-r_FLP | 5′-CGCATTGTTCAGCCGAAG-3′ | |
| | | Phy-r_BLP | 5′-GAATCGACGGTGTTGTGC-3′ | |
| | | Phy-r_FIP | 5′-AGTCATTACCGCCACAGCAGTGTTCGATTCGCGGTA-3′ | |
| | | Phy-r_BIP | 5′-CGTAGCTGTGCAGGGCTTGAACCGCCACTCTACTTC-3′ | |
| | qPCR | PramF | 5′-GCAGGGCTTGGCTTTTGA-3′ | Migliorini et al. (2018) |
| | | PramR | 5′-GCCGAACCGCCACTCTACT-3′ | |
| | | Pram_PR | 5′-FAM-TCGACGGTGTTGTGCG-MGBNFQ-3′ | |
| *Xylella fastidiosa* | LAMP | XF_F3 | 5′-TAGAGTCTTGGACTGAGCC-3′ | This study |
| | | XF_B3 | 5′-ATCGACCCAGTAATACTCGT-3′ | |
| | | XF_FLP | 5′-AGGAGAACGTAATAACCACGG-3′ | |
| | | XF_BLP | 5′-TCCTGGCATCAATGATCGTAAT-3′ | |
| | | XF_FIP | 5′-CACCATTCAACATGGACTCGGTGCGATCTTCCGTTACCAG-3′ | |
| | | XF_BIP | 5′-CTACGAGACTGGCAAGCGTTCGTACCACAGATCGCTTC-3′ | |
| | qPCR | Xf_Fw | 5′-CGGGTACCGAGTCCATGTTG-3′ | This study |
| | | Xf_Rev | 5′-CAATCAAACGCTTGCCAGTCT-3′ | |
| | | Xf_Pr | 5′-FAM-TGGTGCCCGTGGCTA-MGBNFQ-3′ | |
| *Ceratocystis platani* | LAMP | CPL_F3 | 5′-CAGCGAAATGCGATAAGTAATG-3′ | This study |
| | | CPL_B3 | 5′-TTTATACTACACAGGGGAGTTG-3′ | |
| | | CPL_FIP | 5′-AATGACGCTCGGACAGGCTCGAATCTTTGAACGCACA-3′ | |
| | | CPL_BIP | 5′-TGTTCTTGGCGTTGGAGGTCGCAAGTATAACAGCCGATACA-3′ | |
| | | CPL_FLP | 5′-TGCCTGGCAGAATACTGC-3′ | |
| | | CPL_BLP | 5′-GTTCTCCCCTGAACAGGC-3′ | |
| | qPCR | CpITS-F | 5′-GCCTGTCCGAGCGTCATT-3′ | Luchi et al. (2013) |
| | | CpITS-R | 5′-CCTCCAACGCCAAGAACAAA-3′ | |
| | | CpITS-Pr | 5′-FAM-CACCACTCAAGGACTC-MGB-3′ | |
| Cytochrome oxidase (COX) endogenous plant gene | LAMP | COX F3 | 5′-TATGGGAGCCGTTTTTGC-3′ | Tomlinson et al. (2010a, b) |
| | | COX B3 | 5′-AACTGCTAAGRGCATTCC-3′ | |
| | | COX FLP | 5′-ATGTCCGACCAAAGATTTTACC-3′ | |
| | | COX BLP | 5′-GTATGCCACGTCGCATTCC-3′ | |
| | | COX FIP | 5′-ATGGATTTGRCCTAAAGTTTCAGGGCAGGATTTCACTATTGGGT-3′ | |
| | | COX BIP | 5′-TGCATTTCTTAGGGCTTTCGGATCCRGCGTAAGCATCTG-3′ | |

*P. ramorum*). For each plant species, additional healthy plants DNA were also included as negative control.

### Real-time quantitative PCR assay

To validate the LAMP assay, for each target pathogen, DNA samples (from microbial and plant tissue) were also tested by real-time quantitative PCR (qPCR) based on TaqMan chemistry.

Primers and TaqMan® MGB probe for the DNA quantification of *X. fastidiosa* with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Forster City, CA, USA) were designed using Primer Express™ 3.0 software (Applied Biosystems). The DNA sequence of the ribosome maturation factor (RimM) gene (CoDiRO strain) was obtained from the 'National Center for Biotechnology Information' (NCBI) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) (accession number JUJW01000001). The TaqMan® MGB probe was labelled with 6-carboxy-fluorescein (FAM) at the end, and a non-fluorescent quencher (NFQ) with minor groove binder (MGB) ligands, at the 3′ end. Primers and probe are reported in Table 2. The length of the amplification product was 60 bp. The identity of the

amplicon sequence was determined by comparing with other fungal species with the Standard nucleotide–nucleotide BLAST (blast n) of the NCBI.

DNA samples were assayed in MicroAmp Fast 96-well Reaction Plates (0.1 mL) closed with optical adhesive and using the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

The real-time PCR reaction was performed in a final volume of 25 μL. Each tube contained: 300 nM forward primer (Eurofins MWG Operon, Ebersberg, Germany); 300 nM reverse primer (Eurofins MWG Operon); 200 nM fluorogenic probe (Applied Biosystems); 12.5 μL TaqMan™ Universal Master Mix (Applied Biosystems); 5 μL DNA template.

Each DNA sample was assayed in three replicates. Four wells containing 5 μL sterile water each were used for a No-Template Control (NTC) without any nucleic acid. The PCR protocol was 50 °C (2 min); 95 °C (10 min); 40 cycles of 95 °C (30 s), 60 °C (1 min).

For each replicate the Ct value, defined as the point at which the Reporter fluorescent signal first became statistically significant against the background, was utilised to quantify the sample. Measurements of *X. fastidiosa* DNA in unknown samples were achieved by interpolation from a standard curve generated with a DNA standard (Co.Di.Ro. strain), which was amplified in the same PCR run.

Real time PCR protocols for *C. platani* and *P. ramorum* were those described in Luchi et al. (2013) and Migliorini et al. (2018), respectively.

### Statistical analysis

For each 1:5 serial dilution (ranging from 10 ng $\mu L^{-1}$ to 0.128 pg $\mu L^{-1}$) of each target pathogen, the correlation analysis was carried out between amplification time ($t_{amp}$) for LAMP assay and threshold cycle (Ct) for qPCR.

## Results

### Specificity of real-time LAMP assay

For each target pathogen (*X. fastidiosa*, *C. platani* and *P. ramorum*) the nucleotide–nucleotide BLAST ® search showed a complete homology (100%) between the LAMP amplicon sequences designed in the current study and the sequences of the same pathogen available in GenBank database (NCBI).

BLAST ® search did not find sequence identity between the LAMP *X. fastidiosa* amplicon and the other species present in GenBank, while the *P. ramorum* LAMP amplicon showed 99% homology (due to only 2 bases of differences in the ITS region) with *P. lateralis* sequences. Similarly, the *C. platani* LAMP amplicon showed complete homology (100%) with *C. fimbriata* and 99% homology with *C. neglecta*, *C. ecuadoriana* and *C. manginecans*.

LAMP assay was able to detect DNA of each target pathogen (*X. fastidiosa*, *C. platani* and *P. ramorum*) with positive results in the first time of the isothermal amplification ($t_{amp}$ c.a. 7 min for *P. ramorum* and *X. fastidiosa*; c.a. 8 min for *C. platani*) (Fig. 1). All DNA samples of *X. fastidiosa* that include *X. fastidiosa* (Co.Di.Ro), *X. fastidiosa* subsp. *fastidiosa* (Xff) and *X. fastidiosa* subsp. *multiplex* (Xfm) were positively amplified by LAMP assay, and the melting curve showed a specific peak ($T_a$ ranged between 88.78 and 88.98 °C) (Table 1). Bacterial DNA extracted from the other strains were not amplified by LAMP assay (Table 1). LAMP results were also confirmed by qPCR by using the designed primers (Xf_Fw and Xf_Rev) and probe (Xf_Pr) for *X. fastidiosa* (Tables 1, 2).

The real-time LAMP assay designed for *C. platani* was able to detect *C. fimbriata* strains belonging to different hosts and geographic origin (Table 1), whereas the qPCR assay gave negative results for these isolates. Similarly, the LAMP primers designed for *P. ramorum* were able to amplify *P. lateralis* DNA with melting temperatures very close to each other (Table 1). The other *Phytophthora* species included in this work either were not amplified or showed different amplification curves (with different $t_{amp}$) or melting curves (with different $T_a$) (Table 1). For each designed LAMP assay DNA from outgroup species and common host colonizer species were not amplified, as confirmed by qPCR (Table 1).

### Sensitivity of real-time LAMP assays

The values of limit of detection of LAMP assays ($LOD_{LAMP}$) were always very low, ranging from 0.02 pg $\mu L^{-1}$ for *X. fastidiosa* and *C. platani* and 0.128 pg $\mu L^{-1}$ for *P. ramorum*, (Fig. 2; Table 3). *P. ramorum* qPCR assays had the same sensitivity as LAMP ($LOD_{qPCR} = 0.128$ pg $\mu L^{-1}$). The qPCR assays for the other two pathogens were more sensitive than LAMP with lower detection limits (*X. fastidiosa*, $LOD_{qPCR} = 0.001$ pg $\mu L^{-1}$; *C. platani*, $LOD_{qPCR} = 0.005$ pg $\mu L^{-1}$) (Fig. 2).

We also observed a very strong correlation between the $t_{amp}$ of the LAMP assay and Ct value of the qPCR in the same set of DNA samples (*X. fastidiosa*: $R^2 = 0.97$; *C. platani* $R^2 = 0.95$; *P. ramorum* $R^2 = 0.98$) (Fig. 3).

### Real-time LAMP detection in plant samples

LAMP analyses carried out on plant host DNA were further validated by COX gene amplification, showing a specific melting peak at $T_a = 85$ °C for each analysed plant sample (both healthy and infected tissues) (Fig. 1). COX

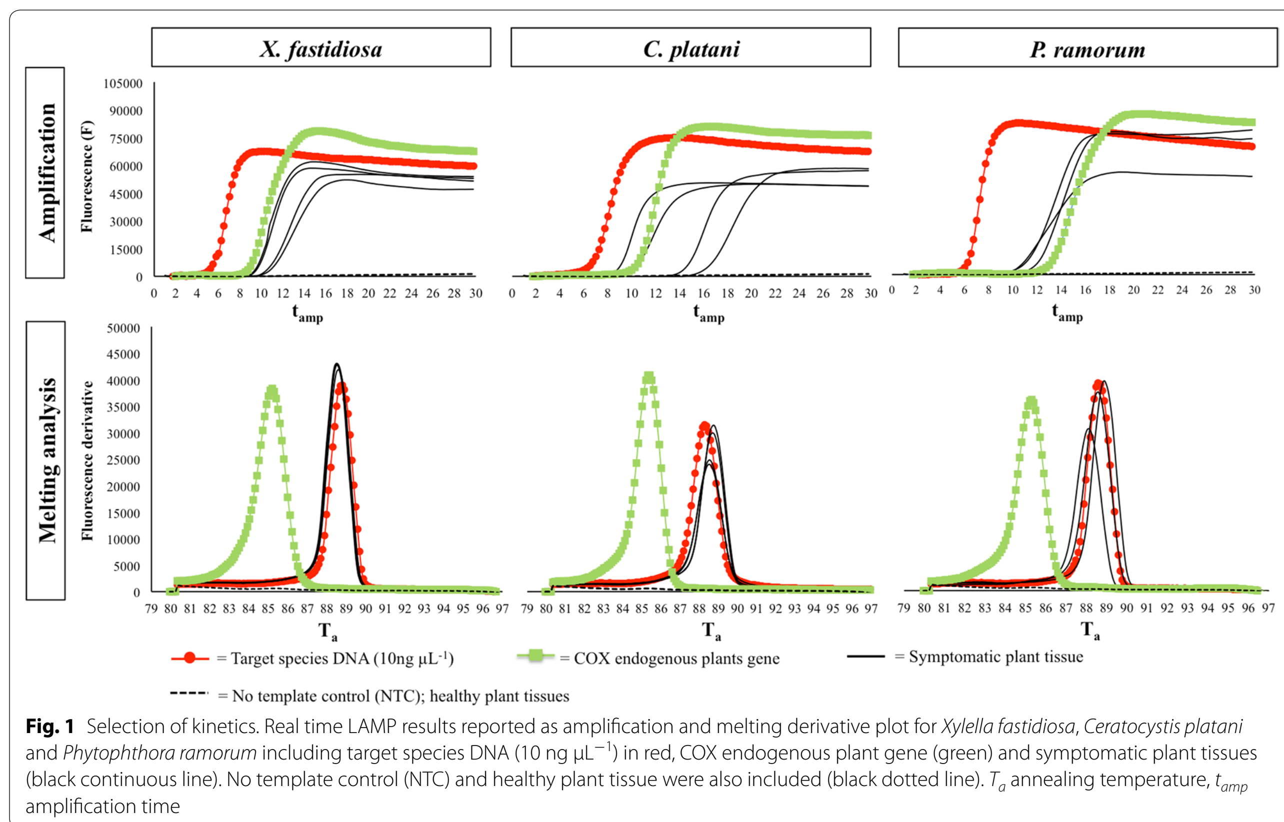


**Fig. 1** Selection of kinetics. Real time LAMP results reported as amplification and melting derivative plot for *Xylella fastidiosa*, *Ceratocystis platani* and *Phytophthora ramorum* including target species DNA (10 ng $\mu L^{-1}$) in red, COX endogenous plant gene (green) and symptomatic plant tissues (black continuous line). No template control (NTC) and healthy plant tissue were also included (black dotted line). $T_a$ annealing temperature, $t_{amp}$ amplification time

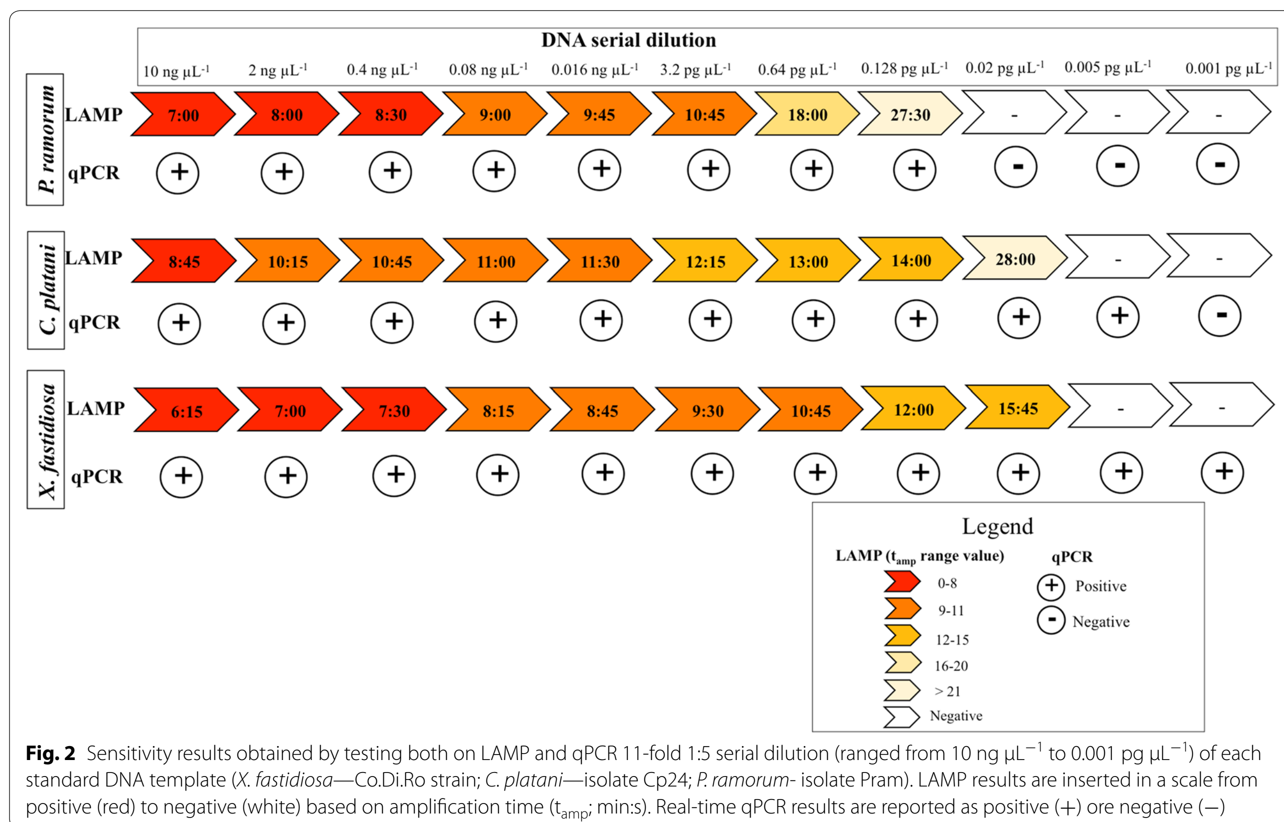


**Fig. 2** Sensitivity results obtained by testing both on LAMP and qPCR 11-fold 1:5 serial dilution (ranged from 10 ng $\mu L^{-1}$ to 0.001 pg $\mu L^{-1}$) of each standard DNA template (*X. fastidiosa*—Co.Di.Ro strain; *C. platani*—isolate Cp24; *P. ramorum*- isolate Pram). LAMP results are inserted in a scale from positive (red) to negative (white) based on amplification time ($t_{amp}$; min:s). Real-time qPCR results are reported as positive (+) ore negative (—)

**Table 3 Comparison of different DNA extraction and LAMP protocols for *Xylella fastidiosa*, *Ceratocystis platani* and *Phytophthora ramorum* detection**

| Protocol | This study | | Tomlinson et al. (2007) | Harper et al. (2010) | |
|---|---|---|---|---|---|
| DNA extraction | | | | | |
| Target pathogen | *Xylella fastidiosa*, *Ceratocystis platani*, *Phytophthora ramorum* | | *Phytophthora ramorum* | *Xylella fastidiosa* | |
| Commercial kit | Plant Material Lysis Kit (OptiGene) | EZNA Plant DNA Kit (Omega Bio-tek) | QuickPick Plant DNA kit (Bio-Nobile) | Invimag Plant DNA Mini Kit (Invitek) | DNeasy Plant Minikit (Qiagen) |
| Use | Field | Laboratory | Field | Laboratory | Laboratory |
| Sample requirement | Fresh plant tissue (80–100 mg) | Fresh plant tissue (80–100 mg) | Fresh plant tissue (15–25 mg) | Lyophilized petiole (200 mg) | Fresh plant tissue (200 mg) |
| Advantages | Rapid and simple protocol with few reagents and steps; no laboratory instruments are required | Protocol kit with spin columns and buffer supplied | Processing up to 24 samples in parallel | Simplified sample processing | Grounding with beads; kit with spin column and buffer supplied |
| Disadvantage | Difficult for large number of samples | Required laboratories facilities for grinding and DNA extraction | Extremely basic equipment is needed | Required laboratories facilities for grinding and for DNA extraction | Required laboratories facilities for grinding and for DNA extraction |
| Time per sample | 5 min | 1 h | 40–50 min | > 30 min | 1 h |
| Isothermal DNA amplification | | | | | |
| Instrument | Genie II (OptiGene) | | Smart Cycler (Cepheid) | ABI 9700 Thermocycler (Applied Biosystems) | |
| Use | Field | | Laboratory | Laboratory | |
| Sensitivity (LOD) | *P. ramorum* (0.128 pg) | | *P. ramorum* (10 pg) | – | |
| | *X. fastidiosa* (0.02 pg) | | – | *X. fastidiosa* (1.4 pg) | |
| | *C. platani* (0.02 pg) | | – | – | |
| Specificity | *P. ramorum* (high specific; *P. lateralis*) | | *P. ramorum* (high specific; *P. lateralis*) | – | |
| | *X. fastidiosa* (very high specific) | | – | *X. fastidiosa* (very high specific) | |
| | *C. platani* (high specific; *C. fimbriata*) | | – | – | |
| Advantages | Rapid detection results; amplification and detection reaction is carried out in the same instrument (16 sample per run) | | High number of samples to be processed | High number of samples to be processed | |
| Disadvantage | Strip tubes with amplification mix need to be prepared before in laboratory | | Additional steps to visualize amplified products (electrophoresis gel, colorimetric detection, fluorescent dye) | Electrophoresis gel to visualize amplified products | |
| Time per sample | 30 min | | > 1 h | > 1 h | |

*LOD* limit of detection

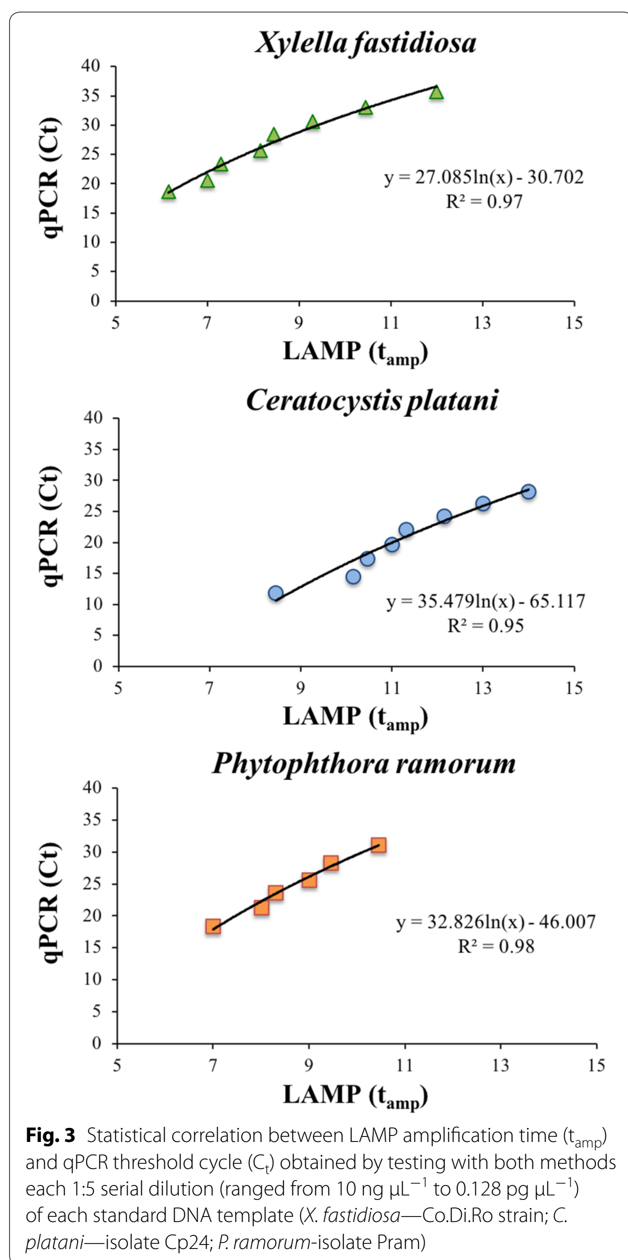


**Fig. 3** Statistical correlation between LAMP amplification time ($t_{amp}$) and qPCR threshold cycle ($C_t$) obtained by testing with both methods each 1:5 serial dilution (ranged from 10 ng $\mu L^{-1}$ to 0.128 pg $\mu L^{-1}$) of each standard DNA template (*X. fastidiosa*—Co.Di.Ro strain; *C. platani*—isolate Cp24; *P. ramorum*-isolate Pram)

gene amplification was a reliable internal positive control, confirming host DNA extractions were successful by using both on-site DNA extraction kit (OptiGene) and laboratory commercial kit (Omega Bio-tek).

All symptomatic host plant samples (*Olea europaea*, *Rhamnus alaternus*, *Calicotome spinosa*, *Cistus incanus*, *S. junceum*, *Prunus dulcis*, *Platanus × acerifolia* and *Viburnum tinus*) were amplified successfully with the LAMP assay designed for each target pathogen (*X. fastidiosa*, *C. platani* and *P. ramorum*, respectively).

Symptomatic plant tissue showed similar $T_a$ obtained from DNA of axenic cultures of each target pathogen (Table 1; Fig. 1), confirming the specificity of each LAMP assay to detect pathogens in infected plant tissues.

No amplification nor melting curve was obtained by applying the LAMP primers to healthy samples confirming the specificity of the LAMP optimized assay.

## Discussion

In this work LAMP assays for detecting *X. fastidiosa*, *P. ramorum* and *C. platani*, optimized for a portable instrument in real time were developed. LAMP-based assays optimized in this study allow a complete analysis (amplification and annealing) in only 30 min, starting to have positive amplification from ca. 7 min (Table 1). To our best knowledge no previous LAMP assay has been developed for *C. platani*. qPCR showed higher sensitivity with respect to LAMP in *X. fastidiosa* and *C. platani* detection, while for *P. ramorum* LOD was the same as that of LAMP.

The opportunity to have an accurate and rapid detection of the three quarantine pathogens considered in this study 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 (Table 3). As an example, the LAMP protocol adopted by EPPO for *X. fastidiosa* detection and developed by Harper et al. (2010), requires ca. 60 min to amplify all the isolates tested by the author and to consistently amplify ca. 250 copies of template for reaction (corresponding to 1.4 pg $\mu L^{-1}$ pathogen DNA) in host (*Vitis vinifera*) DNA. In comparison, the assay developed in the current study requires only ca. 15 min to amplify 0.02 pg $\mu L^{-1}$ of *X. fastidiosa* DNA in dd-water. The use of a simple colour change method to assess the positive result of LAMP-tested samples (e.g. Hydroxynaphtal blue dye used in Harper et al. 2010), could be particularly suited for use in the field, but opening the tube to add the colorimetric dye makes the method extremely vulnerable to carryover contamination due to the very large amount of product generated by LAMP reaction (Tomlinson et al. 2007). Furthermore, some colorimetric dyes reagents can completely inhibit the LAMP reaction at the concentration needed to produce a colour change visible with the naked eye (Tomlinson et al. 2007) and even though they may be possible to observe in a laboratory environment, they are difficult to detect in the field due to the different light conditions at different times of the day (Lau and Botella 2017), leading

to false negative results or to losses in detection sensitivity. In addition, the interpretation of positive results from colour changes in colorimetric dyes is very subjective, requiring experienced staff. On the contrary, the main parameters used to assess the positivity of a sample in a LAMP real-time assay, as the one developed in the present work, are amplification time ($t_{amp}$) and annealing temperature ($T_a$) resulting by fluorescence analysis results provided by the instrument.

The EPPO diagnostic protocol (PM 7/24) for *X. fastidiosa* describes a field LAMP assay based on the paper by Yaseen et al. (2015). In this paper authors optimized the Harper et al. (2010) assay for a portable instrument, but they do not report the sensitivity of the assay, strongly limiting its application due to the risk of false negatives.

LAMP assays developed in this study are specific and able to detect the target species, both from pure DNA and from DNA obtained from plant infected tissues. Some cross reactions have been observed in species genetically closely related to target species (for *C. platani*/*C. fimbriata* and *P. ramorum*/*P. lateralis*); however, their $T_a$ is one-two degrees higher than that of the target organisms (89–90 °C vs. 88 °C), allowing a correct detection (Table 1).

A positive amplification sharing the same $T_a$ of that of *P. ramorum* and *C. platani* (88 °C) was obtained only with *P. lateralis* and *C. fimbriata*, respectively. These species are almost morphologically indistinguishable and phylogenetically very close (De Beer et al. 2014; Kroon et al. 2012; Martin et al. 2014), but they were reported on very different hosts: *P. lateralis* attacks *Chamaecyparis* spp. and other *Cupressaceae* (Hansen et al. 2000; Robin et al. 2011), and *C. fimbriata* is the agent of sweet potato black rot (Okada et al. 2017).

The results of LAMP assays were also validated by those obtained from qPCR assays. The new TaqMan qPCR assay developed in this study for targeting *X. fastidiosa* is able to amplify all the *X. fastidiosa* tested subspecies with high efficiency excluding other tested bacteria species (Table 1). Furthermore, its sensitivity (0.001 pg $\mu L^{-1}$) is much higher than that of the qPCR TaqMan assays developed by Harper et al. (2010) and by Francis et al. (2006) (both EPPO official diagnostic qPCR for *X. fastidiosa*) which has a detection limit of 0.05 pg $\mu L^{-1}$, corresponding to 20 copies of template for reaction.

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, thus allowing a prompt reaction. In conclusion, the results presented in this study show how an advance in technology can provide efficient tools to prevent the introduction or limit the spread of diseases that can have severe economic, ecological and sociological consequences.

#### Abbreviations
*X. fastidiosa*: *Xylella fastidiosa*; *C. platani*: *Ceratocystis platani*; *P. ramorum*: *Phytophthora ramorum*; LAMP: loop mediated isothermal amplification; qPCR: real-time quantitative polymerase chain reaction; Ct: threshold cycle; $t_{amp}$: amplification time; $T_a$: amplicon annealing temperature; LOD: limit of detection.

#### Authors' contributions
NL, AS conceived and designed the experiments. CA, NL, ALP, PB, FP, AS performed the field work and the experiments. AR provided bacterial strains. CA, NL, AS analyzed the data. CA, NL, AS wrote the paper. AR, PC made contribution to the revision of the manuscript. All authors read and approved the final manuscript.


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#### Acknowledgements
The authors wish to thank colleagues who kindly provided DNA and isolates of fungi and bacteria species used in this work: R. Ioos and N. Schenck (ANSES, France), G.P. Barzanti (CREA, Italy), S.O. Cacciola (Università di Catania, Italy), T. Dogmus (Süleyman Demirel University, Isparta, Turkey), T. Jung (University of Algarve, Portugal), S. Leonhard, J. Schumacher (BBA, Germany), A. Pérez-Sierra (Forest Research, UK), C. Robin (INRA, France), P. Tsopelas, (NAGREF, Greece), A. Vettraino (Università della Tuscia, Italy), M. Saponari, D. Boscia (IPSP-CNR, Bari, Italy), G. Marchi (University of Florence, Italy). Authors are also grateful to Tuscany Regional Phytosanitary Service for helping to the sampling in the field. Authors wish to warmly thank Dr. Trudy Paap (FABI, University of Pretoria, South Africa) for the thorough critical review of the text and for English language editing of the manuscript. The authors would like to thank the editor and the anonymous referees for their comments and suggestions that greatly improved the manuscript.


#### Competing interests
The authors declare that they have no competing interests.

#### Availability of data and materials
The data supporting the conclusions of this article are included within the article. Data and materials can also be requested from the corresponding author.

#### Consent for publication
All authors gave their consent for publication.

#### Ethical approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.


#### Funding
This study was funded by European Union's Horizon 2020 Research and Innovation Programme (grant No 771271). Part of this work has been funded by the project "PATINVIVA—Invasive pathogens in nurseries: new tools for the certification of pathogen exemption of material for export"—Fondazione Cassa di Risparmio di Pistoia e Pescia (grant No 255).

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 24 January 2019 Accepted: 5 April 2019
Published online: 24 April 2019

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# PAPER II

# Real-time loop mediated isothermal amplification assay for a rapid detection of Fusarium circinatum



| Journal: | *BioTechniques* |
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| Manuscript ID | Draft |
| Manuscript Type: | Reports |
| Keywords: | Optimization, PCR, Microbiology |
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# Real-time loop mediated isothermal amplification assay for a rapid detection of *Fusarium circinatum*

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## Abstract

*Fusarium circinatum* is the causal agent of pitch canker, a lethal disease of pine and other conifers. Since *F. circinatum* is a quarantine organism, its timely detection could efficiently prevent its introduction in new areas or facilitate its spread management in already infected sites. In this study we have developed a sequence-specific probe LAMP assay for *F. circinatum* using a field-deployable portable instrument. The assay was able to recognize the pathogen in host tissues in just 30 minutes and the sensitivity of assay makes it possible to detect even small amounts of *F. circinatum* DNA (as low as 0.5 pg $\mu L^{-1}$). The high efficiency of this method suggests its use as a standard diagnostic tool during phytosanitary controls.

## Method summary

Here we present real-time loop mediated isothermal amplification based on assimilating probe. This method is rapid, sensitive, specific and field-portable for *F. circinatum* detection.

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**Keywords**

Elongation factor gene; Field-deployable; Invasive species; Isothermal amplification; LAMP; Pine pitch canker;

## Introduction

Pitch canker is a lethal disease of pine trees caused by *Fusarium circinatum* (= *Gibberella circinata*) a quarantine fungal pathogen (1) native to Central America. The pathogen is one of the most economically important diseases and is nowadays established in many parts of the world including North America, Central and South America, east Asia, South Africa and also south-west Europe (2-14).

Symptoms associated to Pitch canker are large cankers on stem and branches oozing a huge amount of resin (15). The disease results particularly damaging in intensively managed plantations of non-native pine species (16) since it drastically reduces the wood yield and inhibits the use of pine species and Douglas fir in the area. All stages of tree development are susceptible and even seeds or plant parts could act as efficient pathways of the disease (16). Since eradication of non-native organisms is generally difficult and very expensive, unless the presence of the pathogen is limited to a restricted number of plants, the only reliable and economic mean of containing the spread of non-native plant pathogens is the early detection. DNA-based detection tools, usually PCR-based, are nowadays preferred for their higher sensitivity and specificity than classical tools, but they need a well-equipped lab and long-time processing data to get a result. Significant advantages concerning prompt response, rapid and sensitive detection can be achieved by using field-deployable portable LAMP-based methods (17,18).

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*Fusarium circinatum*, as a quarantine organism, it is subjected to provisional emergency measures in the EU (19) as in several other countries in the world. A rapid and specific on-site identification method at points of entry, such as ports and airports, as well as in plantations or in nurseries is of primary concern in order to discern as sharply as possible the infected from non-infected material, thus preventing the introduction and spread of this harmful pathogen in a new area, or rapidly applying quarantine regulations as they are requested.

Aim of this paper is to provide a rapid, simple, specific, and sensible LAMP assay to detect *F. circinatum* from infected plant tissue.

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## Materials and methods

### *LAMP primers and fluorescent-assimilating probe design*

Six LAMP primers and the fluorescent-assimilating probe strand were designed for *F. circinatum* using the software LAMP Designer (OptiGene Limited, Horsham, UK) on the basis of the consensus sequence of elongation factor (EF1-α) gene, previously selected by Luchi et al. (20) for a *F. circinatum* qPCR assay. The theoretical specificity of designed LAMP primers was assessed by analyzing the complete amplicon on BLAST® (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST) (21). To increase the assay specificity, a sequence-specific assimilating LAMP probe was designed. The fluorescent-assimilating probe is incorporated into the amplicon and the fluorescence produced by the amplification of the selected specific Loop is given only when the backward loop primer is amplified, increasing the specificity of the assay (22). Due to its high specificity (100% homology only with *F. circinatum*), the backward loop primer (BLP) was selected and used to design the fluorescent-assimilating probe that includes two distinct oligonucleotide strands. A first oligonucleotide strand include a fluorophore and was labeled with FAM (6-carboxy-fluorescein) dye at the 5' end, while the second oligonucleotide strand include a quencher and

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was modified with the BHQ (Black Hole Quencher) at the 3' end (Table 1). LAMP primers and the fluorescent-assimilating probe were synthetized by (Eurofins Genomics, Ebersberg, Germany) (Table 1).

***LAMP assays***

Two different *F. circinatum* assays were developed: i) conventional LAMP (cLAMP) by only using designed primers and ii) quantitative LAMP (qLAMP) that also includes the fluorescent-assimilating probe.

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DNA samples for both assays were tested in Genie ® Strips (OptiGene Limited, Horsham, UK), each one composed by eight 0.2 mL isothermal reaction tubes with a locking cap providing a closed-tube system, using the portable instrument Genie® II (OptiGene Limited, Horsham, UK).

Each isothermal reaction (both for cLAMP and qLAMP) was performed at 65°C for 30 min. The cLAMP assay was followed by a post amplification analysis that allowed the generation of derivatives melting curves and was performed by heating samples from 98 to 80°C with ramping of 0.05°C per second. Differently, when the probe was used (qLAMP assay), reactions were terminated by heating amplification products at 85°C for 5min.

DNA amplification was assayed in duplicate in a final volume of 25 µl. For each run 2 tubes containing diethylpyrocarbonate (DEPC) water were included as No Template Control (NTC). The reaction mixture used for cLAMP was those described by Aglietti et al. (17). The reaction mixture for qLAMP was composed by 15 µl of the Isothermal Master mix without intercalating dyes (ISO-001nd) (OptiGene Limited, Horsham, UK), 6 µl LAMP primer mixture (at final concentration of 0.2 µM for each F3 and B3, 0.8 µM for each FIP and BIP, 0.4 µM for the forward Loop primer (FLP), 0.75 µl DEPC water, 0.25 µl LAMP probe

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3 mixture (fluorescent and quencher strands at a final concentration of respectively 0.04 μM
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5 and 0.06 μM). For each LAMP assay 3 μl DNA was used as template of each reaction, at a
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8 final concentration of 2.5 ng $\mu L^{-1}$.
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12 **Specificity and sensitivity of LAMP assays**
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15 The specificity of LAMP assays (both cLAMP and qLAMP) has been tested by using aliquots
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17 of the same gDNA *Fusarium* samples described in Ioos et al. (23). These samples include *F.*
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19 *circinatum* isolates collected from different geographical areas, as well as phylogenetically
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22 related *Fusarium* species (Table 2).
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24 The sensitivity of both LAMP assays was assessed by testing a 10-fold serial 1:5 dilution
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26 (ranging from 8.5 ng $\mu L^{-1}$ to 4.4 fg $\mu L^{-1}$) of gDNA extracted from the target species (*F.*
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29 *circinatum*-isolate FC096) (Figure 1 A, B), by plotting a standard curve (Figure 1C) .
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31 To further validate the LAMP assays, the same aliquots of each dilution were processed by a
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33 real-time PCR (qPCR) assay developed by Luchi et al. (20). (Figure 1A)
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38 **LAMP assay from pine tissues**
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40 To assess the effectiveness of LAMP assays in pine tissues, *F. circinatum* infected bark and
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42 seedlings samples were collected from symptomatic *Pinus radiata* tree in Cantabria (Spain).
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45 Infection of the challenged tissues was ensured by pathogen isolation with classical methods
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47 in the plant pathology lab in the University of Valladolid. Additional healthy pine samples
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49 were included as negative control. DNA was extracted from small pieces of woody tissues
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52 (c.a. 100 mg) by using Invisorb Spin Plant Mini Kit (Invitek Molecular GMBH, Berlin,
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54 Germany). To assess the effectiveness of DNA extraction all DNA plant samples were tested
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56 by using a previously developed LAMP assay with COX primers (17).
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## Results and discussion

*Fusarium* species show a high genetic similarity, sharing their ITS region (24), which is generally used as barcode sequence for fungal species identification (25). Here a sequence-specific LAMP probe targeting the elongation factor (EF1-α) gene has been developed to circumvent the risk of low specificity and implemented for its use on a portable instrument.

To assess the theoretical specificity of the probe here developed, the amplicon of LAMP primers was paired with other sequences present on GenBank database (NCBI) by the BLAST® software, revealing a complete homology (100%) only with *F. circinatum* sequences.

A high homology, ranging from 97.14 to 97.89 %. was found with other *Fusarium* species (*F. oxysporum, F. quttiforme, F. begonia, F. ananatum, F.fujikuroi, F. bulbicola, F. subglutinans, F. bactridioides, F. anthophilum, F. mexicanum, F. temperatum*).

All tested *F. circinatum* strains were amplified with cLAMP assay showing melting curves with a specific peak ($T_a$ = 88.83°C), despite other *Fusarium* species were also detected (Table 2).

Differently the qLAMP assays resulted more specific than cLAMP to detect *F. circinatum*, where all other *Fusarium* species were not amplified with exception of *F. temperatum* (Table 2). These results were consistent with other studies (20, 23), where a TaqMan MGB probe showed a cross-reaction between *F. circinatum* and *F. temperatum*. However, *F. temperatum* is only present on *Zea mays* (26) and, to our knowledge, it has never been reported on any coniferous host, therefore it is very unlikely that it may yield false positive on pine tissue.

The detection limit of both cLAMP and qLAMP assays here described was as low as 0.5 pg $\mu L^{-1}$. The compared qPCR assay (20) resulted more sensitive allowing to amplify *F. circinatum* DNA until a concentration of 0.06 pg $\mu L^{-1}$.

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LAMP analyses carried out on plant host DNA were further validated by COX gene amplification, showing a specific melting peak at annealing temperature ($T_a$) = 85°C for each analyzed plant sample (both healthy and infected pine tissues). COX gene amplification was a reliable internal positive control confirming that host DNA extractions was successful.

All symptomatic plant samples were successfully amplified with the cLAMP (showing $T_a$ similar to those obtained from DNA of axenic cultures of target pathogen) and with qLAMP assays. No amplification was observed in healthy samples. These assays confirm the reliability of LAMP method to detect *F. circinatum* in infected pine tissues.

The new challenges in molecular diagnostics research focuses around the need to rapidly and accurately identify the causal agent of plant disease. In this context a rapid diagnostic technique is crucial to intercept a new pathogen before its introduction in new ecosystems, to correctly manage the disease, as it plays a relevant role in the prevention of further spreading of pathogens. In this context the classical methods (based on isolation and immunological assay or on lab diagnostics) are time consuming and showed low sensitivity in comparison with a LAMP approach. The LAMP molecular assay developed in the frame of this work could result as an efficient and user-friendly tool that could be used to prevent a further spread of *F. circinatum*.

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**Author contributions**

A.S. and N.L. designed the study. D.S., C.A., A.L.P. and J.J.D.C. performed the experiments and critically revised the data. D.S., A.S. and N.L. wrote the manuscript.

**Acknowledgments**

This study was funded by European Union's Horizon 2020 Research and Innovation Programme (grant No 771271) Homed project and was supported by COST action FP1406 "Strategies for management of *Gibberella circinata* in greenhouses and forests" (PINESTRENGTH).

The authors wish to thank to Dr. R. Ioos (ANSES, France) for providing most of DNA samples of *Fusarium spp.* species used in this work.

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**Compliance with ethical standards**

The authors made sure our manuscript complies to the Ethical Rules applicable for this journal.

**Ethical approval**

This article does not contain any studies with human participants or animals performed.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Table 1.** cLAMP and qLAMP primers and probe designed in this study for *F. circinatum;* [a] FAM = 6-carboxyfluorescein, [b] BHQ = Black Hole Quencher-1 (Eurofins, LUX), [c] The underlined fragment acts as backward Loop primer.

**Table 2.** List of *Fusarium* spp. strains used in the study.

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**Figure 1. A.** Sensitivity results obtained by testing on cLAMP, qLAMP and qPCR 10-fold 1:5 serial dilution (ranged from 8.5 ng $\mu L^{-1}$ to 4.352 fg $\mu L^{-1}$) of standard DNA template *F. circinatum* (isolate 096); LAMP results are inserted in a scale from positive (violet) to negative (white) based on amplification time ($T_{amp}$; min:s). TaqMan qPCR results are reported as positive (+) or negative (−) (20); **B**. qLAMP amplification of serial dilution of standard DNA template *F. circinatum* (isolate 096); **C**. qLAMP standard curve.

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| Primer name | Primer type | Length (bp) | Sequence (5'-3') |
|---|---|---|---|
| **Fctef F3** | F3 | 21 | CATTGAGAAGTTCGAGAAGGT |
| **FctefB3** | B3 | 21 | TGTCGAATGATTAGTGACTGC |
| **Fctef FIP** | FIP | 36 | TTGGTCTCGAGCGGGGTATTTGCCCATCGATTCTCC |
| **FctefBIP** | BIP | 36 | GAGCGATGCGCGTTTCTGTTAACACGTGACGATGCG |
| **Fctef LF** | FLP | 18 | GGCACGTTTCGAGTCGTA |
| **Fctef LB** | BLP | 18 | CCTCCCATTGCCACAACT |
| **Fctef LB probe** | Fluorescent strand | 58 | FAM[a] - ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGACCTCCCATTGCCACAACT[c] |
| **Fctef BHQ** | Quencher strand | 40 | TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT – BHQ[b] |

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| Fungal species | Isolate number | Origin | Host | Collector | cLAMP ($T_a$, °C) (Tamp, mm:ss) | qLAMP | qPCR |
|---|---|---|---|---|---|---|---|
| *F. circinatum* | FcCa02* | Cantabria, Castrourdiales (Spain) | *P. radiata* | J. Diez | 88.93(11:00) | + | + |
| *F. circinatum* | LSVM217* | Côtes d'Armor (France) | *P. radiata* | R. Ioos | 88.88 (9:15) | + | + |
| *F. circinatum* | 2738* | Chile | *P. radiata* | R. Ahumada | 88.83(12:30) | + | + |
| *F. circinatum* | CSF-4* | León (Spain) | *P. radiata* | A. Sanz-Ros | 88.73(10.45) | + | + |
| *F. circinatum* | CSF-8* | Palencia (Spain) | *P. nigra* | A. Sanz-Ros | 88.73(11.00) | + | + |
| *F. circinatum* | CSF-11* | Valladolid (Spain) | *P. nigra* | A. Sanz-Ros | 88.73(11.30) | + | + |
| *F. circinatum* | CSF-12* | Valladolid (Spain) | *P. sylvestris* | A. Sanz-Ros | 88.73(11.00) | + | + |
| *F. circinatum* | CSF-13* | Valladolid (Spain) | *P. pinaster* | A. Sanz-Ros | 88.83(10.45) | + | + |
| *F. circinatum* | 116* | Galicia (Spain) | *P. nigra* | M. Berbegal | 88.83(10.30) | + | + |
| *F. circinatum* | 164* | Asturias (Spain) | *P. sylvestris* | M. Berbegal | 88.73(12.45) | + | + |
| *F. circinatum* | 221* | Cantabria (Spain) | *P. radiata* | M. Berbegal | 88.73(11.15) | + | + |
| *F. circinatum* | 253* | Galicia (Spain) | *P. nigra* | M. Berbegal | 88.83(12.15) | + | + |
| *F. circinatum* | 822* | Galicia (Spain) | *P. pinaster* | M. Berbegal | 88.83(11.30) | + | + |
| *F. circinatum* | 07/0649 1b* | Asturias (Spain) | *P. pinaster* | M. Berbegal | 88.83(12.00) | + | + |
| *F. circinatum* | 310/061* | Asturias (Spain) | *P. palustris* | M. Berbegal | 88.83(11.15) | + | + |
| *F. circinatum* | 2028* | Chile | *P. radiata* | R. Ahumada | 88.73(12.15) | + | + |
| *F. acuminatum* | Do_US_VC_49_1* | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *F. avenaceum* | Do_US_Nat_2_1* | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *F. begoniae* | LSV293* | France | *Begonia elatior* | R. Ioos | 88.53(15:45) | - | - |
| *F. concentricum* | NRRL 25181* | France | unknown | K. O'Donnell | 88.33(20:45) | - | - |
| *F. culmorum* | CSF−14* | Palencia (Spain) | *P. pinea* | A. Sanz-Ros | - | - | - |
| *F. fracticaudum* | CMW 25245 * | Colombia | *P. maximinoi* | G. Fourie | 88.43(18:15) | - | - |
| *F. fractiflexum* | NRRL 28852* | unknown | unknown | K. O'Donnell | - | - | - |
| *F. fujikuroi* | LSV667* | France | *Zea mays* | R. Ioos | 87.83(17:30) | - | - |
| *F. graminearum* | Do-Mur/17–1* | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *F. incarntum-equiseti* species complex | Do_US_Nat_3_1* | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *F. mangiferae* | NRRL 25226* | unknown | unknown | K. O'Donnell | 88.43(23:15) | - | - |
| *F. marasasianum* | CMW 25261 * | Colombia | *Pinus patula* | G. Fourie | 88.33(14:00) | - | - |
| *F. nygamai* | NRRL 13448* | unknown | unknown | K. O'Donnell | - | - | - |
| *F. oxysporum* | CSF−16* | Spain (Palencia) | *P. pinea* | A. Sanz-Ros | - | - | - |
| *F. parvisorum* | CMW 25267* | Colombia | *Pinus patula* | G. Fourie | 88.33(16:00) | - | - |
| *F. pininemorale* | CMW 25243 * | Colombia | *P. tecunumanii* | G. Fourie | 88.53(16:00) | - | - |
| *F. proliferatum* | FGSC 7421* | Dominican Republic | Musa sp. | M Pasquali | - | - | - |
| *F. redolens* | Do-D/11–1* | Switzerland | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *F. reticulatum negundis* | FI-BOS/14–1* | Switzerland | Seed of *Picea* sp. | WSL – Phytopathology | - | - | - |
| *F. sacchari* | NRRL 13999* | unknown | unknown | K. O'Donnell | - | - | - |
| *F. sororula* | CMW 25254 * | Colombia | *Pinus* spp. | G. Fourie | 88.74(15:30) | - | - |
| *F. sporotrichioides* | Do_US_Nat_32_1* | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |

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|---|---|---|---|---|---|---|---|
| *F. subglutinans* | LSVM869* | France | *Z. mays* | R. Ioos | 88.13(20:30) | - | - |
| *F. temperatum* | LSVM870* | France | *Z. mays* | R. Ioos | 88.63(16:45) | + | + |
| *F. thapsinum* | NRRL 22045* | unknown | unknown | K. O'Donnell | - | - | - |
| *F. torulosum* | Do_US_VC_5_1* | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *F. tricinctum species complex* | Do_US_Sno_49_1* | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *F. verticillioides* | LSVM873* | France | *Z. mays* | R. Ioos | - | - | - |

*Isolate provided and assessed in the framework of COST Action FP1406 Pinestrength

Ta - annealing temperature

Tamp - time amplification

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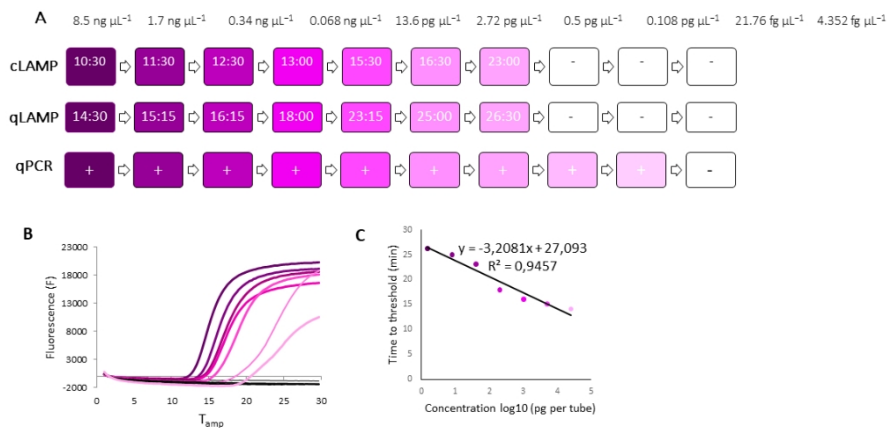


Figure 1

108x60mm (300 x 300 DPI)

# PAPER III

## Development and optimization of sequence-specific LAMP assays to target *Dothistroma pini*, *D. septosporum* and *Lecanosticta acicola* needle blights

Aglietti *et al*.

This work was realized in collaboration with Villari C.[1] and Barnes I.[2]

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### INTRODUCTION

In an ideal situation, the best way to manage a disease is to prevent the introduction of its causal agent (Bulman *et al.,* 2016). For this purpose it is, therefore, important to correctly identify pathogens and to understand the process underpinning introduction into a new environment (Janousek *et al.,* 2016). To understand the origin of a species, population genetics can provide means to study and reconstruct its demographic history (Janousek *et al.,* 2016). However, mode of reproduction plays an essential role in the genetic diversity of ascomycetes and in their infection biology (Janousek *et al.,* 2016). *Dothistroma* and *Lecanosticta* species are known to have both asexual and sexual reproduction: sexual air-born ascospores which are thought to travel considerable distances in air currents and asexual conidia that can be dispersed by rain splash and dew and are usually present in mist and cloud (Bulman *et al.,* 2016; Janousek *et al.,* 2016). The presence of the sexual reproduction in a population can reflect the ability of the pathogen to become invasive in a new environment influencing its ability to adapt to new environments conditions (Janousek *et al.,* 2016). However, a fungus sexual form is quite difficult to detect and to analyze in such populations where it is cryptic or facultative, as reported for *Dothistroma* and *Lecanosticta* species (Janousek *et al.,* 2016). This obstacle can be overcome by analyzing the distribution of maiting type idiomorphs, genes (MAT1-2, MAT1-1-1) that in an heterotallic

population as *Dothistroma*, are both required to have genetic recombination given by sexual reproduction (Drenkhan *et al.,* 2013; Groenwald 2007; Dale *et al.,* 2011). In those countries in which only one mating type belonging to each species was found, like Australia and New Zealand referring to *D. septosporum* (Groenwald *et al.,* 2007), the risk of an introduction of a different mating type is high and could led to a mating or interspecific hybridization (Bulman *et al.,* 2016). The resulting recombination of novel genes could have as result the rise of new haplotypes some of which may increase virulence, overcoming resistance mechanisms or being better suited to new environments (Drenkhan *et al.,* 2016). Further to this, because only *D. septosporum* is known to have a worldwide distribution, the introduction of *D. pini* and *L. acicola* in pest-free countries may have unanticipated impacts on forest health (Bulman *et al.,* 2016). It is important that an attempt is made to prevent the ingress and mixing of plant pathogen populations, even when countries harbor the same species (Bulman *et al.,* 2016). A way to prevent the ingress of such species is to further develop DNA-based tactics for their managing and for limiting the impact of these globally important diseases that could be applied directly on the site of interest (e.g. at the borders) for preventing and controlling the movement of infected planting material between regions and countries (Bulman *et al.,* 2016). Current PCR-based assays available for the detection and quantification of *D. pini*, *D. septosporum*, and *L. acicola* DNA in infected needles (Ioos *et al.,* 2010) require well-equipped laboratories and are time consuming. The possibility to screen for such pathogens directly in the field using a portable instrument represents an advancement in technology that could significantly expedite a rapid response to the threat. The objective of this study was, therefore, to develop and validate a real-time monitoring sequence-specific loop mediated isothermal amplification (LAMP) (Notomi *et al.,* 2000) assay for point-of-care diagnosis of the three species, using a portable LAMP device.

## MATHERIALS AND METHODS

Fungal DNA extraction

A total of 55 fungal strains including 1) different strains of target *Dothistroma pini*, *D. septosporum* and *Lecanosticta acicola*, 2) species phylogenetically related to target and 3) common colonizers of pine needles were used to optimize each LAMP assay (Tab. 1). Mycelium of all the fungi species was grown on 1.5% MEA addicted with cellophane in 90mm Petri dishes and maintained in the dark at 17-22°C, according to species requirement (Mullett and Barnes 2012). After 7-15 days the mycelium of each species was scraped from the cellophane surface and used for DNA extraction following E.Z.N.A.® Fungal DNA mini Kit (Omega, Bio-tek) and the salting out method. The concentration of extracted DNA was measured using Qubit™ Fluorometer (invitrogen™). All the isolates used in this study are listed in Tab. 1.

LAMP primers and probes design

All the available sequences of elongation factor (EF1- α) and beta-tubulin (β-tub2) genes belonging to *D. pini*, *D. septosporum* and *L. acicola* described in Ioos *et al.,* 2010, Quaedvlieg *et al.,* 2012, Janoušek *et al.,* 2016; Van der Nest *et al.,* 2019 in which were included different geographical origins of the target species, were retrieved from GenBank (NCBI) and compared with species reported as phylogenetically near using the multiple alignment server T-COFFEE (online access https://tcoffee.vital-it.ch/apps/tcoffee/index.html) and nucleotide-nucleotide BLAST® (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/BLAST) (Altschul 1990). Sequences regions having the highest genetical variability between target species and phylogenetically related fungi but in which SNP mutations among target strains were not included, were selected and used for primers design. Six LAMP primers, as required by LAMP reaction (Notomi *et al.,* 2000), were designed for each pathogen using Primer Explorer (V.4, Eiken Chemicals, Tokyo, Japan, http://primerexplorer.jp/e/) on the basis of the beta-tubulin (β-

tub2) gene for *D. septosporum* (GenBank Acc. No. FJ467298) and *D. pini* (GenBank Acc. No. FJ467304) and on the elongation-factor (EF1-α) gene for *L. acicola* (GenBank Acc. No. KJ938441), following the specifications of Notomi *et al.,* 2000 and Nagamine *et al.,* 2002. Of every primer set given by the software, those in which primers include strong mismatches (Kwok *et al.,* 1990) between target and genetically related species were selected. To increase the specificity and the fluorescence of each assay, a sequence-specific assimilating LAMP probe was designed for each pathogen following Kubota *et al.,* 2011. Loop primers designed for each target species were aligned on nucleotide-nucleotide BLAST® (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/BLAST). The most specific of the 2 loop primers was selected for each target species and used to design an assimilating probe for each assay following Kubota *et al.,* 2011. To have the possibility of doing multiplex reactions, *L. acicola* and *D. pini* probes were marked with the FAM (6-carboxyfluorescein) dye at the 5' end while *D. septosporum* was marked with TAMRA (carboxytetramethylrhodamine) at the 5' end. All the LAMP primers, the fluorescent and the quencher strands were synthetized by MWG Biotech (Ebersberg, Germany) and are reported in Table 3.

<u>LAMP conditions</u>

LAMP reactions were performed and optimized on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). DNA samples were amplified for 60 minutes in MicroAmp® Fast reaction Tubes (Applied Biosytems) strips at 65°C, measuring fluorescence values in real-time every 30sec. At the end each reaction was terminated by heating at 80°C for 5 min. Except where otherwise noted, each isothermal amplification was performed in duplicate in a final volume of 25 μl. The reaction mixture contained 15 μL Isothermal Master Mix (ISO-001nd) (OptiGene Limited, Horsham, UK), 3.05 μL LAMP primer mixture (at final concentrations of 0.2 μM of each F3 and B3, 0.8 μM of Loop primer without probe and 2.8 μM of each FIP and BIP), 0.6 μl of probe mixture (at final concentrations of 0.08 μM each probe and 0.12 μM for the quencher

strand), 13.5 μL water (Molecular biology grade, Fisher BioReagents™) and 5 μL of template DNA. For each run two tubes including 5 μL water (Molecular biology grade, Fisher BioReagents™) were included as No Template Control (NTC). Reactions were prepared and assembled in the dark using black tubes to not compromise probes functioning.

Sensitivity and specificity of LAMP assays

The limit of detection (LOD) of each LAMP assay was tested in triplicate for each target species (*D. pini*, *D. septosporum* and *L. acicola*) by using an 11-fold 1:5 serial dilution (ranging from 10 ng $\mu L^{-1}$ to 0.001 pg $\mu L^{-1}$) of each standard DNA template (*D. pini* – isolate CMW 29366; *D. septosporum* - isolate WC27 Needle 1 Taiga 626; *L. acicola* – isolate La 9.4). The specificity of each real-time LAMP assay was tested by applying each primer set on the genomic DNA extracted from fungal strains reported in Table 1, each at a final concentration of 2 ng $\mu L^{-1}$. Results were compared to that obtained with the qPCR method by Ioos *et al.,* 2010.

Collection, extraction and detection on needles samples

Pines needles samples were collected and used for testing the developed assays on naturally infected plant materials. In this test were included both symptomatic and asymptomatic needles, including different phases of the disease (needles with fungal fruitbodies, with bands and with incipient symptoms). To assess the capability of each assay to recognize each pathogen in an incipient phase of the disease, samples retrieved from the same symptomatic plants were obtained by taking needles with bands (S) and green needles (A). Health needles samples were included as a negative control. About 80mg of each needle sample (fresh weight) were used for genomic DNA extraction following Nucleospin® Plant II (Macherey-Nagel, Düren, Germany) kit. Plant material was transferred in a 2mL Eppendorf with 2 tungsten balls (Qiagen) and 0.4 mL PL1 lysis buffer, then ground with a Retsch GmbH Retsch mixer mill MM301 (Haan, Germany). DNA was extracted from all samples using the Nucleospin® Plant II (Macherey Nagel) kit. All the obtained

DNA samples were processed with LAMP both with designed primers for *D. pini*, *D. septosporum* and *L. acicola* and with the primers developed by Tomlinson *et al.,* 2010 that amplifies the cytochrome oxidase (COX) plant gene. All the used plant samples are listed in Tab. 2. To further validate results, all the DNA samples were tested with the qPCR assay developed by Ioos *et al.,* 2010.

<u>Isolations and molecular identification on fungi from positive pine needles samples</u>

To further confirm the positive results obtained from needles, isolations were carried out. Following Adamson *et al.,* 2015, needles were rinsed in 96% ethanol, fruiting bodies were taken from needles in sterile conditions under a stereomicroscope and were destroyed in sterile water that was than plated onto 1.5% MEA addicted with 0.15 g/l of streptomycin using a pipette. After 7-15 days colonies were transferred onto 1.5% MEA plus cellophane and, when mycelium was grown, genomic DNA was extracted following the previous described method. The identity of *L. acicola* was confirmed by sequencing the internal transcribed spacer (ITS) genetic region while for *D. septosporum* and *D. pini* EF1-a and Btub-2 genes were used. ITS-PCR was performed using the fungal-specific PCR primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′; Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; Gardes and Bruns 1993). Parts of the β-tubulin gene were amplified using the primer pairs Bt2a/Bt2b and Bt1a/Bt1b (Glass and Donaldson, 1995). The translation elongation factor (EF1-1alpha) gene was amplified using the forward EF1-728F and reverse primer EF1-986R (Carbone and Kohn, 1999). PCR amplicons were purified using ExoSAP-IT (USB Corp.) according to the manufacturer's protocol and sent for Sanger sequencing to StarSEQ® GmbH, Mainz, Germany. Nucleotide sequences were visualized using CHROMAS LITE v. 2.01 (Technelysium) and aligned using MUSCLE as implemented in MEGA 7 (Kumar *et al.*, 2016). Identity searches of the resulting consensus sequences were performed in the GenBank database (National Center for Biotechnology Information (NCBI), Bethesda, MD).

Portable instrument validation

To analyze the capability of the portable instrument to maintain the same results regarding sensibility and environmental samples, the same DNA samples tested on the lab instrument were tested on the field-applicable portable instrument. The 11-fold 1:5 serial dilution (ranging from 10 ng $\mu L^{-1}$ to 0.001 pg $\mu L^{-1}$) DNA belonging to the same isolates (*D. pini* – isolate CMW 29366; *D. septosporum* - isolate WC27 Needle 1 Taiga 626; *L. acicola* – isolate La 9.4) was tested on the portable instrument for field use BioRanger (Diagenetix, INC.) using DNA from the same tubes and maintaining the same conditions. Also, a test with all the plant needles samples previous described (Tab. 2) was made.

Evaluating crude extraction for field application

Preliminary tests for optimizing a crude extraction method from both mycelium and plant samples that can be applied into the field were made. For mycelium, a small amount of fungal tissue was put into a 1.5 ml contained 100 µl pure water and centrifugated at 14000rpm for 1min. Then, the supernatant was discarded and 100 µl of fresh lysis buffer (50mM sodium phosphate at PH 7.4, 1mM EDTA and 5% glycerol) was added. The mixture was then incubated at 85°C for 20-30min. The same method was adopted for needles, adding a first step in which needles were minced by using pestle and mortar and using 1ml of lysis buffer instead of 100 µl. A 1:10 dilution was made and the mixture was then incubated as previous described. To verify the extraction of DNA using this method a classical PCR using ITS4 and ITS5 primers was made (Gardes & Bruns 1993). PCR cycles were set as follow: 5min at 95°C, 45 sec at 95°C, 30 sec at 50°C, 30min at 72°C and 10sec at 72°C to terminate the reaction. PCR products were verified on 1% Agarose gel using 5 µl of the extracted DNA as template.

**RESULTS**

Specificity and sensitivity

For each selected LAMP assimilating probe (*D. pini*, *D. septosporum*, *L. acicola*), the nucleotide–nucleotide BLAST ® search showed a complete homology (100%) only between probe sequences and sequences of target species available on the database. Each LAMP assay was able to detect DNA of each tested strain belonging to each target pathogen (*D. pini*, *D. septosporum*, *L. acicola*) giving results in the first time of the analysis ($t_{amp}$: 10min). No other tested species was amplified by each developed LAMP (Tab. 1). The values of limit of detection of LAMP assays were always very low, ranging from 3.2 pg $\mu L^{-1}$ for *D. septosporum*, 0.64 pg $\mu L^{-1}$ for *D. pini* to 0.128 pg $\mu L^{-1}$ for *L. acicola*, resembling that obtained by Ioos *et al.,* 2010 that assessed a limit of detection of 0.1 pg $\mu L^{-1}$ for the qPCR assay targeting the same species. Fluorescence of TAMRA dye used for the probe of *D. septosporum* were always lower compared to that of FAM dye used for *D. pini* and *L. acicola*.

LAMP detection in naturally infected plant samples

All the DNA samples from needles were correctly amplified with primers that amplifies the COX (cytochrome oxidase) gene confirming the success of the DNA extraction. Of the 9 symptomatic needles samples, 3 were positive to *L. acicola* and 4 to *Dothistroma septosporum* having the same results both from asymptomatic than green needles collected from the same plants (Tab. 2) and obtaining a time of amplification similar to that obtained with pure cultures ($t_{amp}$:10min). No amplification was obtained by applying the LAMP primers to asymptomatic needles samples collected from the health plant that were used as negative control, confirming the specificity of each assay also in plant samples. The same results were obtained by applying on the same samples, the qPCR assay developed by Ioos,*et al.,* 2010, further confirming the efficiency of each LAMP assay.

Identification of isolates from needles

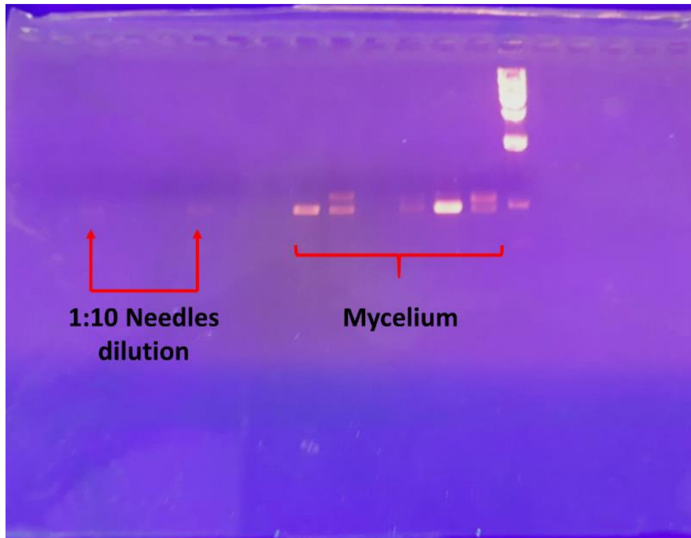
Different kind of fungi were obtained from positive needles samples, including two main morphological types that can match with *Dothistrom*a and *L. acicola* morphological features described by Mullett and Barnes 2012 and Adamson *et al.,* 2015 respectively. By comparing the obtained ITS, Ef-1α and βtub2 DNA sequences of these fungi with those available on GenBank using BLAST® (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/BLAST), 100% homology was obtained with *Dothistroma septosporum* and *L. acicola* species, confirming the efficiency of the developed LAMP assay.

Portable instrument

All the DNA points tested for sensitivity resulted positive also on the portable instrument obtaining the same LOD obtained on the StepOnePlus™ Real-Time PCR System (0.128 pg $\mu L^{-1}$ for *L. acicola*; 0.64 pg $\mu L^{-1}$ for *D. pini* and 3.2 pg $\mu L^{-1}$ for *D. septosporum*). The same results were obtained also from needles samples, validating the use of the portable instrument for a field diagnosis.

Evaluating crude extraction for field application

Positive preliminary results were obtained by applying the crude extraction method on both fungal tissues and pine needles. Big and clear bands were obtained on 1% Agarose gel by processing the PCR products of extracted DNA from all the tested mycelia, confirming that DNA was extracted and amplified. Regarding needles DNA, only weak bands were obtained with the 1:10 dilutions. Even if these needles samples were amplified, a very little product was obtained. This could be due to the presence of much inhibitors in pine needles samples that could inhibit the PCR reaction. However, further work for its optimization is required.




**DISCUSSIONS AND CONCLUSIONS**

Concerning quarantine regulation, molecular diagnostics methods for the rapid identification of intercepted specimens are crucial to prevent the introduction and spread of morphologically indistinguishable pest species (Blaser *et al.,* 2018) as *D. pini*, *D. septosporum* and *L. acicola*. The DNA-based diagnostics is at date very limited for these three species, rely mainly on few classical PCR and qPCR protocols (Ioos *et al.,* 2010), that are based on laborious and time-consuming reactions needing a lab for being apply. However, the possibility to screen for such pathogens directly in the field in an high specific and sensitive way could give many advantages in term of prompt response to threats. For this reason, in this work a field-deployable LAMP-based assay was developed for the early detection of *D. pini*, *D. septosporum* and *L. acicola*. To our best knowledge no others LAMP-based assays were nowadays developed for these three species. Each assay resulted capable to amplify each target species DNA in 30 min starting to have results in ca 10 min, without false positive results that could be given by the amplification of species that have similarity at genetically levels for the target gene regions. The obtained high specificity of the LAMP developed assay (Tab.1), was also due to the insertion of the recently developed probe chemistry (Kubota *et al.,* 2011) by which fluorescence is given only when the sequence-specific selected loop primer is amplified, further reducing the presence of cross-reactions and false

positive results. Results obtained by testing several isolates of the target species coming from different origins showed no false negative results assessing that single nucleotide polymorphisms (SNPs) differences observed in the target gene regions of each target population have no influences in the primer binding and consequently in amplification of *D. septosporum*, *D. pini* and *L. acicola*. In this way was assessed the possibility of using and applying each assay in many countries in which many different haplotypes and mating type belonging to each target species were reported. The opportunity to have an accurate and rapid detection of the three quarantine pathogens considered in this study was further validated by sensitivity results obtained on the StepOnePlus$^{TM}$ Real-Time lab PCR System and confirmed on the BioRanger (Diagenetix, INC.) portable instrument for field use. The detection limit (LOD) of each developed LAMP assay resulted very low (3.2 pg $\mu L^{-1}$ for *D. septosporum*, 0.64 pg $\mu L^{-1}$ for *D. pini*; 0.128 pg $\mu L^{-1}$ for L. *acicola*) but higher than LAMP assays based on the internal transcribed spacer (ITS) gene regions (Aglietti *et al.,* 2019), probably due to the lower copy number of the gene regions of elongation factor (EF1- α) and beta-tubulin (β-tub2) genes that were used in this study as target. However, it was comparable to diagnostics tools based on EF1- α and β-tub2 genes at date developed for these pathogens, as the qPCR one (Ioos *et al.,* 2010) adopted by the European and Mediterranean Plant Protection Organization (EPPO) as an official tool of diagnosis (PM 7/46) for these species that reported a sensitivity of 0.1 pg $\mu L^{-1}$ for each fungus. This sensitivity was also enough for detecting the three pathogens directly on pine needles, obtaining positive results with both instrument (lab and field one) also from asymptomatic needles collected from symptomatic plants, showing the high utility of each assay to detect each pathogen even in a incipient phase of the disease. As the major risk of introduction in new countries is represented by commercial trade and movements of infected planting materials (Bulman *et al.,* 2016), 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, thus allowing a prompt reaction. The most of LAMP-based assay are still elusive regarding integrating the entire process from sample preparation to visualization of results (Lau and Botella 2017), having as the main problem DNA extraction that usually required a lot of reagents and expertise. However, to be applied into the field it should be rapid and simple. In this work preliminary tests to develop a simple and rapid DNA extraction from crude samples (mycelium, pine needles) was included, obtaining preliminary results that could improve each LAMP developed tool. This together with the possibility to apply multiplex reaction showed by preliminary results described below can help in further reducing costs of application. Development and optimization of the three assays are only the first steps toward the use of the assay as a disease management decision supporting tool. Future research will build upon this work and will include testing the suitability of the assay in quantifying pathogen inoculum in naturally infected fields. Such a quantifying specific, rapid and field deployable diagnostic tool could be useful to study each pathogen features (e.g. analyzing the correlation between the airborne spores concentration as determined by the LAMP assay and the environmental parameters to understand and study the occurrence of each disease together with the best management strategies to be applied for each disease control).

**Preliminary multiplexing results**

Methods

Preliminary tests to assess the capability of LAMP to work in multiplexing were made. Maintaining the final reaction volume used in singleplex LAMP reactions, several concentrations of each developed primer set and each probe were tested (data not shown) using in the same tube the following combination of LAMP assay: *D. pini* (FAM dye)/*D. septosporum* (TAMRA dye), *L. acicola* (FAM dye)/*D. septosporum* (TAMRA dye). The DNA of the two target species extracted from pure cultures previous described was inserted in each multiplex reaction at a final concentration of 2 ng $\mu L^{-1}$ that was used for specificity tests performed in singleplex. Due to the best efficiency of amplification (data not shown) the following reaction was selected. Each multiplex reaction was performed in a final volume of 25 µl containing two primer sets and two probes (*D. pini*/*D. septosporum-L.acicola*/*D. septosportum*). Each primer set included a LAMP mixture of 1.79 µl (at a final concentration of 2.8 µM for each FIP and BIP, 0.28 µM for each F3 and B3, 1 µM for Loop primer). Each fluorescent strand composing each probe was inserted in the reaction at a final concentration of 0.08 µM while the quencher strand at 0.26 µM. At this reaction mixture were added 15 µl of Isothermal Master Mix (ISO-001nd) (OptiGene Limited, Horsham, UK) and 0.37 µl water (Molecular biology grade, Fisher BioReagentsTM). For each reaction, 2.5 µl were used as template for each target species. Other LAMP reaction conditions were maintained as previous described. No template control (NTC) including water instead than DNA was added in each LAMP run. To assess the capability of the multiplex optimized LAMP reaction to work on different LAMP assay build with the same probe chemistry, a multiplex test was made using two LAMP primer sets optimized by Villarilab (Warnell School of Forestry & Natural Resources, University of Georgia, USA) targeting respectively the fungus *Raffaelea lauricola* (FAM dye) and the cytochrome oxidase gene that can be found in plant DNA (TAMRA dye). Using these primer sets and maintaining other reaction conditions, first was made a test

including DNA extracted from pure cultures of *R. lauricola* together with asymptomatic plant DNA. Then, a test including DNA of plants that were infected with the fungus was carried out.

Results

Preliminary multiplexing tests applied on *D. pini*, *D. septosporum* and *L. acicola* showed the capability of the qLAMP developed assays to amplify in the same reaction the DNA of two species at one time (*D. pini*/*D. septosporum*-*L. acicola*/*D. septosporum*). All the tested DNA extracted from pure cultures (2 ng $\mu L^{-1}$) were correctly amplified also when were both included in the same reaction as multiplexing. Lower fluorescences were registered in amplification curves for probes marked with the TAMRA dye (2.500 F for *D. septosporum*) compared to that marked with the FAM one (40.000 F for *D. pini* and *L. acicola*). A delay was registered in amplification time when both DNA were included in the same reaction ($t_{amp}$=10min in singleplex, $t_{amp}$=13min in multiplex). The efficiency of the multiplex improvement was also assessed by the positive results obtained when using the same reaction with different LAMP assays working with the same probe chemistry that respectively target the fungus *R. lauricola* (FAM dye) and the cytochrome oxidase gene belonging to plant DNA (TAMRA dye). The optimized reaction used with these different primer sets, resulted capable of amplifying each target when were inserted two different DNA in the same tube (respectively from pure culture and from asymptomatic plant) but also when dealing with DNA extracted from infected plants, showing the same trend of fluorescence and amplification time observed for *D. pini*, *D. septosporum* and *L. acicola* multiplex assay.

Conclusions

Even if the development and optimization of multiplex LAMP reactions are only at the first steps of research needing to be further investigated, described results seems to confirm the capability of LAMP to work in multiplex. The application of multiplex LAMP reaction directly in the field

could further reduce the time needed for a specific and sensitive DNA-based diagnosis also reducing costs of a single analysis.

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| Isolates | Haplotype ITS, Mating type | Host | Origin | Collector | LAMP detection results | | |
|---|---|---|---|---|---|---|---|
| | | | | | ***D. pini*** **assay** | ***D. septosporum*** **assay** | ***L. acicola*** **assay** |
| *D. septosporum (KC19 Needle 1 Taiga 504)* | geno1b; MAT_1_2 | *Pinus contorta var. latifolia* | Canada; BC | UBC_HamelinLab | - | + | - |
| *D. septosporum (CLG 22 Taiga 601)* | geno3; MAT_1_1_1 | *Pinus contorta var. latifolia* | Canada; BC | UBC_HamelinLab | - | + | - |
| *D. septosporum (PG TIS P3 P16 Needle 2 Taiga 460)* | geno2, MAT_1_1_1 | *Pinus contorta var. latifolia* | Canada; BC | UBC_HamelinLab | - | + | - |
| *D. septosporum (WC27 Needle 1 Taiga 626)* | geno3; MAT_1_2 | *Pinus contorta var. latifolia* | Canada; BC | UBC_HamelinLab | - | + | - |
| *D. septosporum (FLNRO 2 19M Needle1 Taiga 486)* | geno1a; MAT_1_1_1 | *Pinus contorta var. latifolia* or *Pinus banksiana* or hybrids of both | Canada; AB | UBC_HamelinLab | - | + | - |
| *D. septosporum (SM 1-4 Needle1 Taiga 484)* | geno1b MAT_1_2 | *Pinus contorta var. latifolia* | Canada; BC | UBC_HamelinLab | - | + | - |
| *D. septosporum (DS 3212)* | MAT1-2 | *Pinus sylvestris* | Estonia, Võru County, Sakurgi | R. Drenkhan | - | + | - |
| *D. septosporum (Ds 57)* | - | *Pinus contorta* | Estonia, Pärnu County, Kaansoo | R. Drenkhan | - | + | - |
| *D. pini (CMW 10951; CBS116487)* | 1 | *Pinus radiata* | Adams G | Stanton, Michigan, USA | + | - | - |
| *D. pini (CMW 37634)* | 1 | *Pinus cembra* | Walla J | Cass County, ND-Hort. Arboretum, Noth Dakota, USA | + | - | - |
| *D. pini (CMW 37786)* | 1 | *Pinus nigra* | Walla J | Indiana, USA | + | - | - |
| *D. pini (CMW 38037)* | 1 | *Pinus ponderosa* | Walla J | Brookings, South Dakota; USA | + | - | - |
| *D. pini (CMW 42947)* | 2 | *Pinus nigra subsp. pallasiana* | Kateryna Davydenko | Tsurupynsk; Kherson; Ukraine; Europe | + | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *D. pini (CMW 43903)* | 2 | *Pinus nigra subsp. Laricio* | Barnes I | 41306 La Ferte Imbault; france; Europe | + | - | - |
| *D. pini (CMW 29366)* | 2 | *Pinus pallasiana D. Don* | Timur SB | Tarasovsky district; Gorodishchensky timber enterprise, Gorodishchenskoye forestry; Russia | + | - | - |
| *D. pini (CMW 37633)* | 3 | *Pinus ponderosa* | Walla J | Cass County, ND-Hort. Arboretum; North Dakota, USA | + | - | - |
| *D. pini (CMW 41496)* | 4 | *Pinus nigra* | Barnes I | France, Europe | + | - | - |
| *D. pini (CMW 50237)* | 6 | *Pinus sp.* | Mullett MS | Little Rock; Arkansas; USA | + | - | - |
| *D. pini (A10)* | - | *P. nigra* | Susa McGowan | Ontario, Canada | + | - | - |
| *D. pini (A11)* | - | *P. nigra* | Susa McGowan | Ontario, Canada | + | - | - |
| *D. pini (A12)* | - | *P. nigra* | Susa McGowan | Ontario, Canada | + | - | - |
| *D. pini (A13)* | - | *P. nigra* | Susa McGowan | Ontario, Canada | + | - | - |
| *D. pini (A14)* | - | *P. nigra* | Susa McGowan | Ontario, Canada | + | - | - |
| *D. pini (A20)* | - | *P. nigra* | Susa McGowan | Ontario, Canada | + | - | - |
| *L. acicola (8496)* | MAT1-1 | *Pinus sylvestris* | Estonia, Tartu County, Kõrveküla | R. Drenkhan | - | - | + |
| *L. acicola (B1599)* | MAT1-2 | *Pinus radiata* | France, Europe | Emmanuel Kersaudy, Renaud Ioos | - | - | + |
| *L. acicola (B1569)* | MAT1-1-1 | *Pinus radiata* | France, Europe | Emmanuel Kersaudy, Renaud Ioos | - | - | + |
| *L. acicola* (La 9) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.1) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *L. acicola* (La 9.3) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.4) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.5) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.8) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.9) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.10) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.11) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.12) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.13) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.15) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.16) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.18) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 9.19) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 10) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *L. acicola* (La 10.1) | - | *P. palustris* | Villarilab | Florida, USA | - | - | + |
| *Leptographium profanum (CV-2017-0072)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
| *Leptographium procerum (CV-2017-311)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
| *Leptographium sp. (CV-2017-0049)* | - | *P. taeda* | Barnes/Gandhi Lab | Georgia, USA | - | - | - |
| *Rhizosphaera sp. (CV-2018-024)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
| *Cladosporium sp. (CV-2018-023)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
| *Alternaria tenuissima (CV-2018-022)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
| *Dothideomycetes sp. (CV-2018-020)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |

| *Leotiomycetes sp. (CV-2018-019)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
|---|---|---|---|---|---|---|---|
| *Nigrospora oryzae (CV-2018-018)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
| *Lophodermium conigeum (CV-2018-002)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |
| *Lophodermium australe (CV-2018-001)* | - | *P. taeda* | Villarilab | Georgia, USA | - | - | - |

Table 1: List of fungal isolates used in this study and specifications

| **Code** | **Plant species** | **Symptomatic (S)/Asymptomatic (A)** | **Origin** | **LAMP detection results** | | | **Isolations** |
|---|---|---|---|---|---|---|---|
| | | | | ***D. pini* assay** | ***D. septosporum* assay** | ***L. acicola* assay** | |
| P20 | *Pinus palustris* | S | Florida, USA | - | - | + | + |
| P12 | *Pinus palustris* | S | Florida, USA | - | - | + | + |
| P19 | *Pinus palustris* | S | Florida, USA | - | - | + | + |
| 1 | *Pinus nigra* var. *laricio* | S | La Sila massif, Calabria, Italy | - | + | - | + |
| 2 | *Pinus radiata* | S | La Sila massif, Calabria, Italy | - | + | - | - |
| 3 | *Pinus nigra* var. *laricio* | S | La Sila massif, Calabria, Italy | - | + | - | + |
| XXX | *Pino mugo* | S | Gardone, BS, Italy | - | - | - | - |
| VAL SAR 3C | *Pinus cembra* | S | Val Sarentino, Bolzano, Italy | - | + | - | + |
| VAL SAR 3Cinc | *Pinus cembra* | A | Val Sarentino, Bolzano, Italy | - | + | - | - |
| H | *Pinus taeda* | A | Athens, Georgia, USA | - | - | - | - |
| VAL SAR 1B | *Pinus mugo* | S | Val Sarentino, Bolzano, Italy | - | - | - | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| VAL SAR 1Binc | *Pinus cembra* | A | Val Sarentino, Bolzano, Italy | - | - | - | - |

Table 2: Pine needles sample tested with LAMP and detection results.

| **Code** | **Sequence 5'→3'** | **Reference** |
|---|---|---|
| LAMP primers-*Dothistroma pini* | | |
| Dp_F3 | GTTGGGATGTATGTGGTGTTA | This study |
| Dp_B3 | CTCCATCGACATCTCCAAGA | |
| Dp_FIP | GAAGTAAACATTCAACCGCTCGCACTCGTGAAGAAAGCTTGTG | |
| Dp_BIP | CGAGGTACGGACTTCACTTCACAGTAAAGTGATGCTGTGCTG | |
| LoopF | CCTCGTATCTGCGAGTCTTC | |
| LAMP primers-*Dothistroma septosporum* | | |
| Ds_F3 | TTTCTGGCAGACCATTTCTG | This study |
| Ds_B3 | ACGGCTCTTTCAAATGACTT | |
| Ds_FIP | GTGCCTTCGTATCTGCATTTCATCCAGGACAGTATGTGGAATCC | |
| Ds_BIP | CGAGAGCGACTGAGTGTCTATTTCGCATAGTGTTGAAGCACTGG | |
| LoopB | GATGAGGTAGGTGCTCCTCT | |
| LAMP primers-*Lecanosticta acicola* | | |
| La_F3 | GTACGCATGGGTCCTCGA | This study |
| La_B3 | GAAATCACGGTGACCAGGAG | |
| La_FIP | CGTACAGTTACGTAATATGAGCGTGAGCGTGGTATC | |
| La_BIP | GGACTCTTCGCTGCCGCCCGATGACCTTTCACGGGTTA | |
| La_LoopB | TCGCTGTCGCAACACCC | |
| Assimilating sequence-specific probes | | |
| *L. acicola* fluorescent strand (La_LFPr)[4] | FAM[1]-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAGGCGTTTCAAACTTCCACAGAG | This study |
| *D. pini* fluorescent strand (DP_LBPr)[4] | FAM[1]-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATTCCAGTGTGCTATGGCAAT | |
| *D. septosporum* fluorescent strand (DS_LFPr)[4] | TAMRA[2]-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAAGTACGAATCTGCATGACGC | |
| Quencher strand | TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT-BHQ[3] | Kubota *et al.,* 2011 |

Table 3: List of LAMP primers and probes optimized in this study

[1]FAM=6-carboxyfluorescein

[2]TAMRA=carboxytetramethylrhodamine

[3]BHQ= Black HoleQuencher-1 (BiosearchTechnologies, Novato, Cal.)
[4] Fluorescent strand of assimilating probe, the underlined fragment act as a Loop primer.

# PAPER IV

Management of Biological Invasions manuscript MBI19-064-ARTEMIS
Short Communication

## Molecular detection of *Dothistroma* Needle Blight in protected pine forests in Italy

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**Abstract**
Widespread and locally severe foliar symptoms resembling Dothistroma Needle Blight (DNB), one of the most important emerging infectious diseases of forest trees worldwide, were recently observed in La Sila Massif, a mountain plateau covered with native forests of Corsican pine in La Sila National Park, Southern Italy. At the same time, DNB symptoms were observed in arolla pine and dwarf mountain pine forests in the Paneveggio Nature Park and in Val Sarentino, Northeastern Italy. Defoliation was extensive at all sites and severe on the majority of plants of affected species, both adult trees and renovation. In particular on arolla pine, the disease was so serious as to locally threaten the species' reproduction and survival, an unusually heavy damage on this host. Species-specific real time PCR diagnostics, based on sequence variation at the beta-tubulin 2 (β-tub2) and translation elongation factor I alpha (EF1-a) genes and recommended by EPPO, was applied to needle samples from these sites and the presence of *Dothistroma septosporum* was ascertained, while *Dothistroma pini*, the morphologically identical congeneric species causing the same disease, was not detected. In addition, a culture independent survey based on the same molecular assays was carried out in other areas of Northern, Central and Southern Italy, where pines of various species were affected by similar symptoms, and gave negative results for both *Dothistroma* species. Results show that *D. septosporum* currently has in Italy a much larger distribution and host range than reported and is associated to life-threatening damage to native pine species growing in established populations for *in situ* conservation of genetic resources, which would require an update of specific conservation actions.

**Keywords**
Emerging diseases, pest surveillance, *Pinus nigra laricio*, *Pinus cembra*, *Pinus mugo*, red band needle blight, TaqMan diagnostics

## Introduction

*Dothistroma* needle blight (DNB) is one of the most important emerging infectious diseases of forest trees in the family Pinaceae. The disease is caused by two morphologically indistinguishable Ascomycete species recently separated using DNA sequence differences (Barnes et al. 2016, 2004; Ioos et al. 2010): *Dothistroma septosporum* (Dorogin) M. Morelet and *Dothistroma pini* Hulbary (Barnes, Crous, Wingfield, & Wingfield, 2004). Both species have been regulated as quarantine organisms in Europe under the collective name *Scirrhia pini* Funk and Parker (EU, 2000). The pathogens generally colonize older needles causing premature defoliation that results in growth reduction, and mortality after repeated attacks especially in young plants. Infected needles develop yellow to red colored spots and bands where dark conidiomata form. The most susceptible species are in genus Pinus, but more than 100 species in the family Pinaceae are hosts with varying susceptibility (Watt et al. 2009, Drenkhan et al. 2016, Mullett et al. 2018).

*D. septosporum* has become infamous as an invasive species mostly on Pinus plantations in the Southern Hemisphere, but both species recently caused unexpected epidemics on pines in the Northern Hemisphere (Mullett et al. 2018, Drenkhan et al. 2016). According to current niche models, the potential geographic range of DNB pathogens in Europe is larger than their current known distribution (Möykkynen et al. 2017), which includes several countries where the pathogens have few occurrences or a restricted distribution (Mullett et al. 2018, EPPO, 2019). In Italy the only published report of DNB dates back to 1977 on nursery plants of the non-native species *Pinus radiata* D. Don at San Pietro di Caridà, in the Aspromonte Massif, Region Calabria (Magnani 1977). The only other mention of DNB fungi in Italy is in the inventory of forest pests and pathogens of Region Friuli Venezia Giulia, which reports the detection of *Mycosphaerella pini* Rostr. Ex Munk (the former name for *D. septosporum*) on *Pinus nigra* J.F. Arnold and *Pinus sylvestris* L. at a single location in Moggio Udinese (Udine, Italy) (Bernardinelli 2016). To our

knowledge, these reports have not been confirmed through specific molecular diagnostics and the actual species involved remain, at least for the report in Southern Italy, uncertain. To date, in the scientific literature as in the global database of pest-specific information maintained by the Secretariat of the European and Mediterranean Plant Protection Organization (EPPO) there are no other reports of *Dothistroma* species in Italy than the one by Magnani (1977).

In 2017 widespread and locally severe foliar symptoms resembling DNB (orangey-red brown distal needle ends, dark red bands, and green bases, with or without black fruiting bodies within the band) were observed in La Sila Massif, a mountain plateau at about 12 hundred meters elevation, covered with forests of the native Corsican Pine (*Pinus nigra* subsp. *laricio* (Poir.) Maire) in La Sila National Park, a protected area for biodiversity conservation in the southernmost continental Italy, about 150 kilometers north-west of the site of first report of *Dothistroma* (Magnani 1977). In 2017, sporadic and moderate symptoms similar to DNB were also observed on arolla pine (*Pinus cembra* L.) growing in natural forests of Paneveggio-Pale di San Martino Nature Park, Region Trentino Alto-Adige, a protected area at the opposite end of Italy, towards the border with Austria. Defoliation became more severe and widespread during 2018 on *P. cembra* and also on the dwarf mountain pine (*Pinus mugo* Turra subsp. *mugo*) growing naturally in the same area. Finally, in late summer 2018 similar symptoms accompanied by heavy defoliation were observed in Val Sarentino, Region Trentino Alto-Adige, on native *P. mugo* and *P. cembra* forests.

Here we report about molecular detection by specific real-time PCR assays of DNB fungi from native pine species in these areas. We also include the results of a culture independent survey that we performed in other areas of Italy where similar symptoms were observed but no conidiomata alike those produced by *Dothistroma* species were found on pine needles.

## Materials and Methods

### *Plant material*

Symptomatic pine needles with or without visible conidiomata from individual trees (100 samples in total, 3-10 trees per site) growing at several sites in Italy in 2017,2018 and 2019 (Table 1) were analyzed. Each sample was received individually packed in hermetically sealed bags and was kept refrigerated at 4 ± 2°C until processing.

### *DNA extraction from needles and Dothistroma detection by real-time PCR*

DNA was extracted from 5-mm-long needle pieces (about 70 mg) with red band symptoms and bearing or not conidiomata. Needle pieces were transferred into 2-ml microcentrifuge tube, frozen at -20°C and ground for 1 min at 30 Hz with two 3-mm sterile steel beads in a Retsch GmbH Retsch mixer mill MM400 (Haan, Germany). Total DNA was extracted using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Total DNA concentrations were estimated using a Thermo Scientific NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). Eluted DNA samples were kept at -20°C until analysis. Each DNA extract was tested by real-time PCR using the TaqMan probe assays for *D. septosporum* and *D. pini* from Ioos et al. (2010). Both *Dothistroma* probes were dual-labelled with FAM-TAMRA and each species-specific assay was performed separately. Prior to testing with the specific assay, amplificability of samples (as such and 1/10 dilution) was checked with the real-time 18S rDNA assay developed by Ioos et al. (2010). Real-time PCR reactions were performed with an ABI 7300 Real-Time PCR system (Applied Biosystems). The master mix contained equal concentrations of the respective forward and reverse primers, and the probe for the target pathogen or for the 18S rDNA. The reaction mixture contained 1 × DreamTaq Green Buffer (Thermo Scientific), 2 × 0.2 mM each dNTP (Thermo Scientific), 2.5 µM each of the two respective forward and reverse primers, 0.2 µM of the respective dual-labeled probe, 1 U

DreamTaq polymerase (Thermo Scientific), 1 µL of template DNA and DNase free water to a final volume of 20 µL. No-template controls were included in all reactions to verify the absence of contamination. The real-time PCR cycling conditions included an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 55 s. The cycle threshold (Ct) value for each reaction was determined using the instrument's software, with automatic setting of the threshold line above the mean baseline fluorescence level.

Conventional PCR was applied on a subsample of positive needle DNA extracts using primers DStub2-F and DStub2-R as described in Ioos et al. (2010) and recommended by EPPO (PM 7/46(3) 2015). PCRs were run on a Biometra Trio thermocycler (Analytik Jena, Jena, Germany) with the following thermal cycling conditions: 1 cycle at 95°C for 10 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, followed by a final extension cycle at 72°C for 10 min. For each sample, 2 µl of PCR amplicon was visualized after electrophoresis in 1% agarose gel (Sigma-Aldrich) in 1 × Tris-acetate-EDTA (TAE) buffer and staining with ethidium bromide (0.5 µg $mL^{-1}$). DNA fragments were purified from the agarose gel by NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sent for Sanger sequencing to StarSEQ® GmbH (Mainz, Germany). Nucleotide sequences were visualized using CHROMAS LITE v. 2.01 (Technelysium, South Brisbane, Australia) and aligned using MUSCLE (Edgar 2004) as implemented in MEGA 7 (Kumar et al., 2016). Sequences were blasted in the GenBank database (National Center for Biotechnology Information (NCBI), Bethesda, MD) to check the correspondence to the expected target.

**Results**

Real-time PCR analysis showed that all DNA extracts from La Sila Massif were amplifiable (Ct values for the 18S assay 15.4-27.1) and that *D. septosporum* had spread in this mountain plateau

in natural forests of Corsican Pine growing at elevation around 12 hundred meters (Ct values for *D. septosporum* assay 25.9-32.7). Defoliation was widespread on *P. nigra laricio* and locally heavy (complete loss of needles other than the current season's ones) in the proximity of lakes, especially the Arvo Lake (Figure 2). In La Sila Massif, besides Corsican pine, which comprised the majority of tested individuals, a few plants belonging to other Pinaceae species, i.e. *Pinus sylvestris* L., *P. radiata*, *Pseudotsuga menziesi* (Mirb.) Franco, and *Cedrus atlantica* (Endl.) Manetti ex Carrière, although not severely damaged, showed red needle bands or dots and resulted positive to *D. septosporum* by real-time PCR. However no fruiting bodies were observed on these species and isolation attempts were unsuccessful. The pathogen was not detected at altitudes below 11 hundred meters nor in the few other areas of Region Calabria, where symptoms similar to DNB were observed on trees of *P. nigra laricio* or other species (*Pinus pinea* L., *Pinus halepensis* Miller, *Abies alba* Mill. ), but fungal conidiomata were missing, including two locations some hundred kilometers southward in Aspromonte Massif (Table 1, Figure 1).

All pine needles extracts from Region Trentino Alto-Adige were amplifiable according to the internal control (Ct values for the 18S assay 15.3-23.3) and real-time PCR results showed that *D. septosporum* infection was widespread on *P. cembra* and *P. mugo* trees growing in natural forests both in the area of Colbricon (Paneveggio,Trento) and at San Martino Reinswald and Valdurna, the two symptomatic sites in Val Sarentino (Bolzano) (Ct values for *D. septosporum* assay 25.9-32.7) (Table 1, Figure 1,3). Defoliation was extensive at both sites and severe on the majority of plants of the two affected species, both adult trees and renovation. Around the Colbricon Ponds and the Valdurna Lake, and along streams in Val Sarentino, defoliation commonly reached life-threatening degrees on *P. cembra* individuals, which appeared almost leafless.

The β-tub2 gene region fragment (231 bp) amplified and sequenced from a subsample of positive needle DNA extracts following the procedure by Ioos et al. (2010) showed 100% identity to *D. septosporum* reference strain Genbank ID: KX364411.1.

The other DNB species *D. pini* was never detected by the species-specific real-time PCR assay. At all other sites in Northern, Central and Southern Italy (Table 1, Figure 1) where a culture independent survey was carried out using the same real-time PCR assays on needles displaying symptoms similar to DNB but bearing no conidiomata, all DNA samples resulted amplifiable (Ct values for the 18S assay 21.1-25.9) and negative to both *Dothistroma* species.

**Discussion**

In this work we have ascertained, by applying species-specific real time PCR diagnostics based on sequence variation at the β-tub2 gene (Ioos et al. 2010), the presence of *D. septosporum* at several sites in Northeastern and Southern Italy, while *D. pini*, the morphologically identical congeneric species causing the same disease in neighboring European countries (Piškur et al. 2013, Piou and Ioos 2014, Queloz et al. 2014, Ondrušková et al. 2018) was not detected. In Italy the only published report of DNB pathogens was about nursery plants of *P. radiata* in the Region Calabria (Magnani 1977) and dates 40 years back, when morphological diagnostics did not enable Dothistroma species discrimination (Barnes et al. 2004). The presence of *M. pini* in Region Friuli Venezia Giulia (Northeastern Italy) results from the regional forest inventory of the year 2015 (Bernardinelli 2016), but there is no specification about diagnostic methods applied. During the EU COST Action FP1102 DIAROD, specific real-time PCR assays carried out at a few locations in Northern and Central Italy, gave negative results (Dello Jacovo, 2014). To date, according to the literature cited by EPPO (2019), Dothistroma in Italy is restricted to *P. radiata* at the only reported location in Calabria.

Our results show that *D. septosporum* currently has in Italy a much larger distribution than reported, which comprises several forest sites both in the South and in the North-East of the country. These new locations lie in some of the areas predicted at the highest risk of infection by *D. septosporum* on the base of climatic suitability (Möykkynen et al. 2017), validating current

predictive models and strengthening the concern that the pathogens' actual range in Italy be still underestimated. At all infected sites, the pathogen spread to native pine species (*P. nigra laricio* in the south and *P. mugo* and *P. cembra* in the north of the country), in naturally regenerating forests as in plantations. All tree species found positive to *D. septosporum* in this work are known hosts of the pathogen (Drenkhan et al. 2016, Mullett et al. 2018). However, while *P. mugo* and *P. nigra laricio,* which were found extensively and severely damaged in this study, are classified as sensitive species, *P. cembra* has rarely been reported as a host and it is believed to suffer minor damage (Bednářová et al. 2005). We found instead that defoliation on the the majority of *P. cembra* individuals, both adult and young trees, was so serious as to locally threaten the species' reproduction and survival. Unusually heavy damage on this host species might be due to repeated occurrence of favorable weather conditions in the study areas, to occurrence of locally conducive climatic conditions because of small-scale changes in topography, to intra-specific variation in host susceptibility (Dvorak et al. 2012, Perry et al. 2016, Woods et al. 2016) or to coinfection by multiple pathogens (Johnson and Hoverman 2012).

At some locations, symptoms resembling those caused by *Dothistroma* infection were observed but conidiomata were not found and real time PCR did not confirm the presence of the fungus. It is known that sap-sucker insect species, such as for instance *Haematoloma dorsatum* (Ahrens), may produce patterns of red discoloration on pine needles similar to initial stages of DNB (Covassi et al. 1989, Sallé and Battisti 2016), which makes it necessary to apply molecular assays for fast, easy and reliable diagnosis of early stages of *Dothistroma* infection. Surveillance would much benefit from availability of specific and rapid molecular tools for *in-situ* detection of *Dothistroma* species, such as a Loop Mediated Isothermal Amplification (LAMP) assay, which we recently developed and are currently optimizing (Aglietti et al. 2019).

Infected forests by *D. septosporum* identified in this study are located within special areas of conservation (La Sila National Park and Paneveggio-Pale di San Martino Nature Park), and

comprise protected sites under Natura2000 Habitats directive (Pal. 42.65 Calabrian laricio pine forests, Pal. 42.32 Eastern Alpine calcicolous larch and arolla forests). They also include tree populations nationally designated for *in situ* conservation of forest genetic resources (Genetic Conservation Units GCUs) for *P. cembra* (GCU ITA00193, ITA00201) and *P. nigra laricio* (GCU ITA00032, ITA00034, 00151, ITA00156), which are conserved under the the European Forest Genetic Resources Programme EUFORGEN. As regards to Corsican pine, the global population of this subspecies in the *P. nigra* complex is naturally separated into disjunct subpopulations, of which the most important outside the island of Corsica (France) are in La Sila Massif. In all, including small scattered populations in Sicily and in the southern Apennines, there are only 7-10 locations, where the specie's genetic diversity is preserved (IUCN 2019). The presence of *D. septosporum* at these sites is impairing, if not compromising, conservation measures for both of these species in Italy and would make it appropriate to revise their conservation status and requirements for conservation action.

**Acknowledgments**
The Authors wish to thank Lorenzo Bonosi and Giuliano Menguzzato for guidance during surveillance campaigns in Trentino Alto Adige and in Calabria, respectively. Renaud Ioos and Rein Drenkhan are acknowledged for kindly providing DNA of Dothistroma species isolates to be used as a positive control in real-time PCR assays. We are indebted to Maria Teresa Ceccherini for scientific and technical advice.

**Funding Declaration** This article is a part of the conference Detection and control of alien forest species in a changing world organized by the project LIFE ARTEMIS (LIFE15 GIE/SI/000770), co funded by the LIFE programme, Ministry of Environment and Spatial planning of the Republic of Slovenia and the Municipality of Ljubljana. The article processing charges were covered by the project LIFE ARTEMIS. Part of this work was also funded by European Union's Horizon 2020 Research and Innovation Programme (grant No 771271 to LG and PC).

**Authors' Contribution**
LG, GM, CA, PC research conceptualization; sample design and methodology; data collection; lab analyses; data analysis and interpretation; MC, AG, EG lab analyses; FL research conceptualization, sample design and methodology; data collection; GM, SM sample design and methodology; LG writing original draft; All Authors writing review & editing. All authors read and approved the final manuscript

**Conflict of Interest/Declaration of Interests**
The authors declare that they have no competing interests

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**Table 1**. Geographic location of sites where symptomatic trees of various Pinaceae species where tested by means of specific real-time PCR assays (Ioos et al. 2010) for *Dothistroma pini* and *Dothistroma septosporum.*

| Samling Site | Region | Latitude | Longitude | Elevation (m asl) | Tree species sampled |
|---|---|---|---|---|---|
| Reggio Calabria | Calabria | 38.10173 | 15.63816 | 18 | *Pinus halepensis* |
| Reggio Calabria - Loisa | Calabria | 38.14299 | 15.76107 | 880 | *Pinus nigra laricio* |
| Santo Stefano in Aspromonte - Gambarie | Calabria | 38.17468 | 15.81612 | 1132 | *Pinus nigra laricio, Abies alba* |
| Squillace - Copanello | Calabria | 38.76745 | 16.56328 | 18 | *Pinus pinea* |
| Aprigliano - Lago Arvo | Calabria | 39.23694 | 16.47111 | 1460 | *Pinus nigra laricio* |
| San Giovanni in Fiore - Rovale | Calabria | 39.24250 | 16.54472 | 1310 | *Pinus nigra laricio* |
| Celico - Lago di Cecita | Calabria | 39.39243 | 16.52822 | 1160 | *Cedrus atlantica, Pinus nigra laricio, Pinus pinaster, Pinus radiata, Pseudotsuga menziesi* |
| Spezzano della Sila - Fossiata | Calabria | 39.39562 | 16.58892 | 1300 | *Pinus nigra laricio, Pinus sylvestris* |
| Cerenzia | Calabria | 39.24875 | 16.77955 | 700 | *Pinus pinea* |
| San Giovanni in Fiore - Bonolegno | Calabria | 39.26596 | 16.66053 | 1160 | *Pinus nigra laricio* |
| Cosenza | Calabria | 39.33120 | 16.23973 | 210 | *Pinus pinea* |
| Bocchigliero | Calabria | 39.36320 | 16.71917 | 1270 | *Pinus nigra laricio* |
| Acri - Moriani | Calabria | 39.459165 | 16.460938 | 1113 | *Pinus nigra laricio* |
| Longobucco - Scanciamoneta | Calabria | 39.504973 | 16.543383 | 1293 | *Pinus nigra laricio* |
| Campo di Giove | Abruzzo | 42.00349 | 14.05602 | 1070 | *Pinus nigra* |
| Reggello - Vallombrosa | Toscana | 43.73145 | 11.55415 | 990 | *Pinus strobus, Pinus murrayana, Pinus nigra laricio* |
| San Marcello Pistoiese - Maresca | Toscana | 44.049191 | 10.838300 | 850 | *Pinus mugo* |
| Gardone Riviera | Lombardia | 45.620430 | 10.562160 | 100 | *Pinus brutia, Pinus halepensis, Pinus mugo, Pinus nigra, Pinus wallichiana* |
| Torri del Benaco - Pai | Veneto | 45.650294 | 10.721644 | 110 | *Pinus brutia, Pinus pinea* |
| Lasino - Lagolo | Trentino-Alto Adige | 46.040814 | 11.008251 | 990 | *Pinus mugo, Pinus sylvestris* |
| Siror-San Martino di Castrozza | Trentino-Alto Adige | 46.26077 | 11.79903 | 1450 | *Pinus mugo* |
| Siror-Colbricon | Trentino-Alto Adige | 46.282650 | 11.766221 | 1920 | *Pinus cembra, Pinus mugo* |
| Sarentino Sarnthein - Reinswald San Martino | Trentino-Alto Adige | 46.681405 | 11.434400 | 1640 | *Pinus cembra, Pinus mugo* |
| Sarentino Sarnthein - Durnholz Valdurna | Trentino-Alto Adige | 46.740827 | 11.443171 | 1560 | *Pinus cembra* |
| Paluzza - Paluzza | Friuli Venezia Giulia | 46.524836 | 13.002392 | 560 | *Pinus mugo* |
| Forni Avoltri - Pietrabec | Friuli Venezia Giulia | 46.619224 | 12.757934 | 1610 | *Picea abies* |

**Figure 1**. Map of locations where symptomatic needles from Pinaceae species tested negative (green) or positive (red) to the specific real-time PCR assay for *Dothistroma septosporum* by Ioos et al. (2010). All of the samples tested negative for *Dothistroma pini*. Map was done using R 3.5.0 (R Core Team, 2019) and rworldmap v1.3-6 (South 2016) package.

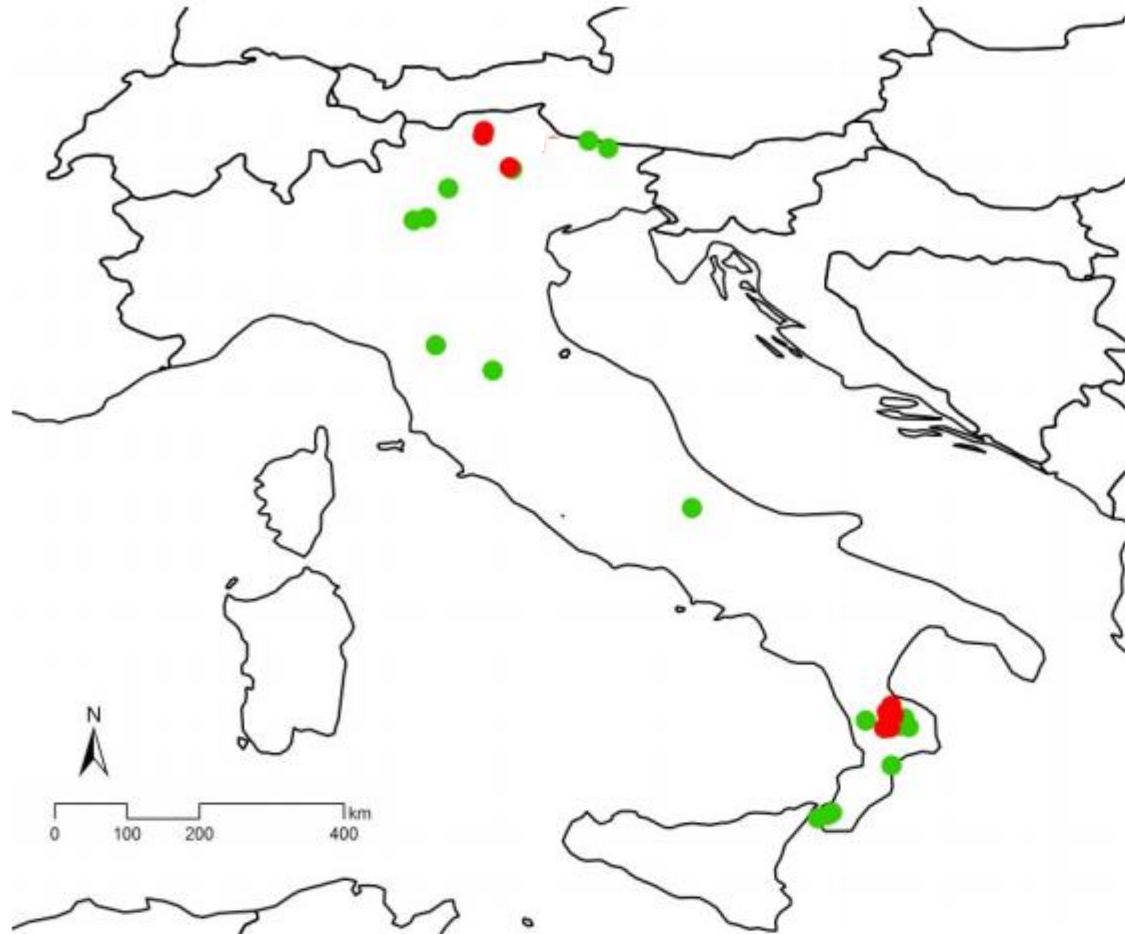

**Figure 2**. Details of symptomatic needles bearing *D. septosporum* conidiomata (top) and symptomatic young plants (bottom) of *Pinus nigra laricio* in naturally regenerating forests in San Giovanni in Fiore (La Sila National Park, Calabria, Southern Italy).

**Figure 3**. Details of symptomatic *Pinus cembra* needles bearing *D. septosporum* conidiomata (top), symptomatic young (middle) and heavily defoliated adult (bottom left) *P. cembra* plants in Val Sarentino (Alto Adige, Northern Italy), and defoliated *Pinus mugo* plants (bottom right) in Colbricon (Paneveggio Nature Park, Trentino, Northern Italy)