













Non-Destructive Evaluation Techniques and What They Tell Us about Wood Property Variation

Volume 10 • Issue 9 | September 2019

Review

Sampling and Detection Strategies for the Pine Pitch Canker (PPC) Disease Pathogen *Fusarium circinatum* in Europe

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Received: 20 June 2019; Accepted: 17 August 2019; Published: 22 August 2019



Abstract: *Fusarium circinatum* Nirenberg & O’Donnell is listed among the species recommended for regulation as quarantine pests in Europe. Over 60 *Pinus* species are susceptible to the pathogen and it also causes disease on Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and species in genera such as *Picea* and *Larix*. The European Food Safety Authority considers the probability of new introductions—via contaminated seeds, wood material, soil and growing substrates, natural means and human activities—into the EU very likely. Due to early detection, constant surveillance and control measures, *F. circinatum* outbreaks have officially been eradicated in Italy and France. However, the global spread of *F. circinatum* suggests that the pathogen will continue to be encountered in new environments in the future. Therefore, continuous surveillance of reproductive material, nurseries and plantations, prompt control measures and realistic contingency plans will be important in Europe and elsewhere to limit disease spread and the “bridgehead effect”, where new introductions of a

tree pathogen become increasingly likely as new environments are invaded, must be considered. Therefore, survey programs already implemented to limit the spread in Europe and that could be helpful for other EU countries are summarized in this review. These surveys include not only countries where pitch canker is present, such as Portugal and Spain, but also several other EU countries where *F. circinatum* is not present. Sampling protocols for seeds, seedlings, twigs, branches, shoots, soil samples, spore traps and insects from different studies are collated and compiled in this review. Likewise, methodology for morphological and molecular identification is herein presented. These include conventional PCR with a target-specific region located in the intergenic spacer region, as well as several real-time PCR protocols, with different levels of specificity and sensitivity. Finally, the global situation and future perspectives are addressed.

Keywords: *Gibberella circinata*; quarantine species; damping-off; survey programs; morphological identification; molecular detection

1. Introduction

Fusarium circinatum Nirenberg & O'Donnel is a serious pathogen which affects certain conifers, and is considered to be native to Mexico, the Caribbean (Haiti) and the southeastern USA. The pathogen has spread from its original distribution area, and has been introduced to South America (Brazil, Chile, Colombia and Uruguay) [1–4] South Africa [5], Asia (Japan and South Korea) [6,7] and Southern Europe [8–11]. The global distribution of *F. circinatum* is mostly limited to Mediterranean and subtropical climates, with some spread into temperate climates [12,13]. The fungus has been found in four European countries (France, Italy, Portugal and Spain), but has established in forests only in the Iberian Peninsula. The European and Mediterranean Plant Protection Organization (EPPO) lists *F. circinatum* among the locally present species recommended for regulation as quarantine pests (A2 list).

F. circinatum induces resinous bleeding cankers on trunks and branches of trees, and is commonly referred to as pine pitch canker [14,15]. On seedlings, the pathogen causes damping off, as well as root disease characterized by the discoloration and disintegration of the root cortex. Over 60 *Pinus* species are susceptible to infection by *F. circinatum*. The most susceptible species is Monterey pine (*Pinus radiata* D. Don), originating from the coast of central California USA and Mexico, which has been planted extensively around the globe, including in northeastern Spain [16–18]. Additionally, *F. circinatum* has been reported to cause disease on Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and species in genera such as *Picea* and *Larix* [18,19].

The European Food Safety Authority (EFSA) panel has conducted a risk assessment of *F. circinatum* for the EU territory [13], and considers the probability of new entry into the EU very likely. The entry pathways include contaminated seeds, wood material, plant material for decorative purposes, soil and growing substrates, natural means (wind, rain, insects and other animals carrying spores) and human activities (travel, vehicles). This risk is mitigated through strict regulations on the importation and movement of plants belonging to the genus *Pinus* and Douglas-fir (including seeds and cones), together with the need for them to be officially inspected (imported plants) and accompanied by phytosanitary documents. In addition, growing media and wood packaging material imports are also regulated. These regulations do not include other conifers, such as Norway spruce (*Picea abies* (L.) Karst.), which could act as a path of entry for the pathogen. Martín-García et al., 2018 [19], demonstrated that asymptomatic Norway spruce seedlings could host latent infections of *F. circinatum*, even 8.5 months after inoculation.

Based on a preliminary pest risk assessment by the French Plant Protection Service [20] and the first report of *F. circinatum* in the EU [21,22], provisional emergency measures were adopted in 2007 to prevent *F. circinatum* introduction into and spread within the Community (Commission Decision 2007/433/EC). This legislation stipulated that Member States of the EU must conduct official annual surveys for *F. circinatum* on their territory and report the results annually to the

European Commission. Any suspected occurrence or confirmed presence of pine pitch canker must be reported to the National Plant Protection Organization (NPPO) of the corresponding country (see https://www.eppo.int/ABOUT_EPPO/eppo_members). In the case of confirmed presence of *F. circinatum*, EU Member States will define demarcated areas (including the infected zone and a buffer zone at least 1 km beyond the infected zone) and take official measures to eradicate the pest and to monitor for its presence.

Since 2015, through Regulation (EU) No 652/2014, the EU has co-financed survey programs for certain pests, including *F. circinatum*. From 14 December 2019, new plant health rules will be applicable in the EU requiring Member States to perform regular surveys for the presence of all quarantine pests, consisting of visual examinations and, if appropriate, collecting and testing samples, based on sound scientific and technical principles (Regulation (EU) 2016/2031 of the European Parliament of the Council of 26 October 2016 on protective measures against pests of plants) [23]. From 31 March 2019, *F. circinatum* is listed in Annex I/A2 of the Plant Health Directive 2000/29/EC.

Sampling for *F. circinatum* is essential in order to prevent its local establishment, spread and movement into disease-free areas, which is influenced by abiotic factors such as forest management and environmental conditions, as well as biotic factors (for example fungal communities and bark beetles) [24]. However, there are no harmonized sampling protocols or even a consensus about the best sampling methods and approaches. This leaves areas where “suboptimal” sampling protocols are used open to disease incursion. In this paper, we collate survey programs used in different countries, climatic zones and disease histories. We also discuss recommendations for sampling and processing plant tissues for the detection of *F. circinatum* as recommended by EPPO and the International Plant Protection Convention (IPPC). The overall aim is thus to provide authorities and pathologists with the relevant knowledge regarding the available approaches for surveys of their forests, plantations and nurseries.

2. Survey Programs in Different Geographical Regions

2.1. European Countries with Existing or Eradicated *F. circinatum* Epidemics

All four of the European countries where *F. circinatum* has been detected are situated in the Mediterranean climatic zone. However, the pathogen is considered successfully eradicated in Italy and France and is currently present only in the Iberian Peninsula (Portugal and Spain). In all these countries, sampling is conducted yearly in forests, nurseries and garden centers. Tables 1–4 present survey data from several countries based on the annual reports submitted to the European Commission by the NPPOs that are represented either by the current authors or collaborators mentioned in the Acknowledgements section.

Table 1. Sampling approaches in production sites (nurseries) for planting material. The data is based on the annual reports submitted to the European Commission by the NPPOs.

Country and Years	Number in the Member State (Total Hectares)	Visually Surveyed Locations (Number/Hectares)	Number of Laboratory Samples Taken	Results for Visually Surveyed Locations +/-	Results for Laboratory Analyses +/-	In case of Positive Findings Size (ha) and Location of Demarcated Area (Infected Zone + Buffer Zone) *
Spain, Castilla y León, 2009–2017	13–121 nurseries of forest reproductive material (<i>Pinus</i> and/or <i>P. menziesii</i>) per year (658 in total)	21–52 per year (304 in total)	106–822 seedlings per year (3499 in total), and 1–165 seed lots per year (371 in total)	1 without symptoms	3 positive/3496 negative	Data not available
Spain, Cantabria 2009–2017	1–3 nurseries of forest reproductive material (<i>Pinus</i> and <i>P. menziesii</i>) per year	1–3 nurseries per year (17 in total)	102 samples in total	all negative	4 positive in total	n.a.
Portugal, 2009–2017	100–224 nurseries of forest reproductive material (<i>Pinus</i> and <i>P. menziesii</i>) per year (1593 in total)	64–209 nurseries of forest reproductive material (<i>Pinus</i> and <i>P. menziesii</i>) per year (1245 in total)	154–259 per year (1908 in total)	4–17 with symptoms per year (79 in total)	0–13 positive, 154–257 negative per year (in total 40 positive, 1869 negative)	500–6269 ha located in 1–3 different regions per year (25,935 ha in total)
Italy, 2010–2017	509–966 nurseries and garden centers of forest and ornamental trees per year	309–481 nurseries and garden centers of forest and ornamental trees per year (3240 in total)	43–309 per year (1111 in total)	all negative	all negative	n.a.
France, 2013–2017	1650–2554 nurseries per year (8187 in total; some of them are inspected every year)	1062–1331 per year (4868 in total)	17–47 per year (0–9 plants, 14–47 seed lots; 152 in total)	all negative	one positive seedlot from USA in 2016; all other years negative	n.a.
Bulgaria, 2015–2016	109 registered nurseries/1583.58 ha (authorized to issue plant passports)	84–89 nurseries (plants for planting) 226–334 ha	3–9	all negative	all negative	n.a.
Northern Macedonia, 2007–2017	25–26 nurseries (total 80 ha) of forest propagation material (<i>Pinus</i> spp. and <i>P. menziesii</i>) + 5 ha of nurseries of ornamental shrubs and trees	25–26 nurseries; 50–52 checks per year (<i>Pinus</i> spp. 30,337,150 seedlings and <i>P. menziesii</i> 1,612,400 seedlings in total)	58–80	all negative	all negative or n.a.	n.a.
Slovenia, 2007–2017	19–45 places of production of plants for planting (per year)	19–45 visually surveyed locations (per year)	0–10 (per year)	all negative	all negative	n.a.
Great Britain, 2007–2017	206–313 Nurseries registered to issue plant passports where plants were inspected	163–323 sites inspected each year	0–84 (per year)	all negative	all negative or n.a.	n.a.
Sweden, 2014–2017	98 registered nurseries for forest propagation material	26–90 nurseries of forest reproductive material + 100 garden centers	0	no occurrence detected in surveys; no further sampling	n.a.	n.a.
Finland, 2007–2017	33–76 nurseries of forest propagation material (<i>Pinus</i> and <i>P. menziesii</i>) + ca. 400 ha of nurseries of ornamental shrubs and trees	0–21 nurseries of forest reproductive material (up to ca. 22 million plants) + 9–77 nurseries of ornamental shrubs and trees	0–11 per year	samples taken from up to 4 locations; all negative	all negative or n.a.	n.a.

* As described in article 6 of the Commission Decision 2007/433/EC. n.a. = not applicable.

Table 2. Sampling approaches in production sites for planting material—demarcated *Pinus* spp. and Douglas-fir (*Pseudotsuga menziesii*) forest sites based on article 1 of the Commission Decision 2007/433/EC (separate, demarcated part of forest where propagating material as seeds and cones were collected). The data is based on the annual reports submitted to the European Commission by the NPPOs.

Country and Years	Total Hectares/Number in the Member State	Visually Surveyed Locations Hectares/Number	Number of Laboratory Samples Taken	Results for Visually Surveyed Locations +/-	Results for Laboratory Analyses +/-	In case of Positive Findings Size (Ha) and Location of Demarcated Area (Infected Zone + Buffer Zone)
Spain, Castilla y León, 2009–2017	3–10 sites per year	50 sites per year (129 sites in total)	23–2329 samples per year (3560 in total)	1–8 sites with symptoms	11 positives (between 2009 and 2011)	2535.7 ha in total
Spain, Cantabria, 2009–2017	data not available	data not available	data not available	data not available	data not available	data not available
Portugal, 2009–2017	183 sites; about 12,000 ha (per year)	161 sites; about 201,802 ha (per year)	1–30 per year (110 in total)	1–26 sites with symptoms per year (79 in total)	2 positive (2016); other years negative	600 ha located in 1 region (2016); other years n.a.
Italy, 2010–2017	17,920–25,279 ha + 0–4 sites (2011–2017) 68 sites (2010) per year	27–1693 + 0–4 sites (2011–2017) 41 sites (2010) per year (total 5120 ha + 53 sites)	0–46 per year (108 in total)	all negative	all negative	n.a.
France, 2013–2017	21,894 ha per year (109,470 ha in total)	included in forest survey	see nurseries and forests	see nurseries and forests	see nurseries and forests	see nurseries and forests
Bulgaria, 2015–2016	3134.52 ha <i>Pinus</i> spp. and <i>P. menziesii</i> forestry stand and seed orchard	6.05–6.50 ha	0–11 (seed samples)	negative	all negative	not applicable
Northern Macedonia, 2007–2017	not available	<i>Pinus</i> spp. 3 ha and <i>P. menziesii</i> 0.5 ha	0	n.a.	all negative	n.a.
Slovenia, 2007–2017	not available	included in forest surveys	included in forest surveys	all negative	all negative	n.a.
Great Britain, 2007–2017	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Sweden, 2014–2017	for <i>Pinus</i> spp. a 628 ha forestry stand and 1080 ha seed orchard; and for <i>P. menziesii</i> a 2 ha forestry stand	0	n.a.	n.a.	n.a.	n.a.
Finland, 2007–2017	1782–2434.2 ha (2007–2010); 0 ha (2011–2018)	0	10 places, 500 seed/place (2008); 0 (other years)	n.a.	all negative	n.a.

n.a. = not applicable.

Table 3. Sampling approaches in *Pinus* spp. and *Pseudotsuga menziesii* forest (including parks, gardens etc.). The data is based on the annual reports submitted to the European Commission by the NPPOs.

Country and Years	Total Hectares/Number in Your Member State	Visually Surveyed Locations Hectares/Number	Number of Laboratory Samples Taken	Results for Visually Surveyed Locations +/-	Results for Laboratory Analyses +/-	In case of Positive Findings Size (ha) and Location of Demarcated Area (Infected Zone + Buffer Zone)
Spain, Castilla y León, 2009–2017	1,291,800 ha per year (11,626,200 in total)	341–5738 sites per year (21,840 in total)	42–212 per year (1222 in total)	5–45 sites with symptoms per year (166 in total)	5 positive (2009–2013)	data not available
Spain, Cantabria, 2009–2017	12,213–33,129 ha	1160–2694 ha per year (12,446 ha in total)	34 in total	53 sites with symptoms	5 positive in total	10,666 ha in 16 demarcated areas since the detection of the pathogen in 2005
Portugal, 2009–2017	794,500–1,015,000 ha per year (8487,616 in total)	677–11,125 sites per year (46,696 in total)	4–77 per year (315 in total)	2–66 sites with symptoms per year (281 in total)	1 positive (2016); other years negative	550 ha located in 1 region (2016); other years n.a.
Italy, 2010–2017	614,000–793,894 ha per year	1799–42,294 ha + 719–12 sites per year (164,184 ha + 1596 sites in total)	30–160 (714 in total)	all negative	all negative	n.a.
France, 2013–2017	3,079,000 ha	48–111 forest sites inspected yearly; 104–191 inspections in parks and gardens yearly (939 sites in total)	3–17 per year	0	0	n.a.
Bulgaria, 2015–2016	240,638 ha productive forests covered by <i>Pinus</i> spp. 7372 ha of <i>P. menziesii</i> plantations. it is also present in mixed forests, parks, gardens etc.	in 263.01–386.30 ha forests covered by <i>Pinus</i> spp. 8–394.55 ha plantations covered by <i>P. menziesii</i>	0–6	all negative	all negative	not applicable
Northern Macedonia, 2007–2017	80,000 ha	20,000 ha (surveyed area) <i>Pinus</i> spp.: 26 locations and <i>P. menziesii</i> : 3 locations	0	n.a.	n.a.	n.a.
Slovenia, 2007–2017	cca. 80,000 ha of <i>Pinus</i> spp. and <i>Pseudotsuga</i> stands (around 6% of total wood stock)	98–900 ha (surveyed area); 28–7781 locations	0–55	all negative	all negative	n.a.
Great Britain, 2007–2017	104 ha (<i>P. radiata</i> only); 416,424–448,284 ha	19–77 sites each year; 40.4–104.257 ha each year	0–88 (per year)	all negative	all negative or n.a.	n.a.
Sweden, 2014–2017	>9.2 million ha productive forests where at least 70% of the area is covered by <i>Pinus</i> spp.	0	0	n.a.	n.a.	n.a.
Finland, 2007–2017	17,000,000 ha	0	0	n.a.	n.a.	n.a.

n.a. = not applicable.

Table 4. Sampling approaches in garden centers. The data is based on the annual reports submitted to the European Commission by the NPPOs.

Country and Years	Number of Inspections in Garden Centers	Number of Laboratory Samples Taken	Results for Laboratory Analyses+/-
Spain, Castilla y León, 2009–2017	data not available	data not available	data not available
Spain, Cantabria, 2009–2017	data not available	data not available	data not available
Portugal, 2009–2017	data not available	data not available	data not available
Italy, 2010–2017	included in production sites for planting material—nurseries	included in production sites for planting material—nurseries	included in production sites for planting material - nurseries
France, 2013–2017	616	37	0
Bulgaria, 2015–2016	data not available	data not available	data not available
Northern Macedonia, 2007–2017	included in forest survey	included in forest survey	included in forest survey
Slovenia, 2007–2017	from 6 to 32	0–23 (per year; some samples taken from plants for planting, originating from other Member States)	n.a. or negative
Great Britain, 2007–2017	71–308	included in production sites for planting material—nursery surveys	all negative or n.a.
Sweden, 2014–2017	included in production sites for planting material—nurseries	0	n.a.
Finland, 2007–2017	0–186	0	n.a.

n.a. = not applicable.

In Spain, the survey programs used in nurseries and forests vary across the seventeen Autonomous Communities. In Tables 1–4, sampling data are presented separately from two such regions with ongoing *F. circinatum* epidemics. As an example, in Castilla y León, forest nurseries and garden centers (all those growing susceptible species) are sampled annually by taking at least one sample per 20,000 plants in large nurseries (i.e., those producing millions of plants); the sampling frequency is higher for smaller nurseries. It is compulsory for the nurseries to declare their entire stock of plant material (including seed lots and the traceability of all plant material), which is taken into consideration when determining the minimum number of samples. Samples are taken preferably from symptomatic plants, but in the absence of symptoms, asymptomatic plants are also sampled. All nurseries that store seed lots are sampled every year, taking 500 seeds per seed lot (The Government of Spain; Real Decreto 637/2006 and 65/2010). Annual surveys of forests are carried out, with areas of susceptible tree species first being located and mapped. For example, in the Castilla y León region, besides the ICP Forests network (8 × 8 km grid and 4 × 4 km grid in protected areas), a specific 2 × 2 km grid has been established for *F. circinatum* in all Monterey pine plantations, also including surrounding maritime pine (*P. pinaster* Aiton) areas. In addition, the presence of *F. circinatum* symptoms is recorded in all stands monitored for the pine wood nematode (PWN; *Bursaphelenchus xylophilus* (Steiner & Buhner) Nickle) (3000–5000 stands per year), and samples are taken for analysis when symptoms are observed. Moreover, nearly 800 environmental agents working in forests conducting different tasks (such as hunting, fishing, wood harvesting, etc.) monitor *F. circinatum* disease symptoms and collect samples from suspect plants. In contrast, in the Navarra region, besides three different grids (16 × 16 km, 8 × 8 km and 2 × 2 for the demarcated areas), roadsides and wood industries are also surveyed, taking advantage of ongoing PWN monitoring and the presence of seed orchards.

In Portugal, all nurseries planting susceptible host species (including forest nurseries) must be inspected once a year by the phytosanitary inspection service before trade commences. If symptoms of *F. circinatum* are observed, samples are taken preferably from symptomatic plants according EPPO guidelines (<https://gd.eppo.int/taxon/GIBBCI/documents>). The following sampling scheme is used: 25 plants are collected from lots with <1000 seedlings, and 60 plants from lots with ≥ 1000 seedlings. The same number of asymptomatic plants is collected randomly if no symptoms are visible. Forest surveys are based on visual inspection of trees at field points classified as “coniferous forest cover” (>2170 points with an area of at least 500 m² each) from a 2 × 2 km grid developed by the Portuguese Forest Inventory (NFI). The grid is shifted annually, with reference to one of the four cardinal points, to maximize the detection of pests and pathogens [25]. Samples are taken only from plants that show symptoms characteristic of *F. circinatum*, focusing on: (i) areas surveyed for PWN (in the 20 km buffer-zone with the Spanish border); (ii) at least 25% of the forest points from the remaining territory; (iii) within 5 km around all commercial nurseries producing forest and ornamental conifer trees; (iv) in the 500 × 500 m zones in the demarcated areas established around nurseries and/or forest traders which had positive results for *F. circinatum*; and (v) in orchards (forest areas for seed production) inspected every three years and before collecting cones. All seed lots are analyzed before trade: a subsample of 500–1000 seeds per lot is subjected to laboratory analysis, and graft material of stone pine is also analyzed before trade.

In Italy, surveillance activities are carried out independently by each Regional unit of the Phytosanitary Services on the basis of a coordinated National Surveillance Plan, which is updated yearly by the National Phytosanitary Committee (D.Lgs 214/05). Surveys are prescribed in four site types where host species of *F. circinatum* (all *Pinus* species and Douglas-fir) are found: (i) plant nurseries and commercial gardens; (ii) forests; (iii) forest areas for seed production; and (iv) green recreation areas including urban forests, parks and city gardens. Each surveillance site is permanent and corresponds to a circular area of about 30 m radius around a Global Positioning System (GPS) point where a visual inspection of symptoms on aerial plant parts is performed. Each inspected hectare should include 3 points. Plants with clear symptoms of infection are sampled for laboratory analysis. In addition, a minimum number of asymptomatic samples (i.e., 2 samples from at least 15% of the inspected sites)

is currently recommended to be sent for molecular analyses (real-time PCR) both in forests and in forests for seed production. Twigs, branches or entire seedlings are usually sampled, while cones are collected at asymptomatic forest sites if crowns are not easily reached. Soil is not routinely sampled. There is considerable variation in the intensity and detail of planned surveillance among Italian regions depending on risk evaluation (climatic conditions, type, structure and age of forests, and the volume of local plant trade) [26,27]. With regard to forests and forest areas for seed production, Italian Regional Phytosanitary Services have reported their surveillance activities according to either inspected area (ha) or number of inspected sites, which makes it difficult to compare data among regions and to get a consistent picture at the national level. Instead, surveillance in nurseries is reported only as the number of inspected sites. From 2010 to 2017 in Italy about 54% (ca. 400 nurseries) of plant nurseries and commercial gardens growing *F. circinatum* host tree species were inspected yearly on average, with small differences from one year to another. In the same period, about 3% (ca. 21,000 ha) of Italian forests (aggregated data with green recreation areas) containing *F. circinatum* host species and about 2% (ca. 700 ha) of the area covered by demarcated forests for seed production, which are present in only a few regions, were inspected yearly on average. Data indicate year-to-year variation of the forest area inspected with a clear reduction after 2014, while the number of inspected nurseries remained quite stable. Since 2010, the number of samples collected for lab analyses has increased both in forests and in nurseries.

In France, all nurseries with susceptible host species must be inspected once a year by the phytosanitary inspection services before trade commences. Only symptomatic plants and plant parts are sampled and sent for laboratory analysis using isolation techniques, followed by barcode sequence analysis of the fungus in pure culture. Forest sites are surveyed based on visual inspection, and samples are taken only from plants that show symptoms compatible with *F. circinatum*. The surveys emphasize high risk regions, e.g., those that are close to the Spanish border and/or show favorable climatic conditions (coastal areas of Languedoc Roussillon, Basque country, Landes, coastal areas of Charente-Maritime). Surveys are undertaken in parks and private gardens, forests and forest plantations (a minimum of 125 inspection visits in parks and 125 visits in forests per annum). Selected forest areas for seed production must be inspected every year before cone collection, during the same year when the harvest of seeds is scheduled. Moreover, all seed lots are analyzed before trade: a sub-sample of 1000 seeds per lot is taken and sent for laboratory analysis by real-time PCR.

2.2. European Countries without *F. circinatum* Outbreaks

In European countries with temperate or boreal climatic conditions, the occurrence of symptoms compatible with *F. circinatum* is investigated in forests, nurseries and garden centers, as well as in gardens and parks in urban areas, and random samples are also sometimes taken from asymptomatic plants. Tables 1–4 present survey data based on the annual reports submitted to the European Commission by the NPPOs for six exemplar countries: Bulgaria, North Macedonia and Slovenia represent mostly humid continental climatic zones, Great Britain represents the Oceanic climate zone and Sweden and Finland represent Nordic countries mostly located in the boreal forest zone.

In Bulgaria, the diverse continental-Mediterranean climatic conditions and landscape patterns are considered likely to be favorable for the establishment *F. circinatum* [13]. The total area of established *Pinus* stands in forests and urban zones is 240,638 ha, and the current area of Douglas-fir plantations totals about 7372 ha. A survey to ascertain the presence of *F. circinatum* is carried out annually by phytosanitary inspectors from the Bulgarian Food Safety Agency (BFSA) at the Ministry of Agriculture and Food (2007–2017). Samples are only taken of symptomatic plants and are sent to the Central Laboratory of Quarantine Plants at BFSA. During 2015–2016, the number of nurseries in which *Pinus* and Douglas-fir were investigated was 84–89, and 394–658 hectares of forests and urban areas were also examined. In addition to these surveys, phytopathologists from the Forest Research Institute – BAS and Forest Protection Stations implement annual monitoring in order to assess the health status of stands and plantations of Scots pine (*Pinus sylvestris* L.), Austrian pine (*P. nigra* Arnold), Monterey pine and Douglas-fir.

In Northern Macedonia, all forest nurseries are inspected yearly by the Department of Forest and Wood Protection (Faculty of Forestry in Skopje), which is the official body responsible for mandatory plant health checks in all forest nurseries. In the period from 2007 to 2017, more than 30 million (30,337,150) *Pinus* seedlings and 1.6 million (1,612,400) Douglas-fir seedlings were checked, by analyzing symptomatic and asymptomatic plants and plant parts. An annual survey of 26 forest stands of *Pinus* spp. and three of Douglas-fir is undertaken by the same Department, which also hosts the Service for Diagnosis and Prognosis (IDP) of Forest Plant Pests and Diseases for Northern Macedonia. Selected forest areas for seed production are also inspected every year, with samples only being taken if symptoms are observed.

In Slovenia, the number of *Pinus* nurseries investigated annually in 2007–2017 varied from 19 to 45, and laboratory samples were taken regularly. Laboratory samples have also been collected at forest sites, parks and gardens (28–7781 locations and typically 98–900 ha per year). Inspection and sampling was also conducted in 6–32 garden centers yearly. Samples are taken from both symptomatic and asymptomatic trees. Seeds are randomly sampled and analyzed for the presence of *F. circinatum*. The surveys are performed by institutions authorized by the Slovenian NPPO: the Slovenian Forestry Institute and Slovenia Forest Service. Monitoring at the border, in nurseries and on trade is done by the Inspection for Food Safety, Veterinary Sector and Plant Health. In addition, Forest Inspectors can perform the surveys.

In Great Britain, all susceptible species (all *Pinus* spp. and Douglas-fir) in all British forest seedling producing nurseries (currently 9 in Scotland and 7 in England/Wales) have been surveyed for *F. circinatum* from 2015. These surveys are carried out at the same time as surveys for *Dothistroma* spp. and stock is assessed on the basis of visual symptoms. Symptomatic plants are collected from the nurseries and assessed by both classical isolation methods and real-time PCR techniques at Forest Research laboratories. Surveys of garden centers and ornamental nurseries have been undertaken since 2007 by the Scottish Government's Horticulture and Marketing Unit and Plant Health and Seed Inspectors within England's Animal and Plant Health Agency. Additionally, an annual survey of 18 forest stands of Monterey pine, four of Corsican pine (*P. nigra* subsp. *laricio* (Poir.) Maire) and seven of Douglas-fir has been undertaken since 2007 by Forest Research. The survey comprises a visual inspection, and samples are only taken if symptoms are observed.

In the Nordic countries (Fennoscandia), the climate is considered unsuitable for *F. circinatum* [13,28]. In addition, Scots pine, the native species in the Boreal region of Europe, is considered to acquire age-related resistance against *F. circinatum* [18]. Therefore, *F. circinatum* surveys are not carried out in forests. However, as the pathogen is capable of surviving in forest nurseries and garden centers throughout Europe, these are inspected yearly, although no samples are usually taken, given the lack of visual symptoms observed. In Sweden (situated in the boreal and hemiboreal climate zones), the inspection of nurseries (mainly forest propagation material) is limited to those that are considered to be at the highest risk according to an assessment made by the Swedish Agricultural Board (90 nurseries were surveyed in both 2016 and 2017; no samples were taken from asymptomatic plants). In Finland (also situated in the boreal zone), only symptomatic plants are sampled. The number of forest nurseries inspected yearly has varied between 0–21 during 2007–2017, and 9–77 nurseries of ornamental shrubs and trees have been inspected yearly. Production sites have been sampled for seed samples only sporadically. The number of garden centers inspected in Finland was up to 186 per year (2007–2017).

3. Protocols Used for Sampling in Nurseries

General recommendations for sampling seedlings for the presence of *F. circinatum* are given by EPPO and IPPC [29,30]. In a production nursery, both symptomatic and asymptomatic seedlings should be collected. This is because seedlings of *Pinus* spp. are commonly infected with *F. circinatum*, yet they display not symptoms. Such apparently healthy but infected seedlings often only induce disease in their hosts once they are planted in the field, where the primary symptoms are root and

collar rot. Collected seedlings are ideally transferred in paper bags to the laboratory where they are processed immediately.

When young nursery seedlings are sampled, the entire plant is sent to the laboratory. In the case of potted plants, this includes the substrate with roots. Plants from bare roots nurseries are collected without soil. From older plants, only portions from the stem and root system, including some surrounding soil or peat moss, are sent for analysis (see Section 4 for sampling of soil). The plants are labelled, packed individually in paper envelopes inserted in sealed plastic bags and kept cool until being sent to the laboratory. Disinfection of hands or gloves is carried out between samples, although cross contamination is not likely to occur as surface disinfection is conducted when the samples are taken to the laboratory (see Section 5.2). A document including collection data and a sample code is attached to each sample. This document includes the environmental conditions under which plants are growing, as well as any phytosanitary treatments that were carried out. Once in the laboratory, the samples must be kept in a refrigerator until analysis, and are analyzed within eight days of arrival [29].

4. Protocols Used for Sampling in Forests and Plantations

EPP0 and IPPC have given general recommendations for sampling plant tissues for the presence of *F. circinatum* [29,30]. As described in the previous section, all plant material should be processed as fast as possible or refrigerated in order to avoid colonization by other fungal species (i.e., secondary pathogens and saprophytes) [29–31].

4.1. Soil Samplings

As most EPP0 countries prohibit the import of soil and restrict the import of plants with soil from other continents [29], regular sampling of soil to detect *F. circinatum* is not currently required from the plant health officials in Europe. However, it is recommended that soil be collected from the plant rhizosphere and from the first 30 cm of soil below the surface [31]. The standard technique is to collect soil samples in paper bags unless the soil sample is too wet (then a plastic bag is preferable). Note, however, that long-term survival of *F. circinatum* conidia in soil has not been reported, and in artificially inoculated soil, the fungus could not be detected after eight months at temperatures of 20 and 30 °C [32].

4.2. Cone and Seed Samplings

F. circinatum can be present in cones and seeds without visual signs and symptoms, both externally and internally [33,34], and seeds from affected areas are an important pathway for long-distance dispersal of the pathogen. While asymptomatic infections are not typically found in Monterey pine, they are a particularly serious problem in maritime pine (see the last paragraph of this section).

There are no strict rules for sampling cones or seeds [35]. Nevertheless, as they may be asymptomatic, cones and seeds should be sampled in areas with and without symptoms. However, non-symptomatic areas may represent a huge extension to surveyed areas; thus, sampling areas can be chosen on the basis of different criteria, such as their location with respect to possible pathogen sources (for example known infected areas or the proximity of nurseries) and the susceptibility of *Pinus* species (Monterey pine stands should always be sampled, as should the maritime pine stands which are close to them). González-Peñalta et al. [36] found *F. circinatum* in ca. 45% and 32% of the seed lots from forest stands and “sequeros”, which are incubation sites where seeds are kept to foster cone opening. The authors found *F. circinatum* in both Monterey pines and maritime pine. Each lot had a minimum of 100 seeds from 25 different cones for the forest stands, and a minimum of 500 seeds for each “sequero”. However, since seeds are typically collected yearly by different institutions (including governmental, research centers, nurseries, etc.), the analysis of all these seeds represents a good opportunity to examine the health of their source forests.

The sampled cones should be grouped into lots (with a lot defined in International Standards for Phytosanitary Measures (ISPM) 5: as a number of units of a single commodity, identifiable by its homogeneity of composition, origin, etc., forming part of a consignment) that must be packed in sealed plastic bags and labelled separately. Cones should be taken from the tree (not from the ground) using adequate tools which should be disinfected between trees. Cones from symptomatic branches of infected trees will have a higher probability of containing infected seeds that reproduce symptoms after germination, while symptomless branches of infected trees may produce cryptically-infected seeds [34]. Nonetheless, when a tree shows symptoms in particular branches, samples should be collected from them. Cones should be mature, i.e., having fully developed, ripe seeds. In this regard, the sampling of each pine species should be planned for the most suitable period to collect samples in each area/forest/plantation.

Serotinous cones may turn out to be problematic during laboratory processing because the temperatures needed for them to open may damage any present *F. circinatum* propagules, leading to false negative results when molecular detection is not used. However, cone sampling is usually accompanied by the sampling of other tree tissues, such as trunk or twigs. In all likelihood, a sampling strategy based only on cones will not be very effective for most pine species. An exception to this is maritime pine, in which an almost cryptic infection has been described [37,38], thus representing a high risk of uncontrolled pathogen spread via seeds. However, in some cases, the presence of *F. circinatum* can be quite high in seeds. For example, González-Peralta et al. [36] detected *F. circinatum* in 40% of 372 seed lots (139 from orchards and 233 from cones of asymptomatic trees in the forest) of maritime pine, Monterey pine and Scots pine in North-Western Spain.

4.3. Twig and Branch Samplings

Samples from twigs, shoots or branches are collected following a visual inspection to find some of the symptoms produced by the pathogen. The sample should include not only the lesion edge, but also a few centimeters of healthy-looking tissue ahead of the lesion. It is recommended that the pieces of tissue be wrapped in sheets of paper and placed in a sealed plastic bag [29,30]. As *F. circinatum* is a hemibiotroph capable of living in dead tissues, isolation of this species from dead seedlings is usually feasible even from totally necrotic tissue and seedlings that have died weeks ago, and it may also displace other fungi (Jorge Martín, personal communication). It was also pointed out by González-Peñalta et al. [36] that *F. circinatum* was exclusively isolated from ca. 33% samples, whereas other *Fusarium* species (e.g., *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, *F. verticillioides* (Sacc.) Nirenberg, *F. oxysporum* Schltdl. or *F. solani* (Mart.) Sacc.) ranged between 1%–14%. Moreover, Martínez-Álvarez et al. [39] were able to isolate *F. circinatum* from naturally infected Monterey pine twigs, but the isolation frequency obtained for this tissue was very low compared with the value obtained from the xylem inside the cankers of the trunk (close to 100%).

4.4. Canker Samplings

To sample cankers from trunks or branches, the inner bark in the area directly around the visible lesion should be cut away with a sterile blade until the canker margin is observed. Pieces of tissue, including phloem and xylem, should be removed to try to collect portions of the lesion edge, where the fungus is most active (Figure 1). During collection, the pieces of tissue should be wrapped in sheets of paper and placed in a sealed plastic bag [29,30].

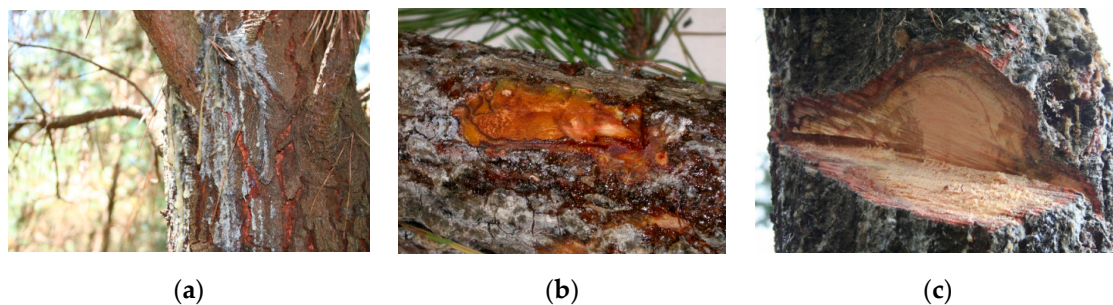


Figure 1. Sampling of cankers caused by *F. circinatum* (a) A canker on Monterey pine (*Pinus radiata*) in the forest (b) collecting wood pieces for culture isolation (c) Gap after wood sampling using a chainsaw. Dark discoloration indicates the progression areas of *F. circinatum*.

Samples collected in such a way are most likely to yield the fungus, as indicated by Martínez-Álvarez et al. [39]. These authors demonstrated that the relative isolation frequency was highest when the sample was collected from a canker in the trunk. Furthermore, the sample extraction method was also important, with the frequency of isolation being higher from samples extracted using an axe or chainsaw than from those extracted using a Pressler borer. The reason for the difference in isolation frequency between the two techniques may be the subsequent surface disinfection of the sample in the laboratory. Samples collected with the Pressler borer are typically thinner than those collected with an axe or chainsaw, leaving the vessels open, and therefore, making it easier for the alcohol and the bleach used in the surface disinfection process to penetrate deeper into the tissues and kill the fungal community, including the target pathogen.

4.5. Root Samplings

Roots should be sampled at an approximate distance of 1 m from the collar region of the tree and obtained from the surface at 20–30 cm depth. The sampling of roots can be done at the same time as sampling of the rhizosphere soil. Root diameters can vary from 0.2 to 5 mm, and roots can be apparently healthy, regardless of whether they belong to non-symptomatic or symptomatic trees. Samples should be kept in paper bags during transport and storage [40].

4.6. Spore Trapping

Spore traps can be used to monitor the presence and means of dispersal (mainly air and rain splash) of fungal pathogens or to study their life cycle and temporal dynamics. Although temporal patterns of *F. circinatum* spore dispersal have not been investigated in detail, it is known that they are dispersed by air, rain and insect vectors [15,41], and have a limited natural dispersal distance [42].

A number of different protocols have been used to trap spores of *F. circinatum*. The first spore sampling study of *F. circinatum* was done in California by Schweigkofler et al. [43] using filter paper as spore traps. DNA of the trapped spores was extracted and amplification by a real-time PCR using specific primers (CIRC1A-CIRC4A, which amplifies 360-bp from IGS region) to detect and quantify the presence of inoculum in the air. Garbelotto et al. [42], also working in northern California, performed the first continuous long-term measurement of spore deposition rates of *F. circinatum*. They studied the life cycle of the pathogen throughout the year with spore traps being collected every two weeks, using the method of Schweigkofler et al. [43]. The same approach was used in South Africa by Fourie et al. [44], who measured spore deposition rates within a commercial seedling nursery. Most recently, Quesada et al. [45] developed a new low-cost spore trap for collecting *F. circinatum* spores, based on a rotating motor that holds a metal rod and two petroleum jelly-coated microscope slides, which they also subjected to the quantification using the method of Schweigkofler et al. [43].

In Europe, the first (and as yet only) study of temporal dynamics of spore dispersal was carried out by Dvořák et al. [46] using an actively rotating arm spore trap ROTTRAP 120 (Dvořák, Boršov

and Vltavou, Czech Republic). Over one year, spore traps were run for 48 h twice per month. They also monitored the air inoculum splashed by raindrops by sampling of rain splash close to the soil surface using an open Petri dish during the 48-h sampling period. After extraction of DNA from the samples, real-time PCR was used to detect and quantify the pathogen using the specific primers of Ioos et al. [47].

4.7. Sampling of Insect Vectors of *F. circinatum*

One of the possible pathways of *F. circinatum* spread is by insects (bark beetles, wood borers, shoot and foliage-feeders, cone insects, predatory insects, sap-feeding insects), which can carry propagules from diseased to not yet infected trees [15]. Some of the species reported to transport *F. circinatum* propagules are: *Pityophthorus* spp. (*P. setosus* Blackman, *P. nitidulus* (currently *P. nigrigans* Blandford), *P. carmeli* Swaine, *P. pubescens* Swaine), *Ips* spp. (*I. mexicanus* Hopkins, *I. paraconfusus* Lanier, *I. plastographus* LeConte, *I. sexdentatus* Börner), *Tomicus piniperda* L., *Conophthorus radiatae* Hopkins, *Ernobius punctulatus* LeConte, *Hylurgops palliatus* Gyllenhal, *Hypothenemus eruditus* Westwood, *Hylastes attenuates* Erichson, *Orthotomicus erosus* Wollaston [48] and the references within]. In Europe, only *T. piniperda* has been shown to be an effective vector of *F. circinatum* [49].

In studies that have considered the associations between *F. circinatum* and its vectors, potential vectors have been trapped using baiting logs [50,51], sliding funnel traps baited with (E)-pityol, ethanol and α -pinene [49,52,53], flight-intercept slit traps baited with ipsdienol pheromone [54] or ethanol and α -pinene [55], crosstraps baited with Galloprotec Pack [54], and multiple funnel traps baited with pheromones [56].

5. Recommendations for Sample Processing in the Laboratory

General recommendations for processing samples in the laboratory to detect *F. circinatum* have been given by EPPO and IPPC [29,30]. It should be noted that the pitch canker fungus is capable of infecting all organs of the host plant, and conidial spores of the fungus are also able to survive for several months in the soil [32]. Optimal processing of the samples in the laboratory is crucial for successful isolation or molecular detection of the fungus.

5.1. Media for the Isolation of *F. circinatum*

A large array of media may be used for the isolation of *F. circinatum* from the samples collected in the field. These include general isolation media such as PDA (Potato Dextrose Agar) and a variety of semi-selective growth media (Table 5). Those used most commonly are PDA supplemented with streptomycin (PDAS) [29], PDAspd (PDA supplemented with dichloran, streptomycin and pentachloronitrobenzene), dichloran chloramphenicol peptone agar (DCPA) [57,58], Komada's medium [59], *Fusarium* Selective Medium (FSM) [60] or modified FSM [38].

Plant material collected in the field, such as branches, pine cones or wood chips from cankers is disinfected prior to plating on agar medium in the laboratory. The surface disinfection protocol includes successive submerging in ethanol (50%–70%) and/or sodium hypochlorite (1.5%–7%) for 1–2 min, followed by rinsing twice in sterile distilled water. Inoculated plates are incubated at 20–25 °C in the dark for approximately one week, after which growth from suspected *Fusarium* colonies is transferred to water agar in order to obtain pure cultures originating from single conidia. The resulting cultures are then transferred to an appropriate growth medium used for identification of *F. circinatum* (described in Section 6.1 and Table 5).

Table 5. Composition of the various growth media used for the isolation and identification of the pitch canker fungus, *Fusarium circinatum*. See the text for references.

Medium Name	Abbreviated Name	Purpose	Ingredients for 1 L of Medium
Potato dextrose agar	PDA	Isolation, identification and routine culturing	20 g dextrose, 20 g agar, broth from 250 g white potatoes, distilled water to 1 L (a commercial preparation of PDA can also be used)
Potato dextrose agar supplemented with streptomycin	PDAS	Isolation	PDA ingredients, 0.5 g streptomycin, distilled water to 1 L
Potato dextrose agar supplemented dichloran, streptomycin and pentachloronitrobenzene	PDAspd	Isolation	PDA ingredients, 2 mg dichloran, 0.5 g streptomycin, 0.2 g pentachloronitrobenzene (PCNB), distilled water to 1 L
Dichloran chloramphenicol peptone agar	DCPA	Isolation	15 g peptone, 1 g KH ₂ PO ₄ , 0.5 g MgSO ₄ ·7H ₂ O, 20 g agar, 2 mg dichloran (2,6-dichloro-4-nitroanilin), 0.2 g chloramphenicol, 0.05 g violet crystal, distilled water to 1 L
Komada's medium	-	Isolation	1 g K ₂ HPO ₄ , 0.5 g KCl, 0.5 g MgSO ₄ ·7H ₂ O, 10 mg Fe-Na-EDTA, 2 g L-Asparagine, 20 g D-Galactose, 15 g agar, 0.5 g PCNB, 0.25 g oxgall, 0.2 g chloramphenicol (or 0.15 g streptomycin), distilled water to 1 L (pH 3.8)
<i>Fusarium</i> selective medium	FSM	Isolation	1.0 g KH ₂ PO ₄ , 0.5 g MgSO ₄ ·7H ₂ O, 0.2 g PCNB, 10 mL of a streptomycin sulfate stock solution (30 mg/mL), 15.0 g peptone, 20.0 g agar, distilled water to 1 L
Modified <i>Fusarium</i> selective medium	Modified FSM	Isolation	The same as FSM, but containing 3 g streptomycin, 50 mg Rose Bengal, 1 g PCNB, and 1 g neomycin
Spezieller-Nährstoffarmer agar	SNA	Morphological identification	1 g KH ₂ PO ₄ , 1 g KNO ₃ , 0.5 g MgSO ₄ ·7H ₂ O, 0.5 g KCl, 0.3 D-glucose, 0.2 sucrose, 20 g agar, distilled water to 1 L
Water agar	WA	Identification	20 g agar, distilled water to 1 L
Carnation leaf agar	CLA	Morphological identification	WA overlaid with sterile pieces of carnation leaves
Complete medium	CM	Sporulation medium	30 g sucrose, 2 g NaNO ₃ , 2.5 g N-Z Amine, 1 g yeast extract 1 g, Vitamin stock solution 10 mL, 1 L of distilled water (Vitamin stock: 4 g inositol, 200 mg Ca pantothenate, 200 mg Choline-Cl, 100 mg thiamine, 75 mg pyridoxine, 75 mg nicotinamide, 50 mg ascorbic acid, 30 mg riboflavin, 5 mg <i>p</i> -aminobenzoic acid, 5 mg folic acid, 5 mg biotin, 50:50 ethanol:H ₂ O to 1 L)
Carrot agar	CA	Sexual compatibility tests	400 g peeled and diced carrots, 20 g agar, distilled water to 1 L

5.2. Samples from Branches, Twigs, Cankers, Roots and Seedlings

When examining samples from trees, the xylem surrounding the canker is the best part from which to isolate the fungus [39], but it can also be isolated from the phloem. The recommended size of the wood xylem sample is a lamina of 4–5 mm in width, to keep the internal parts of the mycelium viable during the surface disinfection treatment. The sample is then cut into small pieces after removing borders necrotized by the aseptic compounds used for surface disinfection. A similar approach is used for other plant organs such as roots, cones and branches.

In the case of nursery seedlings, entire plants, including roots, are usually sampled, unless the seedlings are particularly large. In the laboratory, the seedlings are washed thoroughly with water to remove soil before isolation is carried out. In the case of symptomatic plants, isolation is made from the edge of lesions found beneath the affected bark, mostly from stem bases or from the roots. In asymptomatic plants, different parts of the root, root collar and stem base are plated randomly, including roots of different diameters, from main root to fine roots.

5.3. Seed Samples

Seeds are not surface disinfected because they can be colonized by *F. circinatum* both internally and/or externally. Seeds should be sampled randomly, as no symptoms are visible. The number of seeds that are sampled per seed lot varies in different countries (e.g., 500 in Spain, 1000 in France and Portugal),

and this number may be determined as described by the IPPC (ISPM No. 31) [61]. Accordingly, there is also no standard protocol on how to process seed in the laboratory. In Spain, however, half of each sample (i.e., 250 seeds) is incubated on PDAS medium and the other half on Komada's medium (Table 5) (The Government of Spain; Real Decreto 637/2006). Methods based on in vitro cultures focus mainly on the detection of *F. circinatum* externally. However, a preliminary biological enrichment step can be conducted prior to molecular detection of the pathogen by conventional or real-time PCR analysis in order to enhance the sensitivity of the detection for low levels of infection [47].

In certain cases, a seed lot can be composed of millions of seeds, and stored in several containers. In these cases, seeds should be sampled representatively; thus, one sample should contain seeds from each container and from different depths. In general, rules for seed testing are described by ISTA [62]. Occasionally, the percentage of infection in the lot is very low (1–5 seeds per 500), making analyses difficult and the introduction of the fungus due to detection failure more likely.

5.4. Soil Samples

In the laboratory, the collected samples of rhizosphere soil are air-dried in a laminar flow hood for 24 to 48 h, and then ground in order to generate soil particles of a consistent size for use in soil dilutions; finally, they are plated onto a growth medium [31]. Alternatively, the method described by Hernandez-Escribano et al. [38] can be used for detecting the presence of *F. circinatum* in the collected soil.

Briefly, approximately 500 g within 15 cm of the soil surface is collected per soil sample and any plant debris is removed from the soil, after which it passed through a 2 mm sieve. A soil suspension is then prepared by adding 10 g of the sieved soil to 100 mL sterile distilled water and shaking at 150 rpm for 30 min. From this suspension, three serial 10-fold dilutions are prepared, of which 400 µL of each suspension is plated onto Petri plates containing modified FSM (Table 5). The inoculated plates are then incubated in the dark at 25 °C and monitored for 3 weeks. Putative colonies of *F. circinatum* are transferred to medium for identification (described in Section 6.1 and Table 5).

5.5. Insect Samples

Trapped insects are directly squashed onto *F. circinatum* selective medium (e.g., 50, 51) or subjected to sonication with subsequent plating onto one of the *F. circinatum* selective media [49,55]. *F. circinatum* isolates are identified based on micromorphological characteristics and DNA sequencing. Fourrier et al. [54] developed a method for the rapid detection of as few as 10 conidia of *F. circinatum* from a trapped beetle using a real-time PCR approach (described in Section 6.7).

6. *F. circinatum* Morphological Identification

EPPO and IPPC have given general instructions for the identification of *F. circinatum* based on morphological features of mycelia [29,30].

6.1. Culture Media for the Identification of *F. circinatum*

In order to morphologically identify *F. circinatum* and to differentiate it from other species in the *Fusarium fujikuroi* species complex (FFSC) [63,64], isolates should be grown on several media (Table 5). These include Spezieller-Nährstoffarmer agar (SNA) [65] and Carnation Leaf Agar (CLA) [66,67]. For the examination of pigmentation and colony morphology, PDA is used, while complete medium and Carrot agar are typically used for sexual compatibility tests (Table 5) [31].

6.2. Characters Associated with the Sexual Stage of *F. circinatum*

All fungi in the FFSC, including *F. circinatum*, are heterothallic and require isolates of opposite mating type for completing the sexual stage [68]. All isolates of the fungus harbor either the MAT1-1 or MAT1-2 "idiomorphs" at the mating type locus; the mating type of an isolate can thus be diagnosed

using PCR-based approaches [69,70]. A fertile cross between an isolate presumed to be *F. circinatum* with a standard tester strain for the species further provides strong evidence of conspecificity [31]. Because these tester strains are hermaphrodites, they may be used routinely as the female-fertile partner in laboratory crosses with field isolates that are commonly male-fertile [31]. Spores produced by the presumptive strain grown on Complete medium are typically used to fertilize a Carrot agar culture of the mating tester strain (Table 5) [31].

When cultivated on Carrot agar, perithecia produced by *F. circinatum* are ovoidal to obpyriform, non-papillate and slightly warted around the apex. They can be superficial or immersed, non-stromatic and have a dark purple color in water and 3% KOH, becoming red in 100% lactic acid. Neither ostiolar canals nor periphyses are formed. The cylindrical asci have an apex with a shallow, refractive ring. The ascospores are ellipsoidal to fusiform, smooth, hyaline and septate, with additional septa developing after discharge when they turn pale brown [63,71]. It should be noted, however, that the sexual stage of *F. circinatum* has never been observed under field conditions.

6.3. Characters Associated with the Asexual Stage of *F. circinatum*

For identification purposes, *Fusarium* (*F. circinatum* and other *Fusarium* species) colonies are sub-cultured on SNA medium [29,65] that may be supplemented with two pieces of sterile paper [72]. This medium is used to produce phialides, microconidia and sterile hyphae (distinctly or not distinctly coiled), while CLA can be used to produce uniform macroconidia and the evaluation of sporodochial color [31]. These structures are produced in SNA after ca. 10 days, and on CLA after ca. 20 days, at 25 °C in the dark and are visible using bright-field microscopy at 400× magnification.

In order to study colony morphology and pigmentation of the fungus, isolates should be grown on PDA and incubated at 22 °C ± 6 °C under near UV light or in daylight for ten days [30]. On PDA, *F. circinatum* grows quite rapidly, approx. 4.7 mm/day at 20 °C [63] but ranging from 2.5 to 9.0 mm/day [73,74]. The mycelium produced has entire margins, and white cottony or whitish aerial mycelium with sometimes a salmon-colored tinge in the middle but often producing a pigment in the agar that could range from purple to dark violet or even yellow [13,30,31].

Asexual structures (i.e., macro- and microconidia, Figure 2a–c,e and conidiogenous cells; polyphialides and monophialides, Figure 2a,b) are produced by *F. circinatum* both in culture and on plant tissues. Phialides (i.e., specialized conidiogenous cells that produce conidia in chain succession) of the aerial conidiophores are cylindrical and can be both mono- and poly- phialidic [75,76]. In order to study the type and shape of asexual structures, isolates must be grown on SNA for 10 days at 22 °C ± 6 °C under near UV light or in daylight. After that time, isolates are examined and compared to the *F. circinatum* descriptions of Nirenberg and O'Donnell [63], Britz et al. [71] and Leslie and Summerell [31].

On SNA, sclerotia are absent. Microconidia are aggregated in false heads (not in chains), with branched and erect conidiophores that can end in one or two phialides (Figure 2e). The microconidia in the aerial mycelium are usually obovoid, seldom oval and mostly non-septate or occasionally one-septate: (7.0–)8.5 – 9.7 – 10.9(–12.0) × (2.5–)2.8 – 3.2 – 3.6(–3.9) μm [63]. Macroconidia are cylindrical, mostly three-septate, with slightly curved walls, an apical cell that narrows to an inwardly (i.e., toward the ventral side) curved tip, and a foot-shaped basal cell: (32.0–)33.7 – 38.2–42.7(–48.0) × (3.2–)3.4–3.6—3.7(–3.8) μm [63]. Chlamydospores are absent [13,29,30,63], although many other *Fusarium* species have chlamydospores. However, some *F. circinatum* isolates produce swollen hyphae that may be confused superficially with chlamydospores or pseudochlamydospores [31].

On CLA, sporodochia with macroconidia are sparse and may be difficult to find. When found, they are usually on carnation leaf pieces and are pale orange in color. Macroconidia are slender, thin-walled, usually three-septate and typical of those produced by most species in the FFSC. Microconidia are abundant in the aerial mycelia, being obovoid, oval or allantoid in shape, non-septate and borne on mono- and poly- phialides which may proliferate extensively [31].

F. circinatum produces coiled sterile hyphae on both SNA and CLA during incubation in the dark [63,77], but these structures are more easily and reliably observed on SNA [13] (Figure 2d). The species' epithet "*circinatum*" refers to these structures. The production of these distinctive flexuous/sinuuous sterile hyphae used to distinguish *F. circinatum* from other species in the FFSC. However, a number of *F. circinatum* isolates from a wide geographical range (Europe, America, Africa, and Asia) and different mating types were recently found with sterile hyphae which were not distinctively coiled or absent, indicating that coiled hyphae are not a fully reliable attribute for the diagnostic of the species [72,74]. Thus, it is recommended that the morphological identification of *F. circinatum* is verified with molecular tools [30].

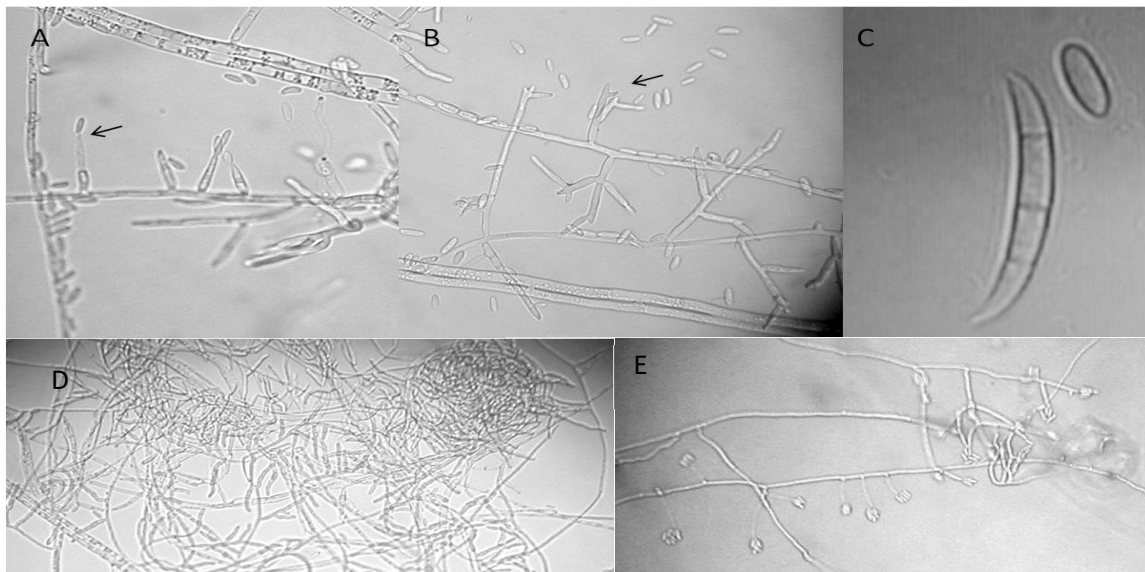


Figure 2. Morphological characters of *F. circinatum* (A) monophialides on Spezieller-Nährstoffarmer Agar (SNA) (B) polyphialides on SNA (C) micro- and macroconidium on Carnation Leaf Agar (CLA) (microconidia are also visible in a, b and e) (D) *Fusarium circinatum* coiled sterile hyphae on SNA (E) *Fusarium circinatum* microconidia growing in false heads on SNA. Spore sizes are given in the text.

6.4. Maintenance and Conservation of *F. circinatum* Isolates

As in the case of most filamentous fungi, *F. circinatum* can be maintained on artificial media as a short-term preservation method, but losses in virulence, pathogenicity and sporulation may occur with repetitive subculturing (e.g., [78,79]). For long-term preservation of fungal cultures, immersion of the mycelium or spores of the fungus in sterile distilled water is one of the simplest methods [80]. However, since the stability of fungal cells was not ensured by this simple procedure [81], other methods have been suggested. For instance, the maintenance of cultures under a layer of mineral oil has been used for many years as a method to reduce growth and extend the time interval between transfers to fresh media [82]. This technique could be a good option for fungi that do not tolerate freeze drying, or where cryogenic storage is not possible [78].

There are other methods which are more appropriate and with higher success rates. For example, Windels et al. [83] succeeded in preserving different *Fusarium* spp. on soil for 5 years. The authors suggested that the storage temperature of 4.5 °C rather than room temperature explained the higher viability of the isolates after the preservation period. One of the inconveniences of storage in soil is that *Fusarium* cultures colonize the substrate, and there is consequently a chance of cultural variation [84]. For this reason, using silica gel rather than soil is preferred, since cultures do not colonize this alternative substrate [83]. Furthermore, it has been reported that not all *Fusarium* spp. remain viable in soil [66]. Although the silica gel technique is not as effective as freeze-drying or liquid-nitrogen storage (see the last paragraph of this section), it is considered a useful alternative for laboratories with limited

resources or those requiring a simple technique [82]. Cereal grains are recognized as another suitable substrate for the preservation of fungal cultures. However, as is the case with soil, they do not prevent fungal metabolism and growth, and thus, risks of physiological and genetic change exist [83].

Although a variety of methods can be used to store *Fusarium* spp. and other fungal cultures, cryopreservation in liquid nitrogen and lyophilization are the methods recommended and used in most recognized laboratories, such as the American Type Culture Collection [81]. The colder the storage temperature and the higher the temperature stability, the greater the likelihood for long-term viability [78,85]. To increase the viability of the cultures after preservation by lyophilization, isolates should be grown on CLA in Petri dishes for 7–10 days. Several colonized carnation leaf pieces are then transferred to sterile vials, and 0.5 mL of sterile skim milk can be added to each vial as an additional cryoprotective substance before lyophilization [66]. The recommended storage temperature for lyophilized cultures is 5 °C or lower, but some fungus cultures are stored at temperatures as low as –52 °C [66]. A simpler and less expensive alternative to conventional freeze-drying methods, but one that is similar in efficacy, particularly with *Fusarium* spp., is the modified filter paper method described by Fong et al. [86]. This methodology was found to be very effective (100% of isolates were viable after four years) for preservation of *F. oxysporum* cultures at 19 °C. *F. circinatum* can also be preserved using cellulose paper (personal communication by J. Diez).

6.5. Molecular Methods for Pathogen Detection

The symptoms caused by *F. circinatum*, particularly in seedlings, stems and shoots may be similar to those caused by several other pathogenic fungi, such as *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton, *Gremmeniella abietina* (Lagerb.) M. Morelet, *Pachyramichloridium pini* (de Hoog & Rahman) C. Nakash., Videira & Crous, *Crumenulopsis sororia* (P. Karst.) J. W. Groves, *Cenangium ferruginosum* Fr. and *Caliciopsis pinea* Peck. In addition, needle symptoms may resemble those caused by species of *Dothistroma*, *Cyclaneusma*, *Lecanosticta*, *Lophodermium* and *Lophodermella* (although the defoliation caused by these species is not as severe as in the case of *F. circinatum* that causes dieback of whole branches and shoots). *Botrytis* spp. also cause similar needle blight in pine seedlings. It is consequently important to confirm the agent causing symptoms resembling those of *F. circinatum* by laboratory analysis. The Pine pitch canker field guide of the EU COST Action FP1406 PINESTRENGTH describes the above-mentioned pathogens causing symptoms similar to *F. circinatum* in more detail (<http://www.pinestrength.eu/2018/04/12/disseminationpitch-canker-field-guide-wg-1-pamphlet/>).

When strains suspected to be *F. circinatum* are obtained after isolation from plant tissue, their identity can be determined by species-specific PCR [43,72,87] or by sequence and phylogenetic analysis of several markers, such as the Translation Elongation Factor 1 alpha (Tef1) gene [88]. As an alternative to isolation in pure culture, *F. circinatum* can be detected directly in planta by conventional PCR and real-time or quantitative PCR (qPCR) using SYBR Green or hydrolysis probes (see Sections 6.6 and 6.7). Similar techniques have been also described to detect trapped airborne propagules of *F. circinatum* [43,44]. A recent collaborative study joined 23 partners to assess the transferability and performance of nine different protocols (conventional PCR and real-time PCR) using a wide panel of *F. circinatum* and other *Fusarium* spp. DNA [89].

6.6. Diagnosis Using Conventional PCR

The first species-specific PCR test for the detection of *F. circinatum* was developed by Schweigkofler et al. [43]. The PCR primers target specific regions located in the ribosomal RNA (rRNA) Intergenic Spacer (IGS). The test was initially designed to be used in a real-time PCR protocol using SYBRGreen as a DNA staining dye, but could alternatively be used in conventional PCR. Although the test proved to be specific at the time of the study, the authors recommended sequencing the PCR amplicon to check for cross reactions with DNA of as yet unknown but closely related cryptic species. Annex 4 of the EPPO diagnostic protocol PM7/91 [29] for *F. circinatum* proposed a modified version of the Schweigkofler et al. test in which the final MgCl₂ concentration of the PCR reaction is

decreased to 2 mM, instead of the 5 mM used in the original article, and for which the test is run in conventional PCR. Despite a lower final MgCl₂ concentration increasing the specificity of the reaction, the modified protocol proposed in PM7/91 also recommended that any positive result should be confirmed by amplicon sequencing and comparison to reference sequences by BLAST analysis on GenBank. PM7/91 recommends this modified protocol for the direct detection of *F. circinatum* in plant tissue, including seeds.

Another conventional PCR assay was developed by Ramsfield et al. [87]. This test works in a multiplex fashion, and two loci of unknown function are targeted to be co-amplified if *F. circinatum* DNA is present. This test may be used to detect *F. circinatum* in plant, soil or potting mix. Since the test requires the simultaneous amplification of two unrelated but specific regions of the *F. circinatum* genome, it has been suggested that it brings additional reliability. The conventional PCR tests described above are easy to set up and need less expensive equipment than qPCR methods, but require an additional electrophoresis step, and thus, more time to obtain a result [89].

6.7. Diagnosis Using qPCR

Several protocols targeting *F. circinatum* have been developed in the past ten years which take advantage of real-time PCR technologies and reduce turnaround time for analysis. Whether using a double-stranded DNA staining dye such as SYBR Green or a specific hydrolysis probe (also called “double-dye”, “TaqMan[®]”, or “dual-labelled”), these technologies bring an additional level of specificity due to specific binding of the probe or melting curve analysis of the SYBR Green stained DNA of the PCR product.

Dreaden et al. [90] and Fourie et al. [44] proposed a real-time PCR protocol targeting specific regions of the IGS of the rDNA. The test of Fourie et al. [44] was particularly optimized to detect *F. circinatum* conidia in traps set up in nurseries and to measure airborne inoculum pressure. These protocols work in real-time but require an additional melting curve analysis of the PCR products at the end of the run. The melting temperature (T_m) of the amplicons (i.e., the so-called denaturation peaks) is measured and compared to that of the positive control. The T_m is a function of the size and the sequence composition of the amplicon; therefore, by comparing the T_m of the positive control to those of the unknown DNA samples, it is possible to confirm a positive result. SYBR Green real-time PCR requires real-time PCR equipment, but it involves less costly reagents than hydrolysis probe technology. Nevertheless, interpretation of the data is not always straightforward, and the T_m of the denaturation peaks is sometimes variable.

An additional level of specificity was achieved by Ioos et al. [47] using a probe that was initially designed in the IGS region to detect minute amounts of *F. circinatum* DNA in pine seeds, after a biological enrichment step. This test was later adapted to detect *F. circinatum* from potential insect vectors [54]. Lamarche et al. [91] proposed an alternative assay also targeting the IGS region, which was validated with seeds, woody tissue and insects. Another locus in the *F. circinatum* genome was targeted by Luchi et al. [92], who used the Tef1 gene to develop a duplex real-time PCR test for the simultaneous detection of the pine pitch canker pathogen and *Caliciopsis pinea* in pine woody tissues. This test may be useful since both pathogens share the same ecological niche on Monterey pine causing similar bleeding canker symptoms, especially at the early stage of colonization.

6.8. Specificity of DNA-Based Tests

Given the importance of *F. circinatum*, a quarantine fungus for several countries, diagnostic tests utilized must provide reliable results, with a particular emphasis on specificity. *F. circinatum* belongs to the American clade of the FFSC [64,76], which accommodates many genetically- and morphologically-related species. New species in this clade are continuously being described, such as the new pine pathogenic species *F. marasasianum* Herron, Marinc. & M. J. Wingf., *F. parvisorum* Herron, Marinc. & M. J. Wingf., and *F. sororula* Herron, Marinc. & M. J. Wingf. [93]. It is therefore of great importance that the specificity of each test published is continuously re-assessed in light of

the current genetic diversity of the FFSC. It is also recommended that several tools targeting specific, but different parts of the *F. circinatum* genome are used in a complementary way in order to confirm the diagnosis, and/or that the specific amplicons are sequenced to verify the result. In addition to existing tests targeting known taxonomic markers, several other *F. circinatum*-specific regions have been unraveled by in silico analysis, and these putative genes should be used for the development of new detection tools, either by PCR, real-time PCR or LAMP analysis [94]. Inclusivity (i.e., ability to target all *F. circinatum* strains) and specificity (i.e., ability not to cross-react with non-target species) of the currently available molecular detection tools targeting *F. circinatum* have been recently assessed and compared [89]. Overall, the study demonstrated that current diagnostic protocols for the detection of *F. circinatum* is not easily transferable, and that end-users should perform validation tests, especially under modified conditions, such as different reagents and/or equipment, prior to use.

7. Global Situation

Due to early detection, constant surveillance and control measures, *F. circinatum* outbreaks are considered officially eradicated in France and Italy (<https://gd.eppo.int/taxon/GIBBCI/distribution>). The outbreaks in France occurred on *P. menziesii* and *Pinus* spp. nurseries and gardens [10,95–97], and in Italy in urban parks on Aleppo pine (*P. halepensis* Mill.) and Italian stone pine (*P. pinea* L.) [9]. *F. circinatum* is widely established in northern Spain (Galicia, Cantabria, Navarra and Basque Country), mainly affecting Monterey pine plantations, but it is also present in maritime pine stands (Drenkhan et al., unpublished). In Portugal, *F. circinatum* was exclusively reported from nurseries for years, but was also recently detected in mature trees from a Monterey pine plantation. However, all trees were destroyed and the surrounding area intensively surveyed, and the pathogen has not been detected since (H. Bragança, personal communication). From an epidemiological point of view, it is highly problematic that *F. circinatum* can persist as a latent endophyte in several plant species, including conifers such as *Picea* and *Larix* that are not regulated in the EU legislation [19].

The introduction of *F. circinatum* into new environments has in some countries led to more severe epidemics. For example in South Africa, *F. circinatum* was detected in 1990 from a single nursery in the Mpumalanga province [5]. Since then, it has been detected in various forestry nurseries across South Africa, causing seedling disease as well as field establishment problems [98–100]. Fifteen years later, in 2005, *F. circinatum* was reported on established plantation trees for the first time [101]. Today, it is a problem in the coastal and mountain regions of the Western and Eastern Cape, as well as in the KwaZulu-Natal midlands, causing disease on Monterey pine and Gregg's pine (*P. greggii* Engelm. Ex Parl.) [102,103]. More recently, however, pitch canker was also detected in the Limpopo province on mature *P. patula* [104]. This report is of significance to South Africa, since Mexican weeping pine (*P. patula* Schiede ex Schltdl. & Cham.) is the most widely planted species in the country. If one considers the origin and movement of *F. circinatum* by studying the genetic makeup of populations, it is clear that populations appear to be interconnected, but also diverse and different. Overall, the results suggest that multiple introductions and subsequent movement (either between plantations or from planting material) are responsible for the presence of pitch canker in South Africa.

8. Conclusions

F. circinatum is listed among the species recommended for regulation as quarantine pests in Europe, and the EFSA considers the probability of new introductions into the EU very likely. Each country has adapted its survey program according to the presence or absence (and proximity) of the pathogen, tree species distribution and environmental conditions. This results in large differences in the number of surveys and samplings conducted at different sites hosting susceptible species. Laboratory methods used for pathogen detection are also diverse but mostly based on EPPO recommendations.

The global spread of *F. circinatum* in the relatively recent past suggests that the pathogen will continue to be encountered in new environments in the future. The ease with which *F. circinatum* has moved globally also suggests that new genotypes of the pathogen will be introduced to new

areas, including those where the pathogen already occurs. In some areas, such as those where plantation forestry based on non-native species is practiced, this will result in lost productivity. Clearly, continuous surveillance of reproductive material (seed and seedlings), nurseries, plantations and forests, prompt control measures, and realistic contingency plans (PLURIFOR, 2019; <https://plurifor.efi.int/es/chancro-resinoso-del-pino/>) are of the utmost importance in Europe and elsewhere to limit disease spread. It is important to recognize that new disease outbreaks in countries outside Europe are also relevant to European countries. In this regard, the “bridgehead effect”, where new introductions of a tree pathogen become increasingly likely as new environments are invaded, must be considered [105].

Author Contributions: All authors contributed to original draft preparation, review and editing of the article.

Funding: This article is also based upon work carried out during COST Action FP1406 PINESTRENGTH (Pine pitch canker-strategies for management of *Gibberella circinata* in greenhouses and forests), supported by COST (European Cooperation in Science and Technology). This study was supported by project AGL2015-69370-R “Nuevas tecnologías de secuenciación para el estudio de los micovirus en *Fusarium circinatum*” (MINECO/FEDER, UE). This work is a contribution of URGENTpine (PTDC/AGR-FOR/2768/2014) funded by Fundação para a Ciência e a Tecnologia, I.P., through national funds, and the co-funding by the FEDER (POCI-01-0145-FEDER-016785), within the PT2020 Partnership Agreement and Compete 2020. Thanks are due for the financial support to CESAM (UID/AMB/50017/2019), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. FCT also supported J.M.-G. (SFRH/BPD/122928/2016). The Forestry Commission UK provided financial support for M.M. and K.T.

Acknowledgments: Tiziana Baschieri (Servizio Fitosanitario Regione Emilia Romagna), who is the national coordinator for surveillance of *Fusarium circinatum* in Italy, is warmly acknowledged for providing summary data for the Italian territory. Dina Ribeiro from the Portuguese Forest Authority is thanked for providing data from Portugal. Odile Colnard from the French Ministry of Agriculture, is thanked for providing data for France. Kristof Capieau from the Swedish Board of Agriculture is thanked for providing data for Sweden.

Conflicts of Interest: The authors declare no conflict of interest.

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