Ph.D in Agricultural and Environmental sciences-XXXII cycle

UNIVERSITÀ DEGLI STUDI FIRENZE
DAGRI DIPARTIMENTO DI SCIENZE E TECNOLOGIE AGRARIE, ALIMENTARI, AMBIENTALI E FORESTALI

Development of diagnostics techniques for studying quarantine plant pathogens

Ph.D. student: Chiara Aglietti

Ph.D. coordinator: Prof. Giacomo Pietramellara

Supervisor: Prof. Paolo Capretti
Co-supervisors: Dott. Alberto Santini
Dott. Nicola Luchi
Dott.ssa Luisa Ghelardini

2016/2019

Thesis collaboration

This thesis was realized thanks to the collaboration of:

- **Prof. Paolo Capretti and Dott.ssa Luisa Ghelardini (DAGRI, University of Florence)**
- **Dott. Nicola Luchi and Dott. Alberto Santini (IPSP-CNR, Sesto F.no, Firenze)**
- **Dott.ssa Caterina Villari (Warnell school of Forestry and Natural resourches, University of Georgia, Athens, GA, USA)**

A part of the work was made in her lab (stage in Athens, GA, USA for 6 months)

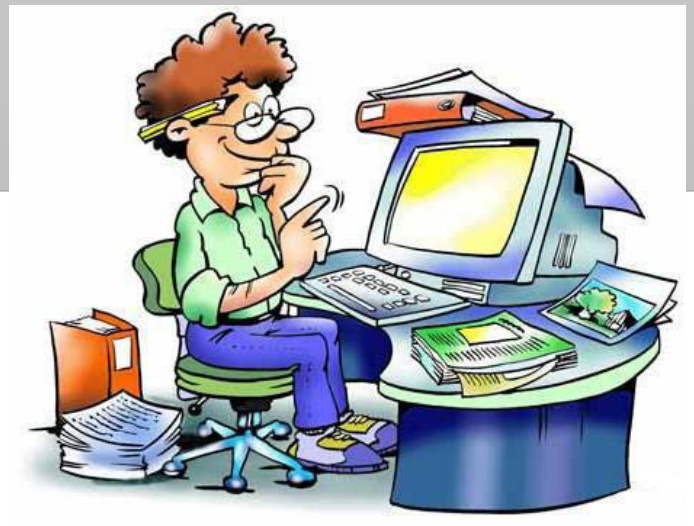
- **Prof.ssa Maria Teresa Ceccherini (DAGRI, University of Florence) who provided lab supplies to finish some parts of this work**

Thesis production

From the work carried out in this thesis:

4 manuscripts were retrieved:

- **1 published**
- **1 in press**
- **1 submitted**
- **1 writing**



General topic of this thesis

The study has dealt with the development and improvement of field-suitable early detection diagnostics tools for the control and management of invasive plant pathogens

Main attention on forest pathogens but also crop pathogens and pathogens damaging nurseries environments were included

Invasive plant pathogens: risks and threats

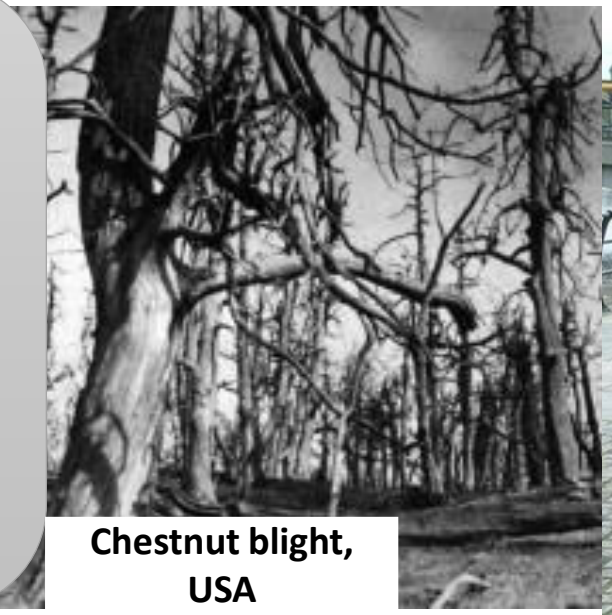
Phytosanitary emergencies caused by invasive pathogens:

Have always conditioned human history

Nineteenth century: Irish potato famine as a consequence of *Phytophthora infestans* introduction from South America

Twentieth century:

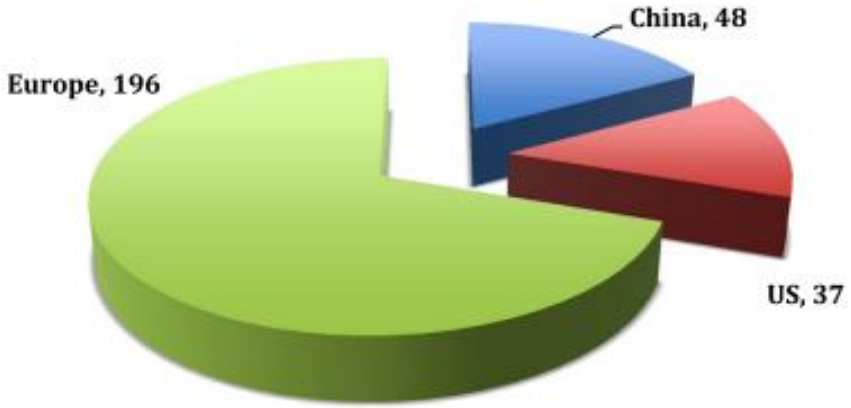
- Dutch elm disease, *Ophiostoma novo-ulmi* subsp. *americana* from North America to Europe
- Chestnut blight, *Cryphonectria parasitica* from Asian chestnut to the east coast of USA



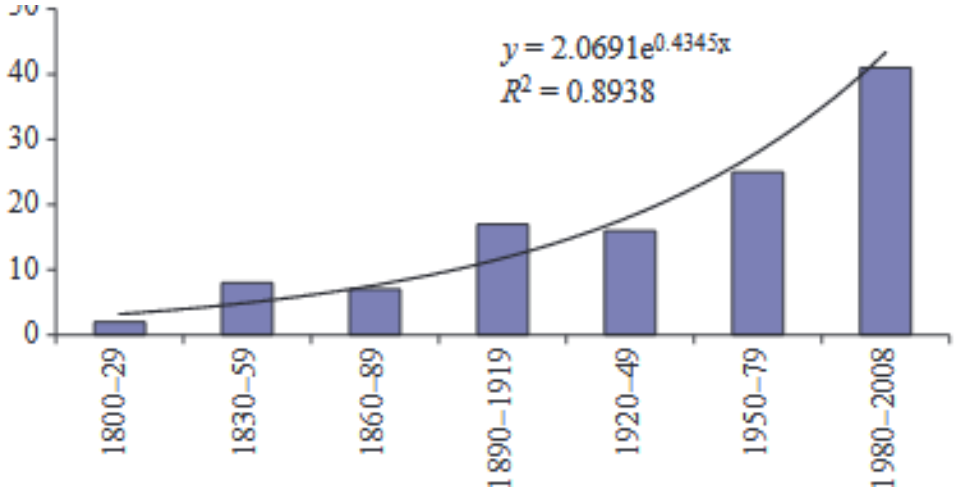
Chestnut blight, USA

Potato Famine Memorial
Dublin, Ireland

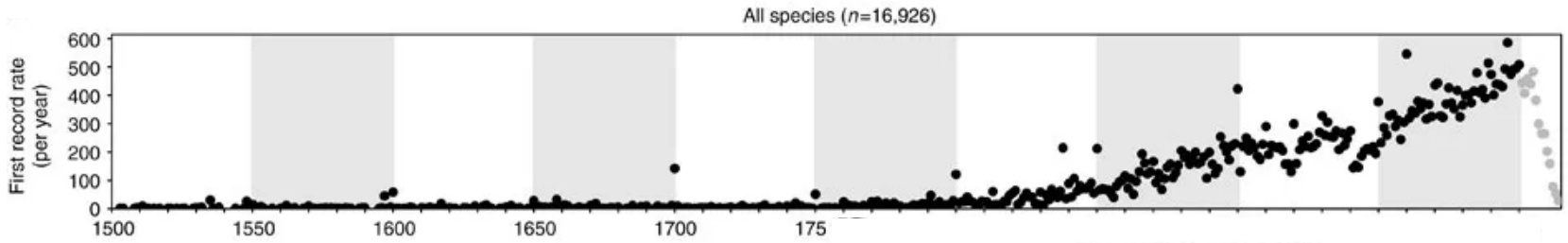
Invasive pathogens emergencies are increasing



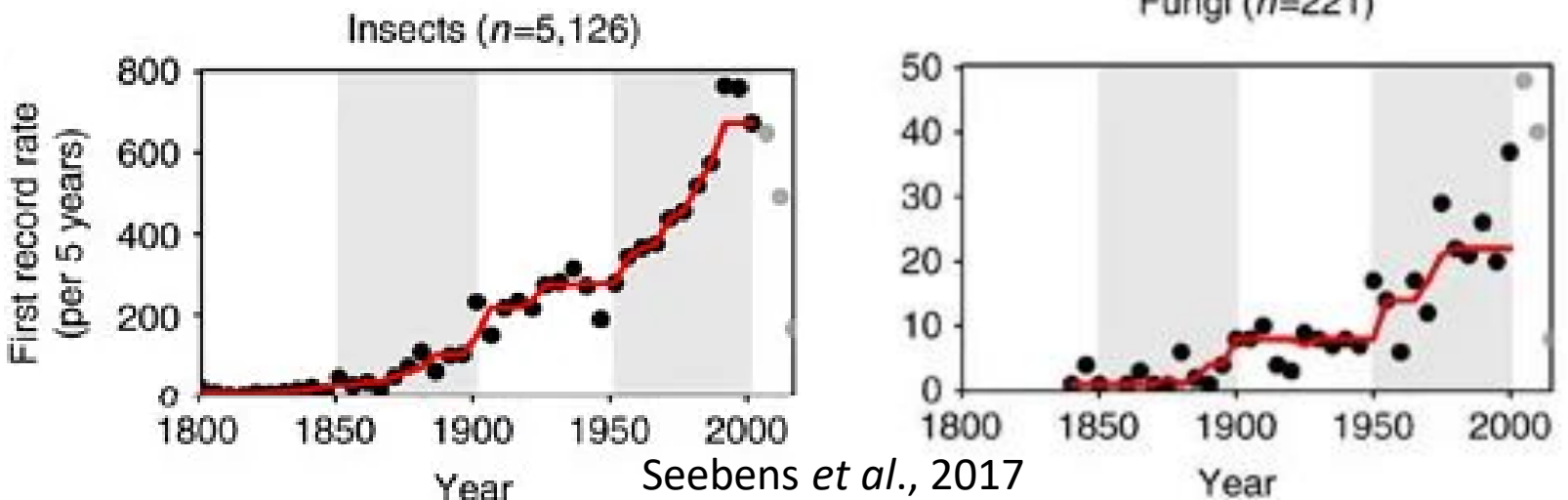
Ghelardini *et al*., 2017 **Number of forest pathogens per country**



Santini *et al*., 2013 **Total number of alien invasive forest pathogens according to time of arrival in Europe**

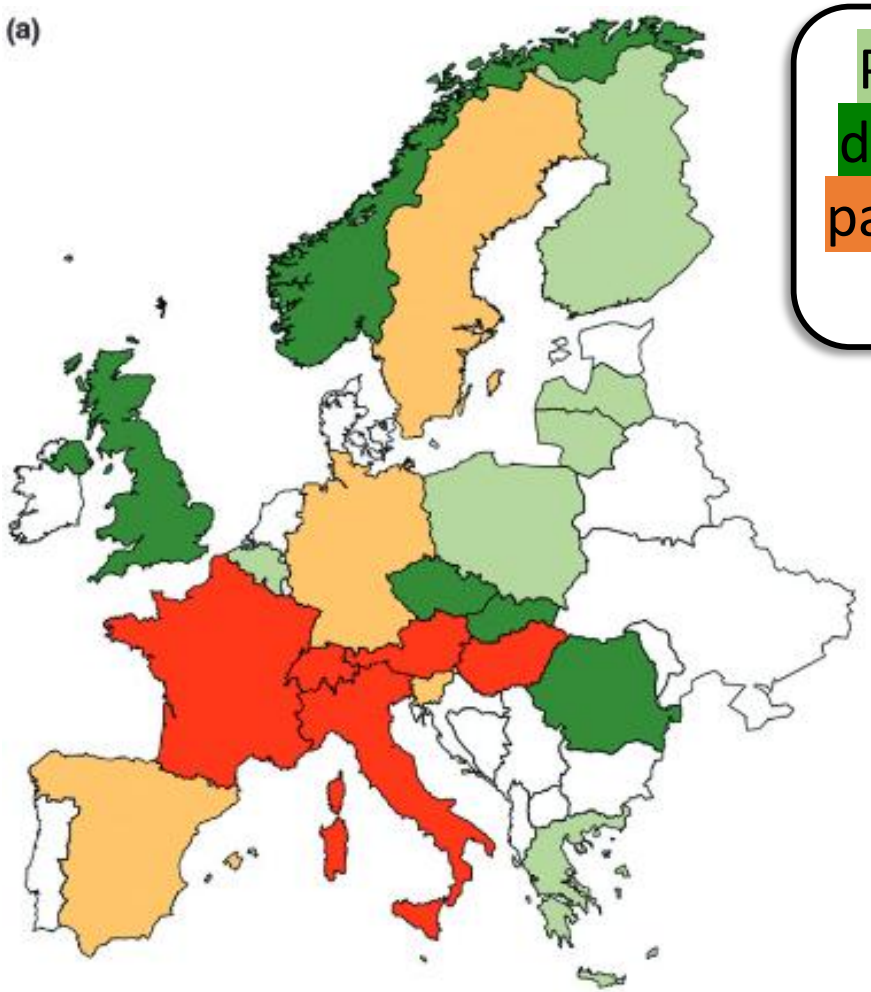


The increase in numbers of alien species does not show any sign of saturation



Seebens *et al*., 2017

According to Daisie-Delivering Alien invasive species in Europe (2018), **Italy** is one of the most damaged countries due to biological invasion with the presence of more than 1500 alien species



Pale green, 1–20;
dark green, 21–30;
pale orange, 31–40;
red,>41

Santini *et al*., 2013 **Numbers of alien invasive forest pathogens in each country**

1) Plants commercial trade and human activities

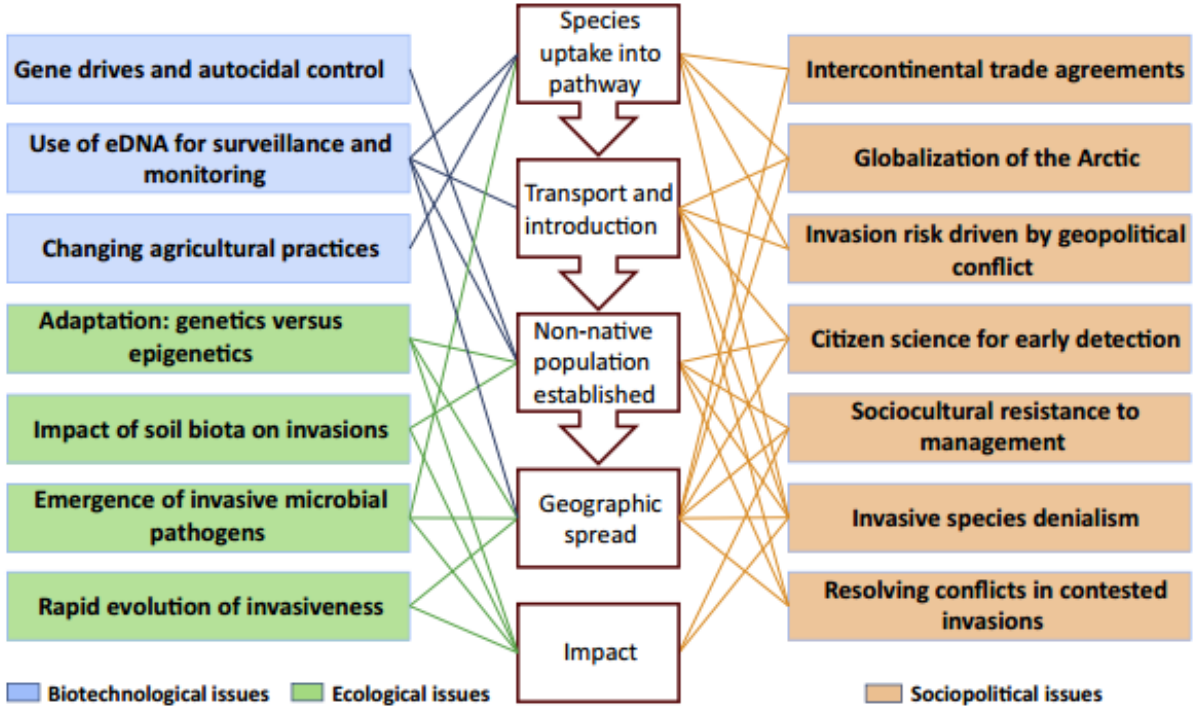
2) No co-evolution between hosts and introduced pathogen

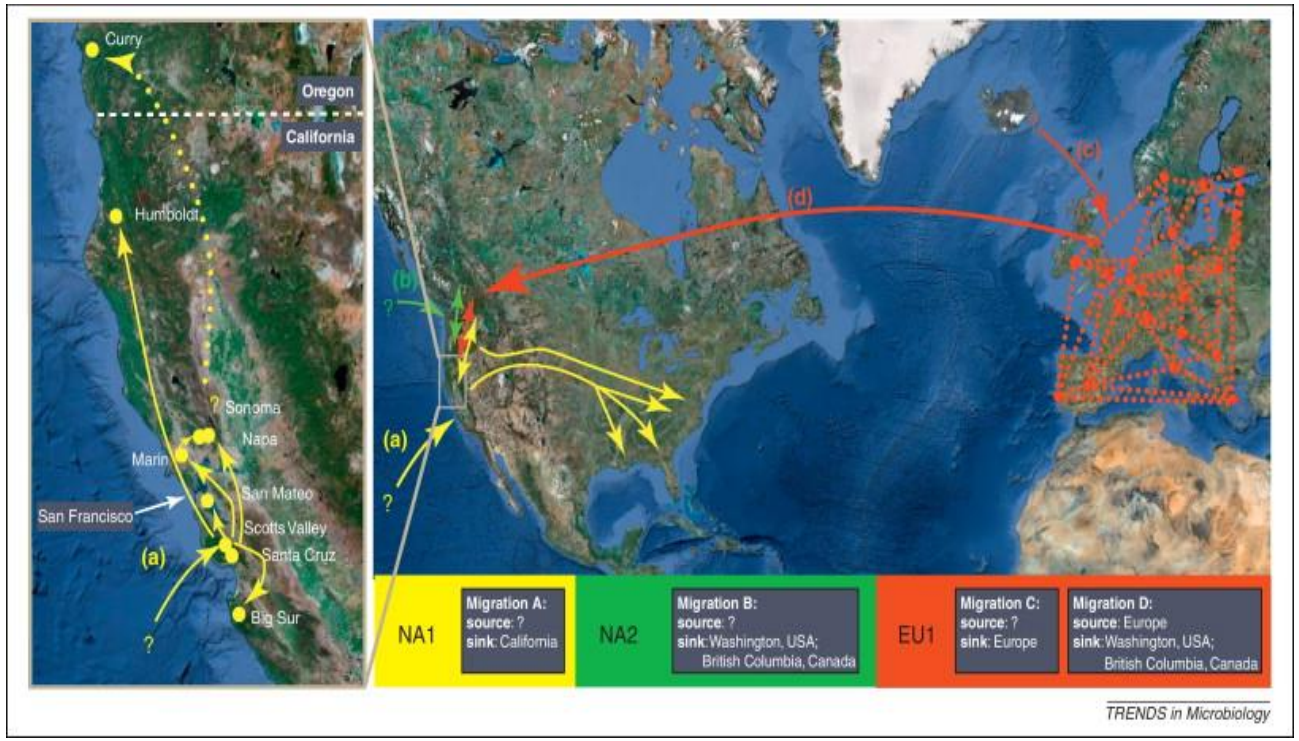
3) Fungi high genetic recombination capability

Pathogens movements

Higher hosts susceptibility in the area of introduction

- Host jumps
- Increasing in virulence





Ricciardi *et al.*, 2017

1) Plants commercial trade and human activities

2) No co-evolution between hosts and introduced pathogen

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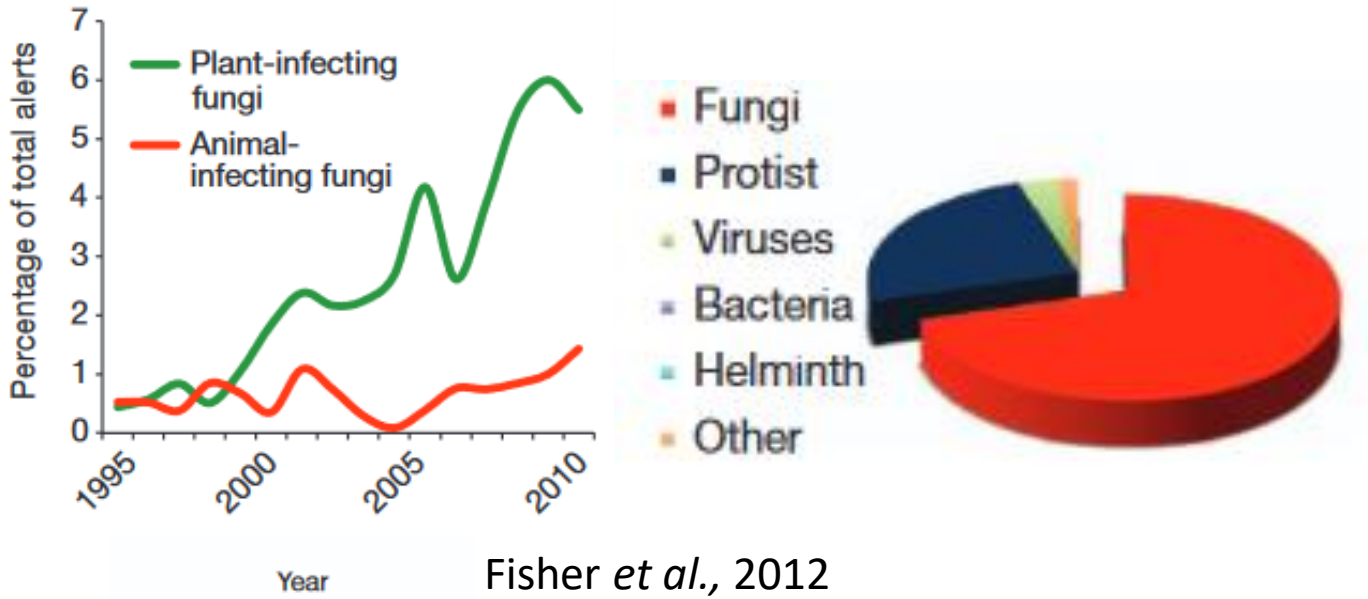
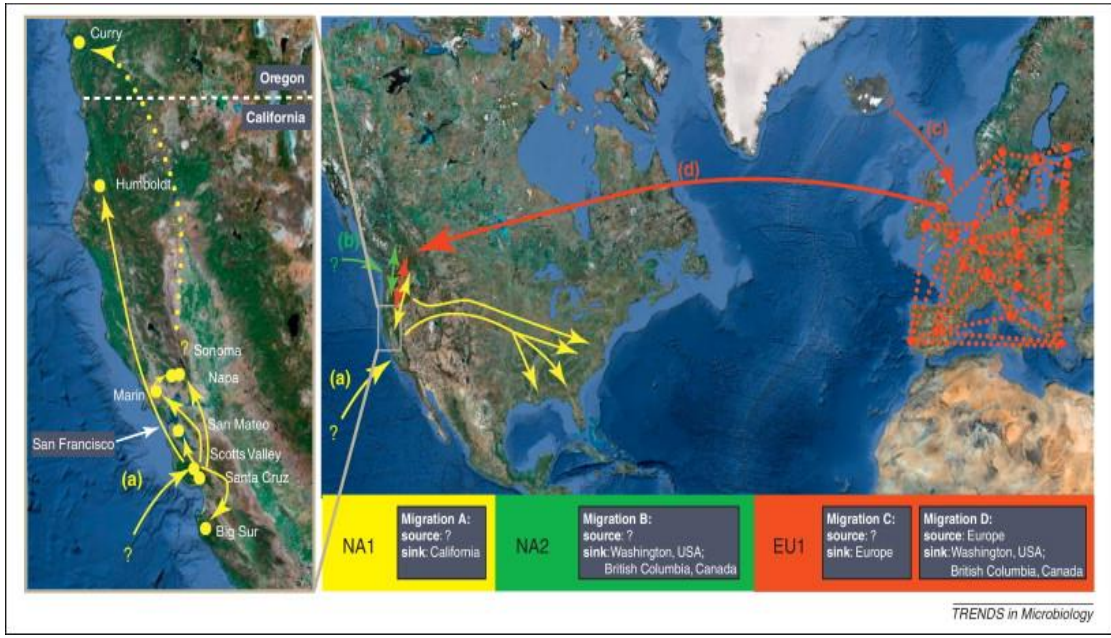
Pathogens movements

Higher hosts susceptibility in the area of introduction

- Host jumps
- Increasing in virulence

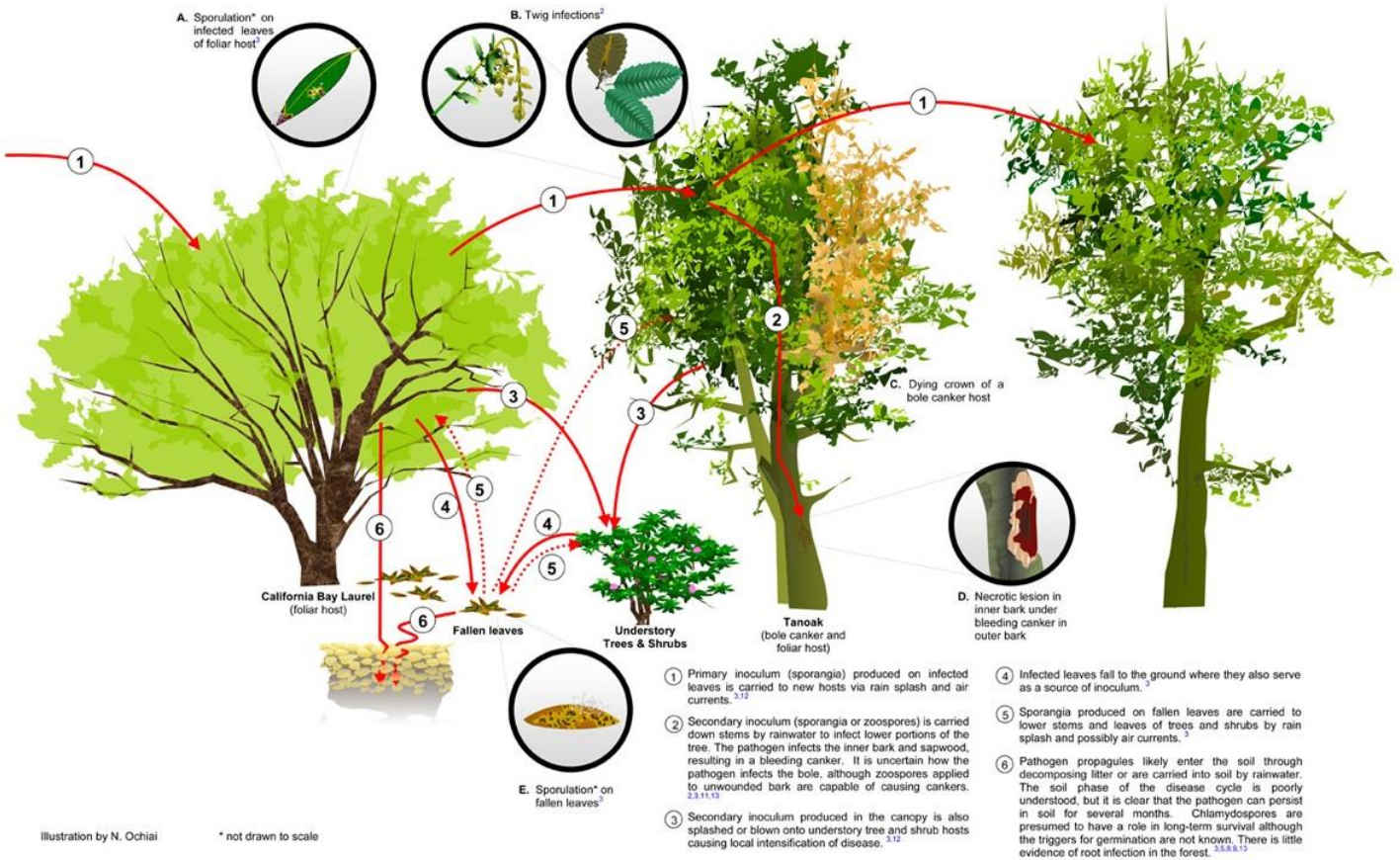


Fisher *et al.*, 2012



1) Spread in natural ecosystems

Ecosystem changes, biodiversity and ecosystem services losses



2) Economic damages

Plant nurseries, forestry, urban forestry, crops

Category	Nonindigenous species	Losses and damages	Control costs	Total
MICROBES	20,000			
Crop plant pathogens		21,000	500	21,500
Plant pathogens in lawns, gardens, golf courses		NA	2000	2000
Forest plant pathogens		2100	NA	2100
Dutch elm disease		NA	100	100

Pimentel *et al*., 2005 **Estimated annual costs associated with some alien species introduction in the United States (x millions of dollars)**

Pathogens have reduced crop productivity causing losses of at least 10% of global food production (Donoso *et al*., 2018)

It was estimated 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)

EU regulation about invasive plant pests

The EU had an open-door phytosanitary system

Any plant that is not specifically regulated was enabled to be imported
(the attention was focused on a small number of pests compared to the high number of regulated organisms)

Inspections were generally limited to visual examinations of aerial parts in few time
(incipient infections were not recognized especially in tissues/soil/roots)

From the 14th of December 2019 this regulation has changed:

All plants (including living parts of plants) need a phytosanitary certificate to enter in the EU

Lists of high risk plants (introduction provisionally prohibited)

Priority pests (selection based on high risk of spreading and establishment)

Improving management: early detection

Statement by Commissioner Andriukaitis on the entry into force of the new Plant Health Regulation

Early detection of plant pests, better action plans for eradication, higher surveillance rules for the import of high risk plants, enhanced rules for the certification of plant products are among the new provisions which will make sure that we deal in a timely and swift manner from the potentially devastating effects of some plant diseases.

These critical points claim for better diagnostic tools!!
Possibly characterized by:

High sensitivity and specificity: facilitate the application of effective control and eradication measures

Rapid, simple and portable: To be applied inspections at ports of entry, nurseries environments, in urban and or natural ecosystems

Diagnostic tools for plant pathogens identification

Observation of symptomatic plants

Laboratory analyses

Pest identification, management application

Bottleneck!

Isolations and culture on selective media

Difficulties:
- **Microorganisms difficult to isolate and to correctly identify ; - Specialized skills; -Time consuming;**

Immunoassays (LFD, ELISA…)

Difficulties:
- Low sensitivity; - Further tests required to identify the pathogen at a species level

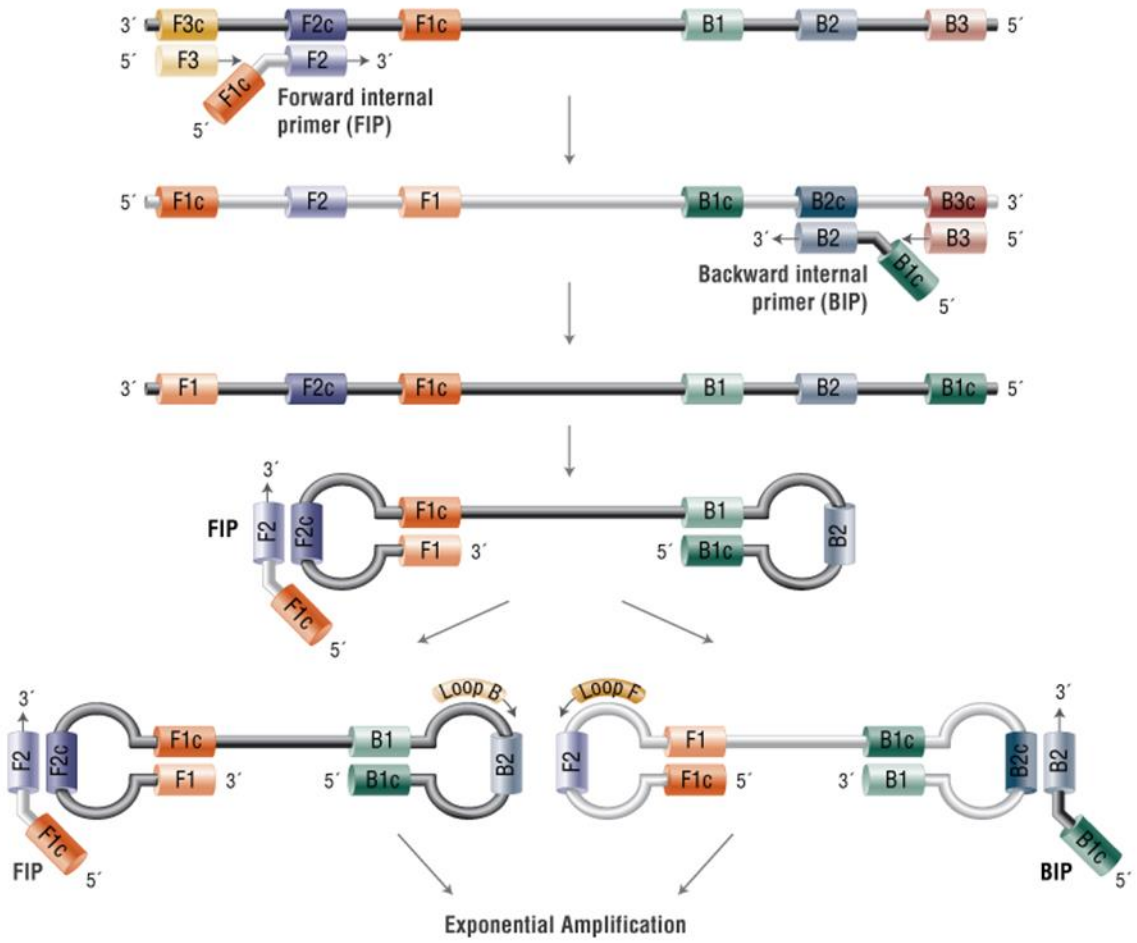
DNA-based methods (PCR…)

- The best as sensitivity and specificity; Difficulties: - They need a lab to be applied; - Specialized skills

Moving from lab into the field using DNA-based methods

- Reduce delays between results obtaining and control measures application;
- Maintaining high sensitivity, specificity and accuracy;
- Applicable in nurseries, at the borders, in forests and cities;
- For preventing, monitoring and controlling pathogens spread

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



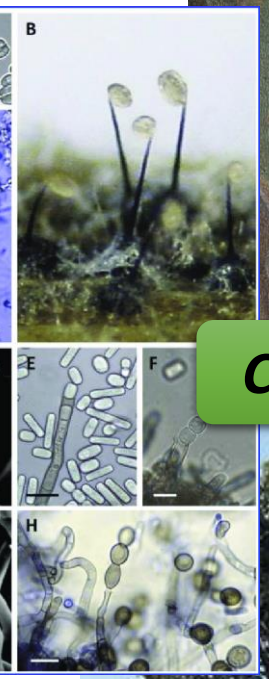
Notomi *et al*., 2000

We can use LAMP

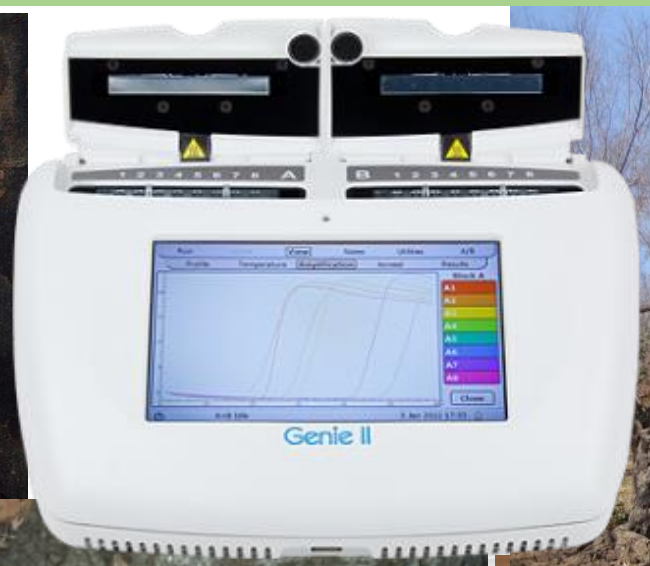
Benefits than a PCR-based method:

- **Isothermal reaction (Constant temperature): no thermocycler needed**
- **Resistant enzymes: it can works with unpurified DNA**
- **Results in 30 minutes**
- **Portable instruments**

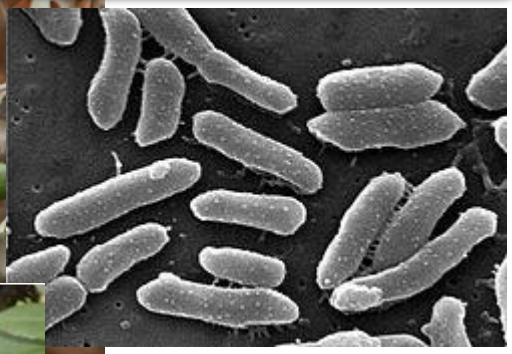
Conventional LAMP reaction optimization: the case of *Ceratocystis platani*, *Phytophthora ramorum* and *Xylella fastidiosa*

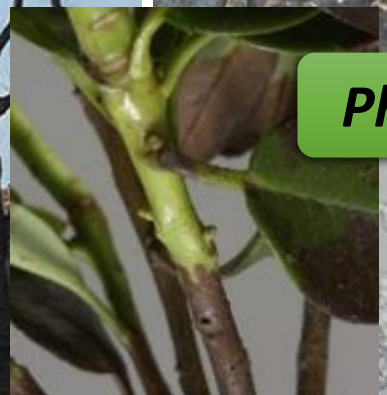


Ceratocystis platani



Xylella fastidiosa





Phytophthora ramorum

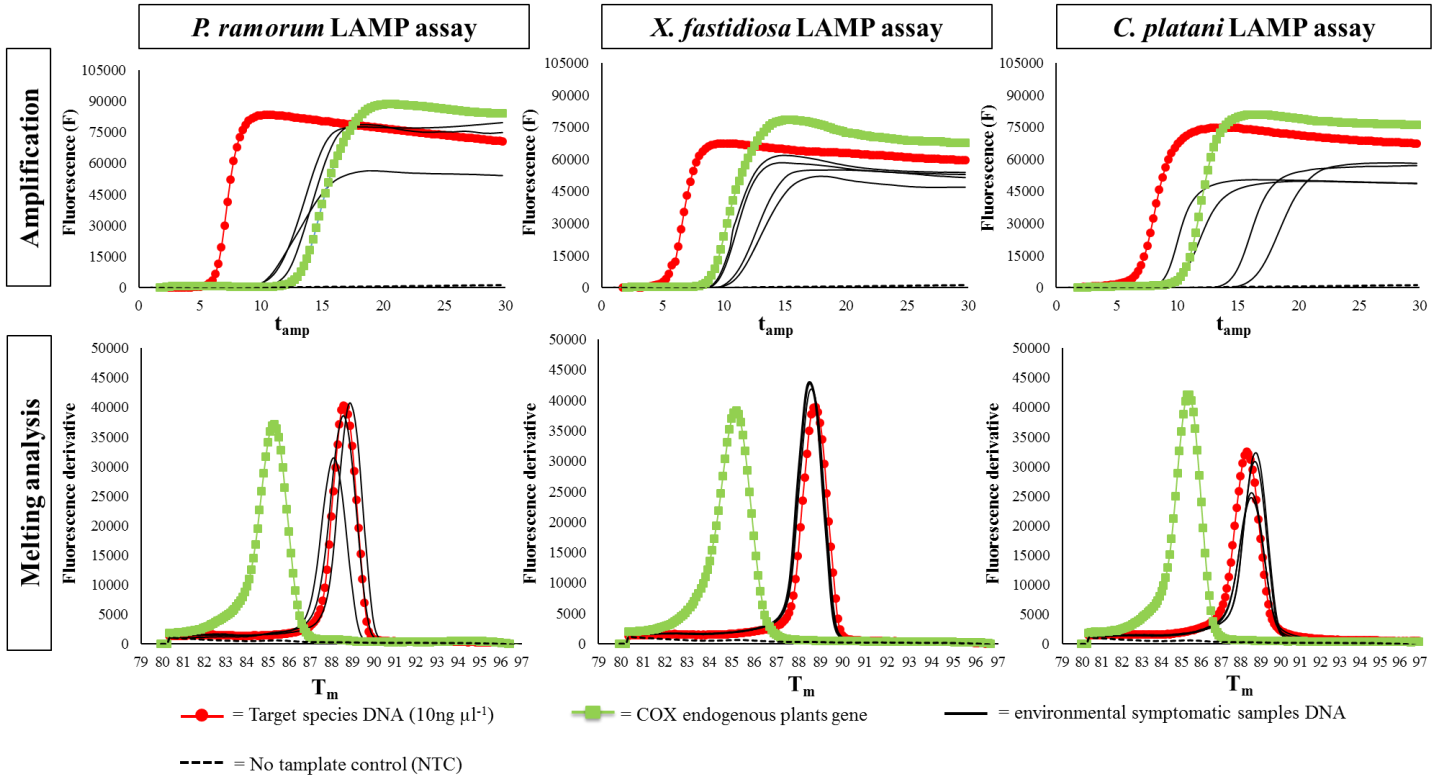
Aglietti *et al. AMB Expr* (2019) 9:50
https://doi.org/10.1186/s13568-019-0774-9

AMB Express

ORIGINAL ARTICLE Open Access

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

Chiara Aglietti[1,2], Nicola Luchi[1*], Alessia Lucia Pepori[1], Paola Bartolini[1], Francesco Pecori[1], Aida Raio[1], Paolo Capretti[2] and Alberto Santini[1]



Main methods:

- Sequences alignments and BLAST analysis
- Target DNA regions selected
- Six LAMP Primers were designed for each species (F3, B3, LoopF, LoopB, FIP, BIP) (Notomi *et al.* 2000; Nagamine *et al.* 2002)
- Sensitivity and specificity tests
- Test on DNA from infected plants

Main results:

- Specific and sensitive
- Rapid (30min) and user-friendly tools for applying diagnosis at point-of-care

Main problems:

- Conventional LAMP reaction uses large amplicons (>200bp)
- Fluorescence measured as a SYBRGREEN
- Can not distinguish between species that differs for few DNA bases (e.g.2)

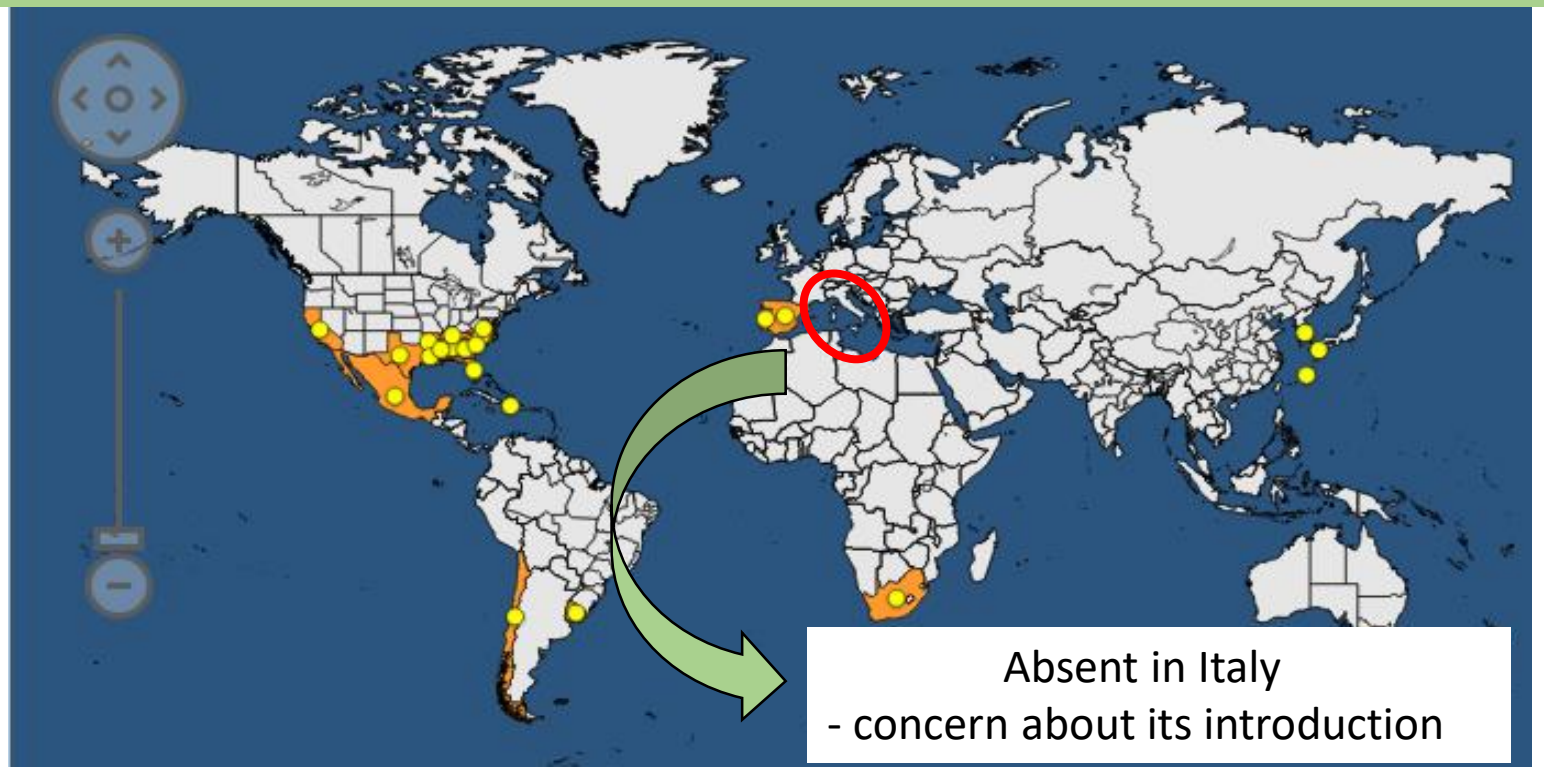
Improving the specificity of a LAMP assay: the case of *F. circinatum*

Copious resin flow

Damping off

Deformations

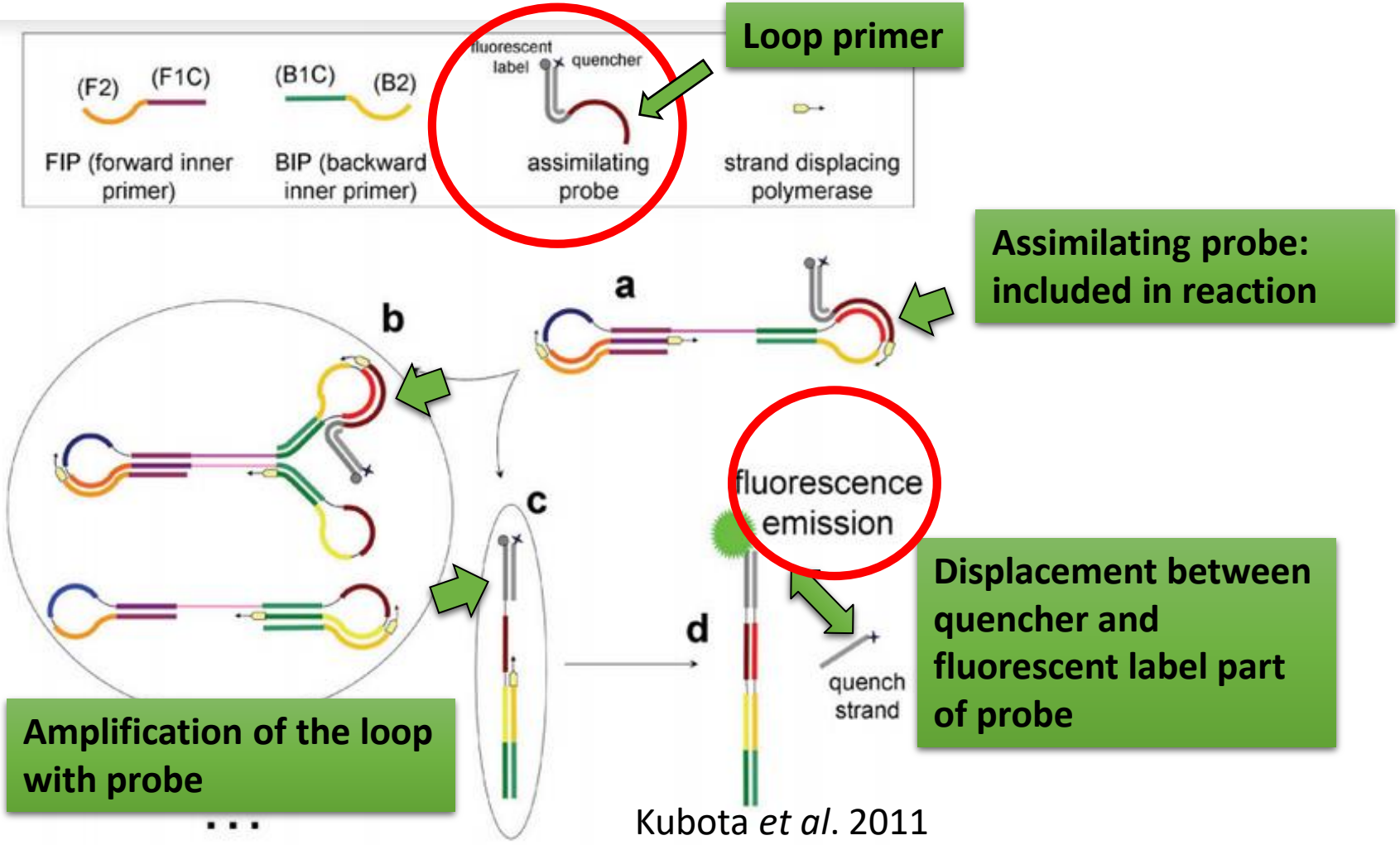
- **Serious threat to pine forests (especially *on Pinus radiata* plantations)**
- **High damages in nurseries**



Main issue for its diagnosis: specificity

- Great deal of *Fusarium* species diversity
- Differentiated one from the other with few DNA nucleotides

Solve the specificity problem using LAMP probes



Kubota *et al*. 2011

Advantages:

- Fluorescence is given only when the short sequence of the selected Loop primer is amplified
- Can distinguish between species that differ for few DNA nucleotides

BioTechniques

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

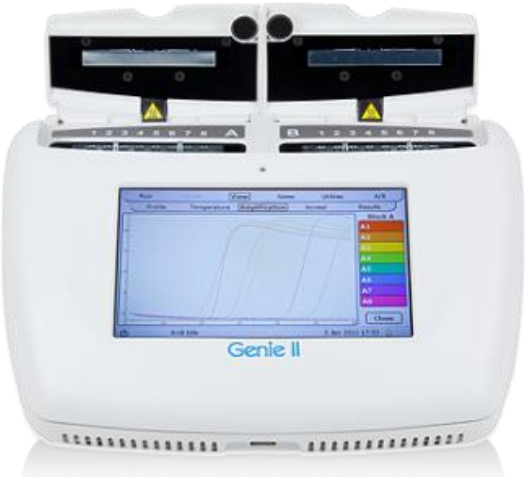
Dagmar Stehlíková[1,2], Nicola Luchi[2], Chiara Aglietti [2,3], Alessia Lucia Pepori[2], Julio Javier Diez Casero[4], Alberto Santini[2]

[1] University of South Bohemia Faculty of Agriculture - Biotechnological Centre Na Sadkach 1780 CZ-37005 Ceske Budejovice
[2] Institute for Sustainable Plant Protection - National Research Council (IPSP-CNR), Via Madonna del Piano 10, 50019, Sesto Fiorentino (Firenze), Italy
[3] Department of Agrifood production and Environmental Sciences (DISPAA), University of Florence, Piazzale delle Cascine 28, 50144, Firenze, Italy
[4] Universidad de Valladolid Escuela Técnica Superior de Ingenierías Agrarias, Campus Yutera Edificio E, despacho 204. 34071, Palencia, Spain

Corresponding author: Nicola Luchi, Institute for Sustainable Plant Protection - National Research Council (IPSP-CNR), Via Madonna del Piano 10, 50019, Sesto Fiorentino (Firenze) Italy. Email: nicola.luchi@ipsp.cnr.it

Main methods:

- Sequences alignments and BLAST analysis
- Target DNA regions selected
- Conventional LAMP design: Six LAMP Primers were designed for each species (F3, B3, LoopF, LoopB, FIP, BIP) (Notomi *et al.* 2000; Nagamine *et al.* 2002)
- Probe-based LAMP design: loop primers specificity analyzed, probe designed following Kubota *et al.*, 2011
- Sensitivity and specificity tests (with and without probe→ comparison)
- Test on DNA from infected plants



Fungal species

cLAMP qLAMP

F. circinatum	*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)		+ +
Other *Fusarium* species	*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. incarnatum-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	-		- -

Other *Fusarium* species	*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

Main results:

- Only *F. circinatum* and *F. temperatum* were amplified with the probe-based developed LAMP (qLAMP)
- With the conventional developed LAMP (cLAMP) many other species were recognized (*F. sororula*, *F. pininemorale*, *F. subglutinans*, *F. parvisorum*, *F. marasasianum*, *F. mangiferae*, *F. fujikuroi*, *F. fracticaudum*, *F. concentricum*, *F. begonia*)

F. temperatum

Application of LAMP probes for multiplexing: the case of *Dothistroma septosporum*, *D. pini* and *Lecanosticta acicola*

Lecanosticta acicola

- Similar symptoms
- Similar morphology
- Co-occurrence
- Quarantine species in many countries
- Severe foliage disease on pine needles

Dothistroma pini, D. septosporum

Screen any combination of two of the three pathogens at the same time
Direct in-field application
Rapid response to threats

PAPER III **Manuscript**

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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2 Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

UNIVERSITÀ DEGLI STUDI FIRENZE
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UNIVERSITY OF GEORGIA
Warnell School of Forestry & Natural Resources

FABI

Credits: Jeff Hamilton

Main methods:

- Development and optimization of qLAMP for *D. septosporum*, *D. pini* and *Lecanosticta acicola* as previous described
- Probes were marked with different dyes (FAM and TAMRA) to allow multiplex reaction: targeting more pathogens in the same reaction
- *Preliminary* multiplexing tests following Kubota *et al.*, 2015

Main results:

- High specific and sensitive (singleplex), also on needles samples
- Efficiency of TAMRA also on the portable instrument
- Preliminary multiplexing results: each DNA was correctly amplified and recognized when included in the same reaction
- Further tests needed for multiplexing optimization.

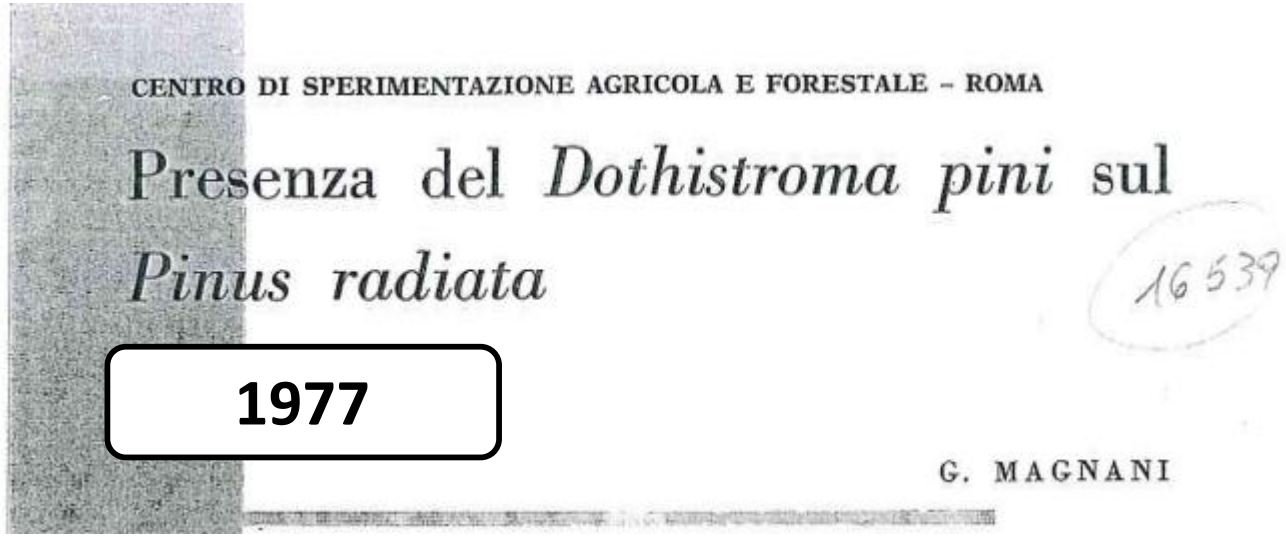
14th -15th March: Crossnore, North Carolina, USA, **Forest pathology and entomology seminar**

September 2019: **Sipav congress, Milano; LIFE ARTEMIS conference Lubiana, Slovenia**

Application of molecular tools to study plant pathogens

Assessing the presence of quarantine pathogens: the case of *Dothistroma septosporum*, *D. pini* and *Lecanosticta acicola* in Italy

The only reports in Italy were based on morphological identification (uncertain)
Models analyzing climatic conditions in Italy found the area suitable for *Dothistroma* spread



CENTRO DI SPERIMENTAZIONE AGRICOLA E FORESTALE – ROMA

Presenza del *Dothistroma pini* sul *Pinus radiata*

16539

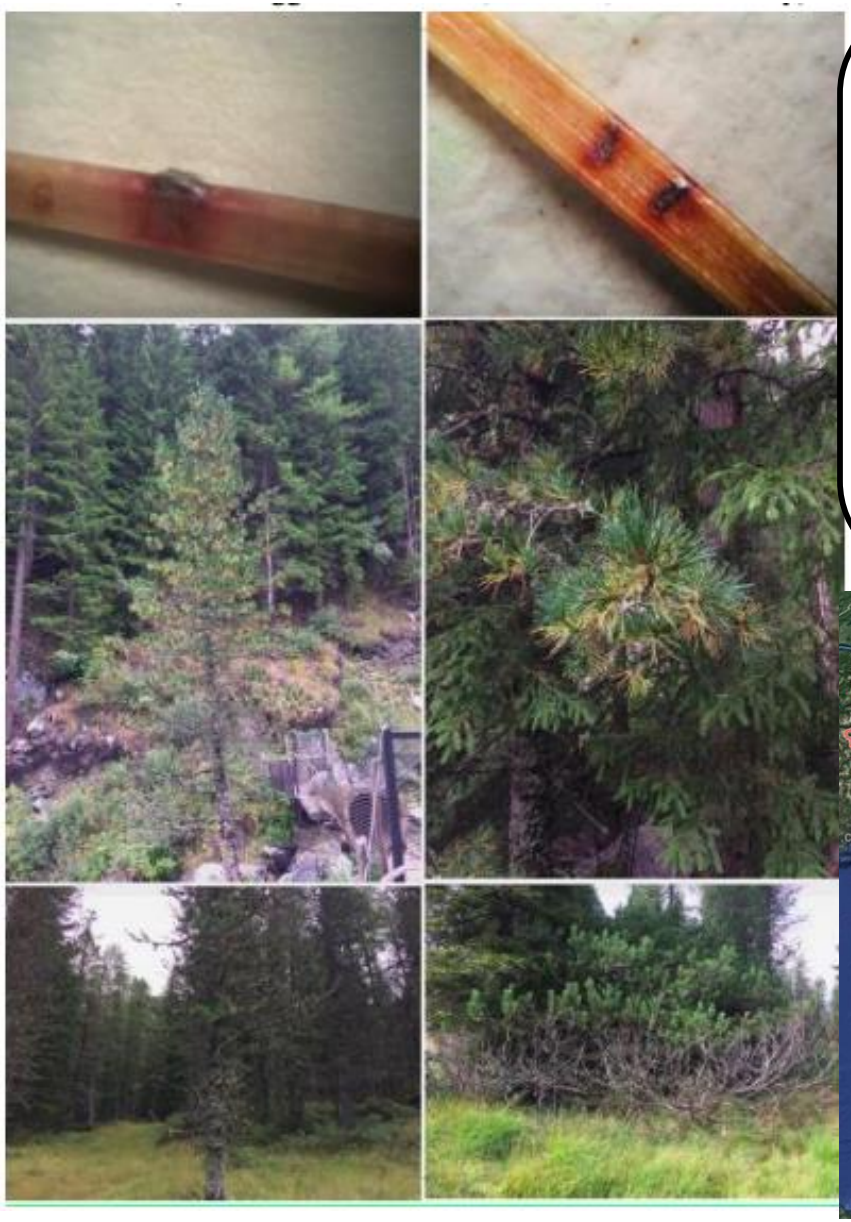
1977

G. MAGNANI

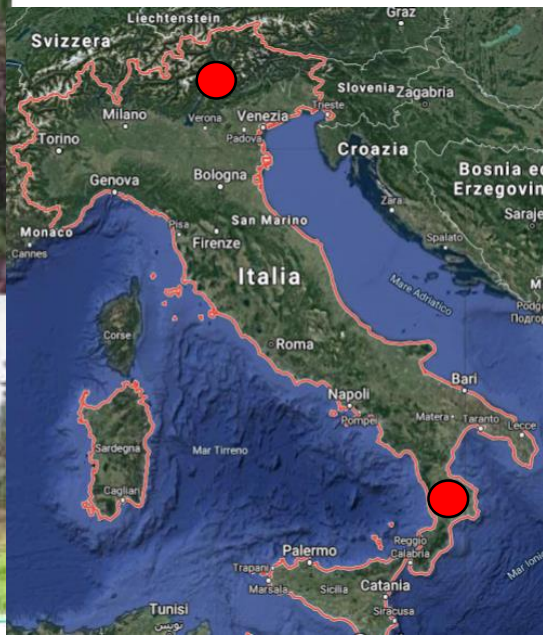
D. septosporum **distribution in Italy (Magnani 1977 & EPPO, 2020)**

Symptoms observed on naturally regenerated forests of *Pinus spp*.

San Giovanni in Fiore
(La Sila National Park, Calabria, Southern Italy)



Val Sarentino (Alto Adige); Colbricon (Paneveggio Nature Park, Trentino) Northern Italy



MANAGEMENT OF BIOLOGICAL INVASIONS
International Journal of Applied Research on Biological Invasions

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

In press

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

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LIFE ARTEMIS

Life

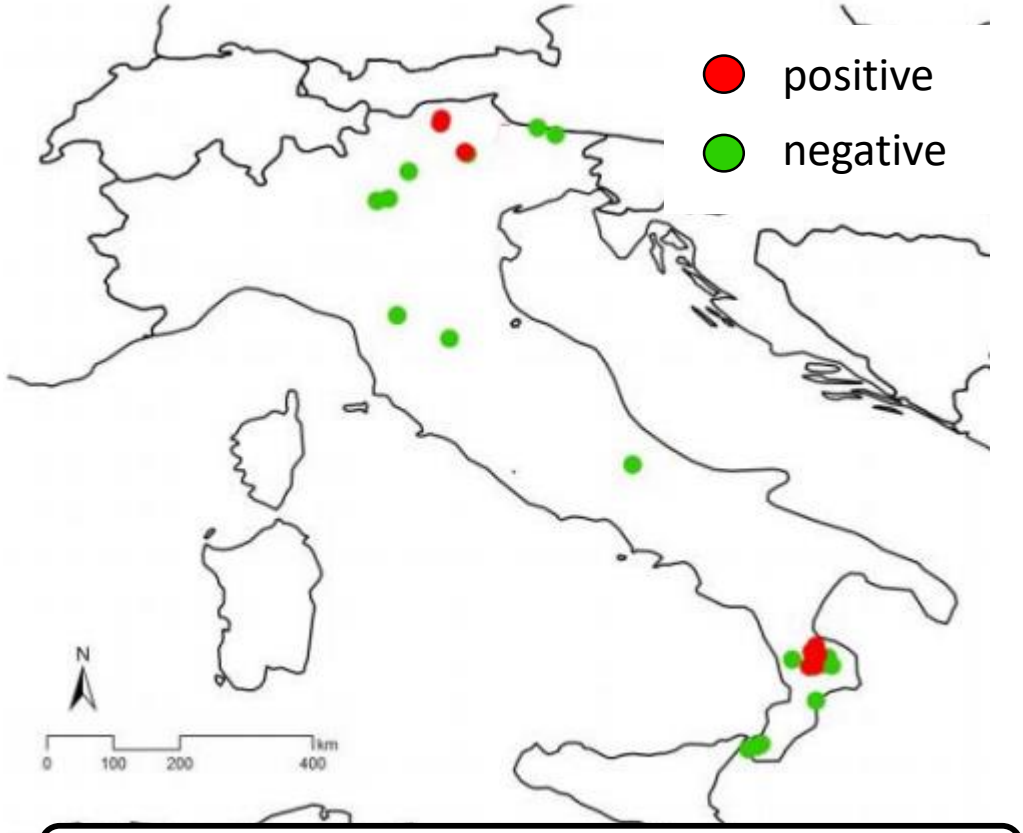
HOMED

Dothistroma septosporum

Main methods:

- Pine needles analysis with species-specific real time PCR (Ioos *et al.*, 2010)

Main results:

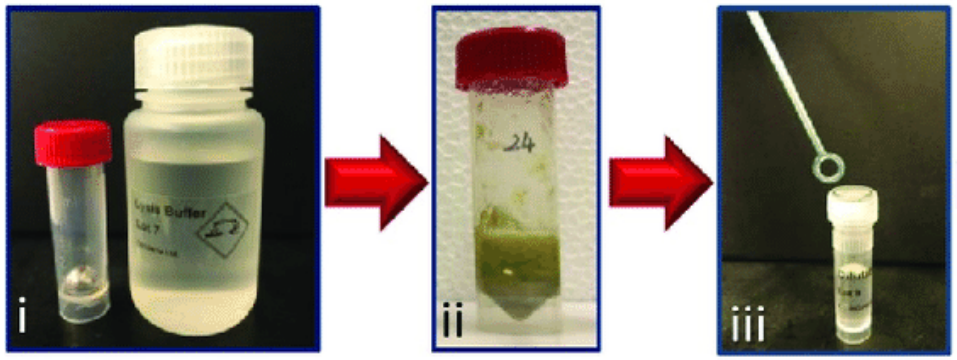


***D. pini* was not found**

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 having as the main problem applying DNA extraction in field conditions

1) Plant Material DNA extraction kit Optigene



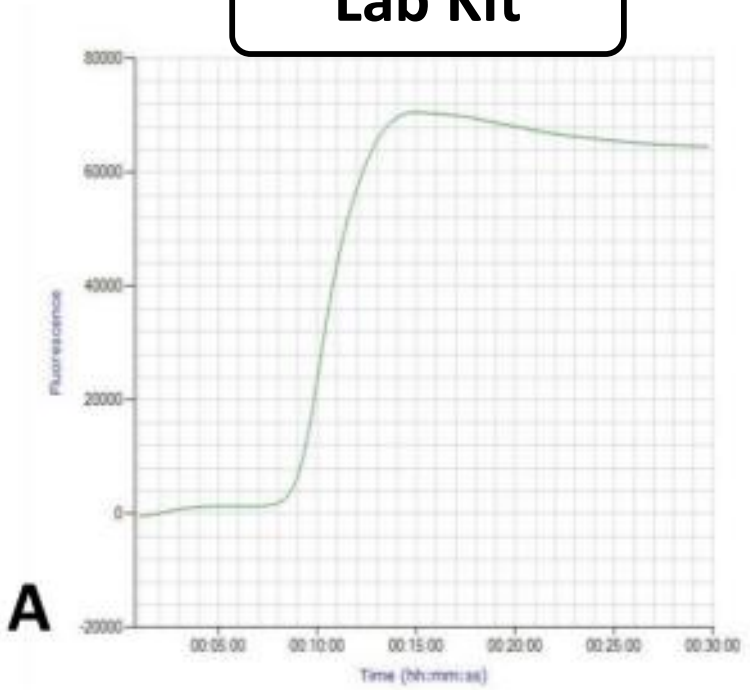
- Rapid (5min); simple: few reagents
- Difficult with a large number of samples

Methods:

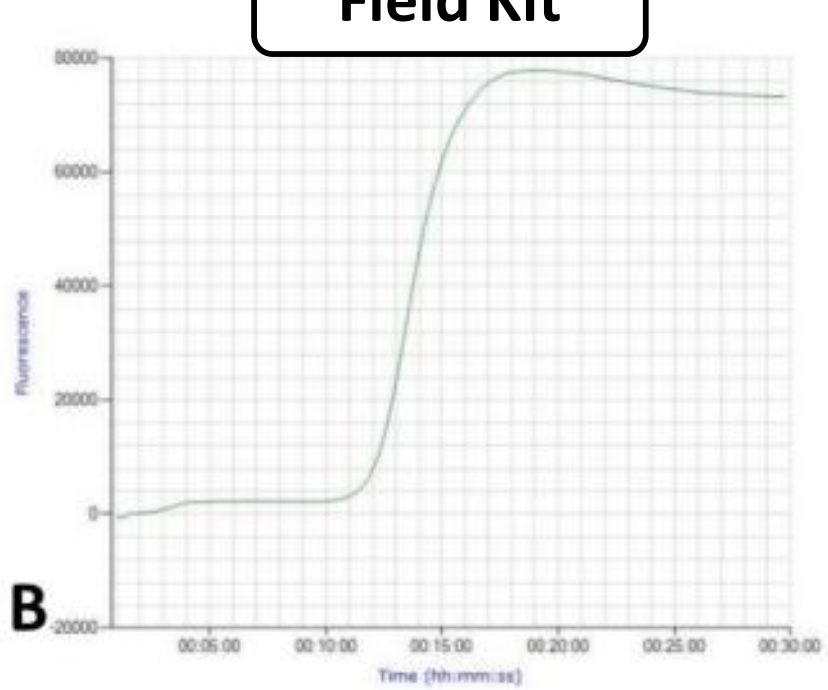
- Extraction with the field kit of the same plant samples used in Paper I and extracted with the lab kit (*X. fastidiosa*, *C. platani*, *P. ramorum* cLAMP optimization)
- Each sample was processed on LAMP with primers detecting COX (cythocrome oxidase) plant gene and primers developed for each species (*X. fastidiosa*, *C. platani*, *P. ramorum*)→ comparison between lab and field kit

Results:

Lab Kit



Field Kit

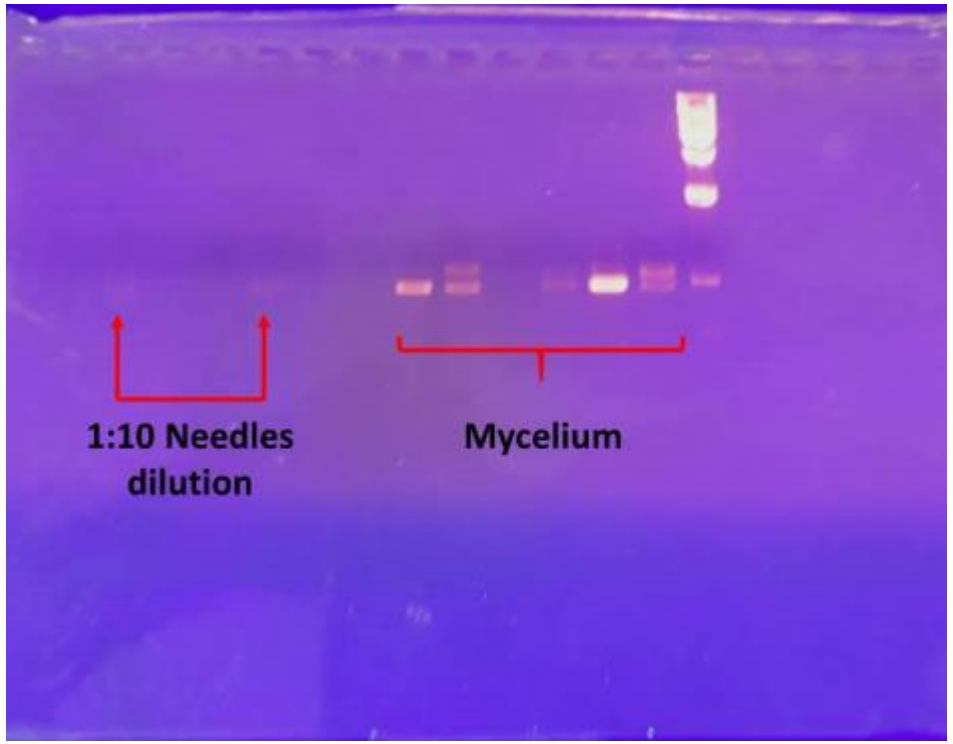


- Same amplification results were obtained by processing on LAMP DNA extracted with both kit
- Same results in shorter time directly in the field (5min vs 1hour)

A critical disadvantage for a field-suitable diagnostic method: the absolute requirement for DNA extraction → difficult to perform in resource limited settings
Omitting DNA extraction step using LAMP: reducing the costs and analysis time

2) Crude extraction optimization, preliminary tests and results

- Mikita *et al.*, 2014 Direct Boil-LAMP; Tomlinson *et al.*, 2013 crude extractions
- Test from minced mycelium and pine needles
- Lysis buffer + incubation at 85°C for 20min
- Amplifiability test by using PCR (ITS4-ITS5) and visualizing products on agarose gel (1%)



- **Good preliminary results**
- **Further research needed to improve efficiency and field-usability**

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Regione Toscana

Conclusions: state of the art

Many field assays were developed to control medical and food safety issues

In plant pathology there are only the first steps toward the use of field-suitable molecular assays as a disease management decision supporting tool

Conclusions: obtained results

7 LAMP assays were developed in this study for important plant pathogens

Ceratocystis platani

Dothistroma pini

D. septosporum

Fusarium circinatum

Lecanosticta acicola

Phytophthora ramorum

Xylella fastidiosa

- **Highly specific and sensitive, improved also with new LAMP technology (assimilated probes)**
- **Rapid (30 min)**
- **Completely field-suitable (from DNA extraction to results analysis), applied on-site in Firenze for the detection of *C. platani* and in Tuscany Mediterranean areas recently assessed as infested by *X. fastidiosa***
- **Phytosanitary controls, check exported-imported plants (nurseries, at borders..)**

Conclusions: future perspectives

Each protocol could be applied to:

- **Prevent and monitor each pathogen introduction/spread**
- **Study epidemiological and ecological features of pathogens**

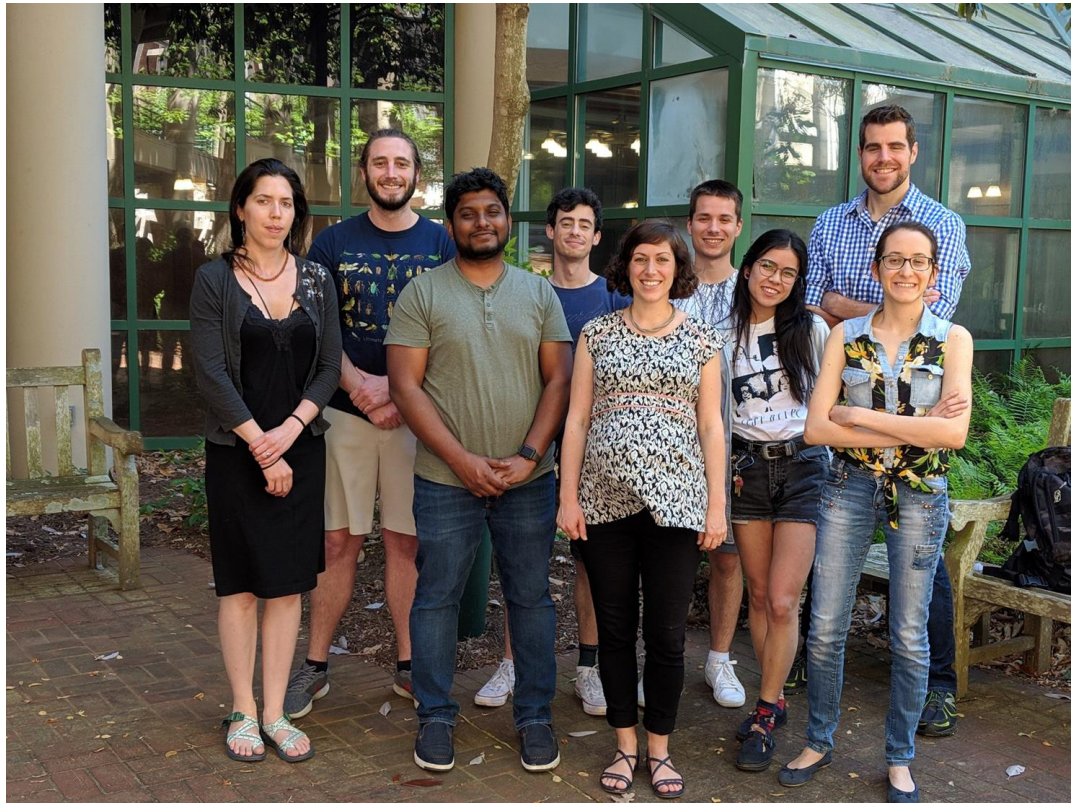
LAMP probes based assays allow to:

1) **Have higher specificity → studying *X. fastidiosa* subspecies distribution (development of subspecies-specific assay)**

2) **Working as a quantitative assay → analysing inoculum in naturally infected sites (e.g. airborne spores concentration for *D. septosporum*….)**

Aknowledgments

- Supervisor and co-supervisors
- Prof.ssa Maria Teresa Ceccherini
- Prof. Guido Marchi
- Dott. Matteo Cerboneschi
- Prof.ssa Caterina Villari
- Dott. Afaq Niyas
- Dott. Jeff Hamilton



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Thanks for your attention