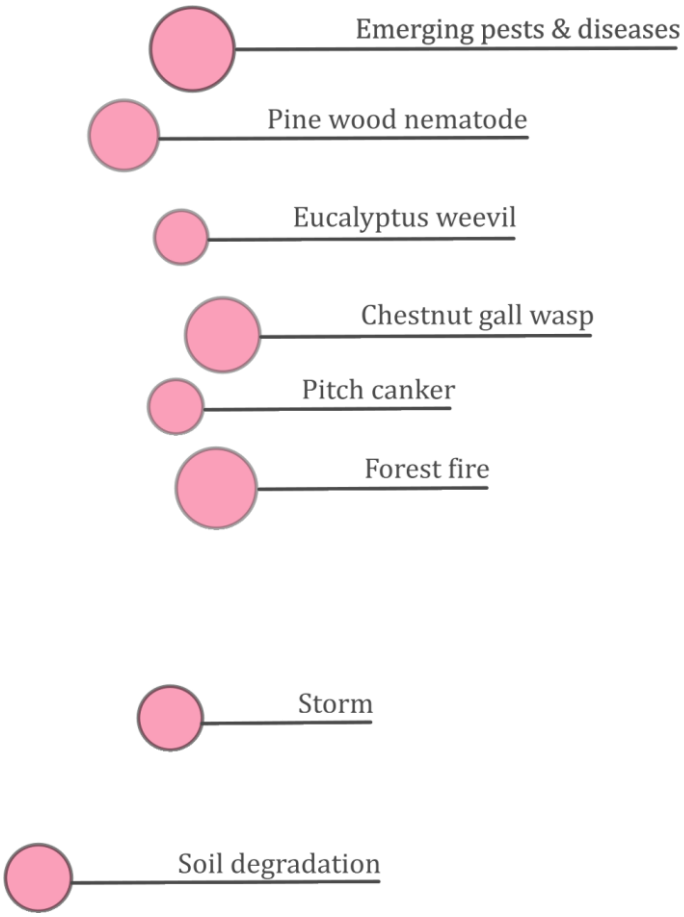
Minutes of the pine pitch canker (*Fusarium circinatum*) workshop

Tools for fast disease diagnostic



RAIZ, Instituto de Investigação da Floresta e Papel
Eixo, Aveiro, Portugal
3 October 2017

Author of the minutes: Eduard Mauri (EFIATLANTIC)

Reviewer of the minutes: Pablo Martínez (UVa)

Workshop organisers: Helena Bragança (INIAV), Julio Javier Diez (UVa)

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Agenda

PLURIFOR PROJECT
PINE PITCH CANKER WORKSHOP:
TOOLS FOR QUICK DISEASE DIAGNOSIS

TUESDAY
3 OCTOBER 2017

Organisers:
Helena Bragança, INIAV, +351 214 46 37 93, helena.braganca@iniav.pt
Julio Javier Diez, UVA, +34 979 108 420, jdcasero@pvs.uva.es

Language: Spanish/English

Venue: RAIZ, Eixo, Aveiro, Portugal

9:45	**Welcome**	**RAIZ**
10:00	**Presentation of PLURIFOR WP2 objectives**	**Julio Diez (UVa)**
10:15	Update of tools used in each region for dealing with *Fusarium circinatum*: monitoring, epidemiology, control. Presentations by different partners: 10-15 min each • Cantabria • Portugal	
11:00	**Participative discussion**: What are the limitations of current tools and the needs for new ones?	
11:30	**Coffee break**	
11:50	**New tools for diagnosing *Fusarium circinatum***	
	Presentation of a new tool using q-PCR for *Fusarium circinatum* spore traps	Helena Bragança (INIAV)
	Presentation of a new tool using Next Generation Sequencing for the monitoring of *Fusarium circinatum* in the REINFFORCE arboreta	Julio Diez (UVa)
	Activities carried out in • Cantabria • Portugal 10 min presentation each	
	Overall preliminary results	Helena Bragança (INIAV) and Julio Diez (UVa)
13:00	**Questions and discussion**	
13:30	**Lunch**	
14:50	**Roundtable** *: discussion about the potential of new tools. Definition of the next steps and products resulting from WP2	
17:00	**Close**	

The morning session will be open to all participants, partners and stakeholders. Places are limited to 50 people. Register for the workshop before 15 September 2017.

* The afternoon session is for partners and associated partners only.

Pine pitch canker WP2 objectives

Pine pitch canker risk partners and associated partners

Region	Organisation	Contact person	Associated partners
Cantabria	UVa	Julio Diez	Gobierno de Cantabria
Portugal	INIAV	Helena Bragança	Altri Florestal Instituto da Conservação da Naturesa e das Florestas RAIZ - Instituto de Investigação da Floresta e Papel

Tools and risk management plans to be developed within PLURIFOR project

As decided by the PLURIFOR Technical committee n°2 meeting (25-26 January 2017 at NEIKER, Parque Tecnológico de Bizkaia, Parcela 812, calle Berreaga 1, Derio, Spain), the following tools and risk management plans will be developed by the pine pitch canker risk team in WP2:

- Test spore traps around nurseries and in forest;
- Early detection of pathogens in REINFFORCE arboreta;
- Assessing the pathway of *Fusarium circinatum* – through review of grey literature.

Attendees

Attendees

First name	Last name	Organisation
Ana	Lança	INIAV
Ana	Fernandes	ICNF
Antunes	Alda	ICNF
Catarina	Goncalves	RAIZ
Dina	Ribeiro	ICNF
Eduard	Mauri	EFIATLANTIC
Edurne	Lacalle Galdeano	Unión de Selvicultores del Sur de Europa
Eugénio	Diogo	INIAV
Francisco José	Lario Leza	TRAGSA
Helena	Marques	ICNF
Joana	Henriques	INIAV
Leire	Salaberria Isasi	Unión de Selvicultores del Sur de Europa
Luis	Caparica	ICNF
Paula	Afonso Pinto	ICNF

Speakers

First name	Last name	Organisation
Eugénia	Andrade	INIAV
Helena	Bragança	INIAV
Julio Javier	Diez Casero	University of Valladolid
Pablo	Martínez Álvarez	University of Valladolid

Organisers

First name	Last name	Organisation
Helena	Bragança	INIAV
Julio Javier	Diez Casero	University of Valladolid

Absent

First name	Last name	Organisation
João	Silva	ICNF / DCNF Alentejo
João	Oliveira	DRAP Norte / DATM
José Manuel	Rodrigues	ICNF
Sónia	Lopes	ICNF/DCNF Centro

Presentation of the tools for fast disease diagnostic

Problematic and WP2 objectives

Julio Diez, University of Valladolid

Two problems will be addressed in WP2 concerning pine pitch canker caused by *Fusarium circinatum*:

- Better understand the pathways of the disease spread: assessing the endophytic state of the fungus, asymptomatic plants harbouring *F. circinatum* (the fungus is within the plant but the plant does not show external symptoms, this has been observed even on pines), and other plants of the understory functioning as reservoir of *F. circinatum* (if this is the case, the destruction of the infested trees is worthless because the fungus remains in the soil and in other plants that are not destroyed).
- Because of this first problem, it is necessary to detect the presence of the fungus *in situ* and quickly. So, the second problem is how to improve the accuracy and the speed of fungal identification: using real-time PCR and Next Generation Sequencing analyses.

According to the regulations, the fungus is considered eradicated from a demarcated area if it is not detected for a period of time (e.g. two years in Portugal). However, because in current regulations the search for *F. circinatum* is done by visual inspection of symptomatic trees (and later detecting the presence of the fungus through laboratory analyses), the presence of asymptomatic plants harbouring *F. circinatum* and other plants of the understory being a reservoir of the fungus make eradication virtually impossible. The regulation in other countries, as in Chili, already assumes these issues. In the Chilean case, in a lot of plants from a tree nursery, if less than 10% of the plants are infected and show no symptoms, the lot is sent to be planted. The lot of plants is only destroyed if the infected plants are more than 10%. Consequently, Chili is accepting to live with the disease in asymptomatic plants and that *F. circinatum* has arrived to stay forever. Mentalities have to switch from eradication to contingency and control.

It is becoming increasingly evident worldwide that there are plants with no symptoms and that the fungus can be dormant for years in soil, in living plants and in wood as a saprophyte. For example, in oak inoculations, the fungus "moves" along the wood up to 4 or 5 cm in a year.

Update of tools

Goal

Introduce updates on tools used in each region (Cantabria and Portugal) to deal with *Fusrium circinatum* concerning monitoring, epidemiology and control of the disease.

Cantabria

Pablo Martínez, University of Valladolid

There is a thigh relationship and collaboration between the University of Valladolid (UVa) and the Government of Cantabria. Pine pitch canker was first detected in Cantabria in 2005, in forest stands and tree nurseries, on pine species. Since 2006, the pine pitch canker and its causing agent (*Fusarium circinatum*) are regulated by law in Spain. In 2008, the Spanish law was adapted to Cantabria as a regional law. The main regulatory points are:

- In natural regenerated forests: a visual inspection grid of 8 x 8 km;
- In pine plantations de and in natural parks: a visual inspection grid of 4 x 4 km;
- In nurseries: two inspections per year;
- Delimitation of demarcated areas: areas affected by the disease and 1 km buffer area. If within the buffer a new case is detected, demarcated area is increased;
- Removal of the infected trees *in situ*;
- To take wood out of a demarcated area it must be debarked and heat treated (56 ºC in the centre of the wood during 30 minutes). This is a very difficult procedure to perform within the demarcated area. It is now allowed the wood to be treated outside the demarcated area.
- It is forbidden to plant any pine species and Douglas fir on demarcated areas.

Currently, all demarcated areas correspond to radiata pine plantations and their respective buffer area. A common practice after harvesting pine trees in a demarcated area is to replace them by eucalyptus.

Research studies on *F. circinatum* performed in Cantabria are:

Susceptibility of different conifer species to *F. circinatum*:

Experimental plantation plots of different coniferous species and provenances have been established for two years near infested pine stands. In parallel, *in vivo* inoculations were practiced in the laboratory. Although most of the conifers were affected by the pathogen in the laboratory tests, only *Pinus radiata*, *P. nigra*, *P. pinaster* and *P. uncinata* were susceptible to the pathogen in the field. *P. radiata* is the most susceptible species, and infection rate in the nurseries may be higher than in the forest.

Use of endophyte fungi to fight against *F. circinatum*:

In vitro, six endophyte fungi naturally occurring on the field were tested. As a result, two species of fungi reduced by 25% the symptoms caused by *F. circinatum*.

Detection of viruses hosted by *F. circinatum*:

In northern Spain, *F. circinatum* most probably comes from a single introduction in Galicia or Asturias and a single introduction in País Vasco or Cantabria. Three viruses attacking *F. circinatum* were discovered in Cantabria. They are common in northern Spain, but unknown in South Africa. Preliminary tests show that they do not reduce the virulence of the fungus, so by the moment they cannot be used as a biological control tool.

Analysis of the role of different insects in relation to the disease:

The introduction of *F. circinatum* in a forest through infested plants from nurseries is a well know mechanism of dissemination. However, studies have to be carried out to assess the way of dissemination from stand to stand (insects, wind, rain, pruning tools…). The pine shoot beetle *Tomicus piniperda* is one of the vector insects carrying spores from the bark galleries of infected trees to the shots of healthy trees, where it feeds.

Effect of pruning on the disease:

Pruning plays an important role on the dissemination of the disease. In Cantabria, pine plantations at risk are not pruned, or they are pruned during winter to reduce dissemination.

Portugal

Helena Bragança, INIAV

F. circinatum first appeared in Spain, then in Italy (but it was eradicated) and thirdly in Portugal, in 2009. Pine species in Portugal are more abundant in the northern half of the country. As in Spain, radiata pine is the most susceptible species. *P. pinaster*, the most spread pine species in the country (23% of forest area), has an intermediate susceptibility, and *P. pinea* shows a different set of symptoms and is the less affected.

INIAV, along with ICNF and DGAV (phytosanitary authority), set a national action plan that establishes extraordinary phytosanitary protection measures to prevent the introduction and dissemination of the fungus. Its measures are very similar to the Spanish ones: the visual inspection grid measures 2 x 2 km in any type of forest, with more than 8,000 sample plots in 2015. Samples in forested areas are taken when symptoms are observed. In nurseries, samples are always systematically taken and consist in 60 plants and 400 seeds.

When the pitch canker fungus is detected, a demarcated area is established. It is composed of an infested zone and a buffer zone. The buffer zone measures at least 1 km wide around the infested zone. The main control measure is the destruction of seeds, symptomatic seedlings, plants, trees, within the infested places. For the rest of the host plant species without symptoms within the demarcated area, a two-year quarantine is applied. During this period their circulation is forbidden and they are intensively monitored and sampled.

ICNF is the responsible organisation to collect and send plant and seed samples to one of the three laboratories in Portugal and then it collects the results. They are sent to DGAV, who communicates them to the EU.

The analysis procedure is slow and it causes plants and seeds to be immobilized for long periods in the nurseries, as it is necessary to be able to detect the presence of the fungus within the seeds. Protocols are those of the EPPO – Protocol Bulletin 39(3):298–309. On forest plants, bark is removed on branches and stems to search for necrotic tissues and prepare the samples. The first step is to proceed to morphological identification of the genus *Fusarium*. *Fusarium* colonies are subcultured to PDA then by Spezieller-Nährstoffarmer Agar (SNA) medium. The positive cases are then confirmed by biological enrichment and real-time PCR.

In Portugal, first positive results were detected in 2009 on seedlings from nurseries, and in 2015, the first report in seeds from two positive samples of *P. radiata* were detected. In 2016, the first positive results were detected in forest stands, where 1,300 trees were destroyed. ICNF tracked the origin of the positive seeds and the *P. radiata* stand of origin, situated in the north of country, was more intensively surveyed. The same year, a second report on the field showed positive results in young a plantation of *P. radiata* in the centre of the country. However, it is known that there exist infected trees without symptoms.

Needs expressed by forest owners and forest service:

- To understand the pathways of dispersion to implement good management procedures;
- Methods for early detection;
- Risk maps;
- Rehabilitation plan for affected forest areas;
- Compensatory measures should be implemented for laboratory analyses and vegetal material destruction (currently, forest owners support all at their own expense).

Ongoing or further research in Portugal:

- Characterization of Portuguese isolates/epidemiology (INIAV/Valencia University collaboration);
- Improved methods for detection (PLURIFOR & COST Action FP1406 – PINESTRENGTH);
- Improved methods for disinfection of seeds, containers and subtracts (new project approved: +PrevCRP);
- Screening for susceptibility of the two most important pines in Portugal, *P. pinaster* and *P. pinea*, based on the knowledge already gathered in a previous breeding program;
- Evaluation of the potential of insects as passive or active vectors of the fungus in Portuguese forests and nurseries;
- Development of control measures by testing chemicals and/or natural products/organisms (INIAV Master Thesis and +PrevCRP project).

New tools for the diagnostic

Goal

Introduce new tools for the diagnostic of *Fusarium circinatum* that are being developed or improved by the PLURIFOR project in Portugal and Cantabria regions.

Tools for fast disease diagnostic: spore traps combined with real-time PCR for *F. circinatum*

Helena Bragança, INIAV

The objective of this tool is to develop a method for early detection of *Fusarium circinatum* on the field, *in situ*. Field work program is conducted by ICNF and INIAV and will be executed during 2018.

The test sites, in tree nurseries as well as in forests, will be those where the fungus is known to be present. They will be distributed in two Portuguese regions following a climate gradient. In region Centro (warmer), three series of three sites will be sampled, with two spore traps placed during one week in each site. The first site, sampled in January, is closest to the coast, with milder climate conditions. The second site is sampled on March, and the last site is sampled on May. In region Norte (colder), two series of three sites will sampled, with three spore traps placed during one week in each site. The first site, sampled in February, is closest to the coast, with milder climate conditions. The second site is sampled on April, and the last site is sampled on June. Region Centro will be sampled one month earlier because its climate is warmer than region Norte. Thanks to this distribution the spore traps would be present on the field at the same time of the spore release. The spore traps will be place in the buffer are of demarcated areas for *Fusarium circinatum*.

The spore traps are a rotor with two rods with double side adhesive strips where the spores become fixed. The rotor makes the rods turn and capture the spores in the air. The battery lasts one week. After that time the rods are brought to the laboratory for DNA extraction using real-time PCR.

Spore traps and field work are financed by the PLURIFOR project.

Implementation and optimization of molecular methods for the detection/identification of *F. circinatum*

By Eugénia Andrade, INIAV

The available methods are: conventional PCR, real-time PCR using hydrolysis probes (Ioos *et al.*, 2009; Lamarche *et al.*, 2015) and SybrGreen real-time PCR (Schweigkofler *et al.*, 2007; Dreaden *et al.*, 2012).

With real-time PCR using hydrolysis probes (Ioos *et al.*, 2007, PM7/91(1)-Appendix 6) nothing is mentioned concerning sensitivity and sensibility. Consequently, it is not possible to distinguish between *F. circinatum* and *F. subglutinans*. With SyberGreen real-time PCR, Schweigkofler *et al.* (2007) methodology is not good enough to distinguish *F. circinatum*. Dreaden *at al.* (2012) methodology is better and is the one recommended. However, the research of other regions of the genome must continue to be able to distinguish *F. circinatum* more precisely.

Laboratory work is financed by the INIAV.

Presentation of a new tool using Next Generation Sequencing for the monitoring of *F. circinatum* in the REINFFORCE arboreta

By Julio Diez, University of Valladolid

Real-time PCR can detect DNA with little material: one spore is enough. The Next Generation Sequencing (NGS) can even go further. The objective of this tool is to set a methodology to detect *F. circinatum* using NGS. I will be tested on arboreta of the REINFFORCE project established in Castilla y León and Cantabria, as they contain several species of pine, including *Pinus pinaster*.

Sample methods must be improved because *F. cricinatum* has many dispersion pathways. Massive sequencing techniques are proposed, like NGS. This is a very powerful tool, as in just 4 hours the whole genome of a bacterium can be sequenced.

To test this sequencing method, 10 plots of *Pinus pinaster* were sampled, all with dieback symptoms. Ten soil subsamples were collected and mixed to get a representative mycobiota from the soil in order to detect *F. circinatum* in the soil. Total DNA was extracted from the soil samples. NGS was applied using Illumina MiSeq sequencer. The molecular markers were ITS (for fungi) and 16d (for bacteria). The resulting read were clustered by operational taxonomic units (OTUs) in order to manage the millions of reads. OTUs were compared with a specific database of soil microbiome.

This technique shows a high detection rate, with 47 to 423 fungal taxa per plot and 463 to 663 bacterial taxa per plot. The results show that it is necessary to identify which are the genes that allow distinguishing different *Fusarium* species between them; more accurate algorithms are required. Secondarily, the results could be used to detect the presence of other fungi or bacteria that could be useful in the biological control of *F. circinatum* as plant health promoters or fungal antagonists.

Discussion

J. Diez argues that *F. circinatum* would no longer be a quarantine fungus. However, the northern European countries do not want to change its status, as it could reach them more easily. Forest managers have to switch their minds from eradication or control mode towards proactive disease management mode. When *F. circinatum* arrives to a stand, nothing can be done to eradicate it. The best action possible is prevention.

There are many vested interests in plant trade. Some plant trade has little economic importance, much more risk in *F. circinatum* spread but no control for this pathogen, e.g. exotic ornamental plants with soil. This disease needs a more integrated management, covering the whole pathways of spread.

Conclusions

Round table

D. Ribeirio says that, according to current regulation, if a positive case is detected in a lot of plants, the positive must be destroyed and the rest of the lots must remain during two years in quarantine. It makes no sense to continue sampling repeatedly the same lot. The best practice would be to sample the lots since de beginning and commercialise the negative lots.

J. Diez argues that foresters must learn to live with this disease. Two points must be included in the forest management:

- *F. circinatum* as endophyte in other forest plants: eradication makes no sense.
- Some plants may host the fungus without symptoms for long periods: this would suppose long quarantine periods of suspect healthy plants from nurseries. For this reason, inspection should concentrate on healthy plants (so they can be commercialised) and immediately destroy plants with symptoms.

According to E. Andrade, the European and Mediterranean Plant Protection Organization (EPPO) focuses on respect of the evaluation methods and on standard and uniform laboratory norms applied internationally, these are its main roles. EPPO agents are working on an ISO norm to quarantine pathogens. However, lobbies exist in every country that wants to influence this norm.

E. Diogo asks what else can be achieved with the NGS as a diagnostic method. J. Diez answers that NGS offers a wider diagnostic in a single step and a single sample, and the possibility to detect the presence of other pathogens. Nowadays, other genes have to be added incorporated to the analysis to reach the species level of accuracy. H. Marques adds that NGS is a very specific method offered only by few laboratories. E. Andrade warns that with this broader method plant viruses can be detected that would be false positives, as they can be in fact viral RNA inserted in the plant. Care and control must be exercised.

J. Diez asks the participants what can be improved in the current norms. He requires them to send their suggestions to H. Bragança and to him so they can be included in the WP2 improved risk management plan for *F. circinatum*.

General workshop evaluation questionnaire

Questions

Workshop content

		Strongly disagree	Partially disagree	Partially agree	Strongly agree	Not applicable	No opinion
1.	I was well informed about the objectives of this workshop and they were clear to me.		1	5	8		
2.	This workshop fulfilled my expectations.	1		6	7		
3.	The content is relevant to my job tasks concerning forest risks management.	1		4	9		
4.	The quality and depth of knowledge of this workshop were appropriate and represented state-of-the-art tools/technologies.	1		6	7		

Workshop design

		Strongly disagree	Partially disagree	Partially agree	Strongly agree	Not applicable	No opinion
5.	The workshop activities/case studies stimulated my learning.		1	5	8		
6.	The activities/case studies in this workshop gave me sufficient practice and feedback.		3	4	4	2	1
7.	It was easy for me to understand the messages of the professionals/lecturers, they were good communicators.	1		3	10		
8.	The pace of this workshop was appropriate.	1		5	8		

Workshop instructor/facilitator/lecturer

		Strongly disagree	Partially disagree	Partially agree	Strongly agree	Not applicable	No opinion
9.	The instructor/facilitator/lecturer was well prepared.	1		1	12		
10.	The instructor/facilitator/lecturer was helpful.	1		2	11		

Workshop results

		Strongly disagree	Partially disagree	Partially agree	Strongly agree	Not applicable	No opinion
11.	I accomplished the objectives of this workshop.	1		6	6	1	
12.	I would be able to use the tools that I learned in this workshop on my tasks concerning forest risks management.		3	6	4	1	
13.	The exchanges with other professionals/instructors/lecturers were fruitful and will be useful for accomplishing my tasks concerning forest risks management.		1	7	5	1	

Self-paced delivery

		Strongly disagree	Partially disagree	Partially agree	Strongly agree	Not applicable	No opinion
14.	The workshop was a good way for me to learn its content.	1		5	8		

Improvements and values

How would you improve this workshop? (Check all that apply)

___Provide better information before the workshop.
_2_Clarify the workshop objectives.
___Reduce the content covered in the workshop.
_4_Increase the content covered in the workshop.
___Update the content covered in the workshop.
___Improve the instructional methods.
_1_Make workshop activities more stimulating.
___Improve workshop organization.
___Make the workshop less difficult.
___Make the workshop more difficult.
___Slow down the pace of the workshop.
___Speed up the pace of the workshop.
___Allot more time for the workshop.
___Shorten the time for the workshop.
___Improve the tests used in the workshop.
_2_Add (more) video to the workshop.

What other improvements would you recommend in this workshop? *The order of the answers is not relevant.*

The content of some presentations and discussions has been so technical/scientific that it has been difficult to understand it: it should have been also adapted to practitioner level.

What is least valuable about this workshop? *The order of the answers is not relevant.*

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What is most valuable about this workshop? *The order of the answers is not relevant.*

The opportunity to discuss common difficulties and to establish contact with the rest of the participants: ideas exchange, see other points of view, etc. (2 opinions)