



Euphresco

Final Report

COSEPATH
Seed-borne pathogens of conifers

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Contents

1. Research consortium partners	3
2. Short project report.....	7
2.1. Short executive summary.....	7
2.2. Project aims	8
2.3. Description of the main activities.....	9
2.4. 4. Main results	13
2.5. Conclusions and recommendations to policy makers.....	20
2.6. Benefits from trans-national cooperation.....	21
3. Outreach.....	23
3.1. Article(s) for publication in journals.....	23
3.2. Grey literature	23
3.3. Events	23
4. Open Euphresco data	24



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2. Short project report

2.1. Short executive summary

A total of 14 partners from 13 countries (Austria, Belgium, Germany, Italy, New Zealand, Norway, Poland, Portugal, Sweden, Switzerland, Turkey, USA, UK) committed to this project with the aim of gathering current knowledge on seed borne pathogens with a special focus on conifer species of interest for the participant countries and to generate data on the movement of conifer seed, focusing on the most traded conifer genera and/or species. This project also provided the opportunity of comparing and run in parallel testing of seed lots using the traditional and molecular methodologies by participant laboratories with the aim of harmonising methodologies for the detection of pathogens.

An exhaustive review of the literature on seed borne pathogens of conifers was carried out and a total of 153 bibliographic references were collected during the project. The interceptions concerning conifer seeds were extracted for the period 2012-2022 from official sources and a total of 87 "non-compliant" conifer seed interceptions were identified over this period, mostly for administrative problems (incomplete documents). Only one case of interception of *Gibberella circinata* in 2017 on *Pinus taeda* seeds originating from USA. Most of the interceptions concerned *Pinus* seeds.

Knowing the movement in international trade of conifer seed is important to understand the risk of this pathway for invasive alien species. Mapping trade patterns over time helps to understand geopolitical environments that may lead to changes in the risk profile of the transboundary movement of conifer seed. A database was created of the export and import volumes of tree seed internationally to enable an analysis of trade patterns of conifer seed for planting over time and different analyses were performed and one of the analyses indicates that overall North America is a growing exporter of forest tree seed, especially for south Europe and east Asia. The imports to Europe grew from 37,318 during 2005-2009, to 2.3 x 10⁶ kg in 2010-2014 which doubled during 2015-2019. East Asia imported 94,665 during 2005-2009, and around 5 x 10⁵ kg in each of next two time periods (2010-2014 and 2015-2019). Trade from Mediterranean Europe to Western Asia also increased significantly, while trade within Mediterranean Europe declined significantly over these time periods. The largest trading years apparent in the data were 2009 and 2016, when large volumes of seed were exported from Spain to Portugal (2009) and from Portugal to Türkiye (2016).

Seed-associated fungi were recovered from all tree species analysed and with each isolation protocol applied. However, important differences in the incidence of seed-associated fungi as well as in the diversity and composition of the fungal communities were observed among tree species and isolation protocols. In the comparison of culturing-based and HTS methods for detection of conifer seed fungi, our analyses showed that the diversity of seed-borne fungal taxa as revealed by HTS (in our case Illumina MiSeq sequencing) is considerably higher than when assessed by plating the seeds on agar media. Thus, for studies aiming at investigating the diversity of seed-borne fungi a high-throughput amplicon sequencing approach should be applied. However, it should not be forgotten that such an approach does not allow to assess whether the fungi detected are alive or not.

During the duration of this project, researchers from different partners reviewed some scientific articles on seed-borne/transmitted pests on conifers to collaborate with ISTA Reference Pest List project. This work was coordinated with Nicolas Denancé from the Groupe d'Étude et de contrôle des Variétés Et des Semences in France. The information was part of a publication in which researchers who contributed were acknowledged: Denancé N & Grimault V (2022). Seed pathway for pest dissemination: The ISTA Reference Pest List, a bibliographic resource in non-vegetable crops. EPPO Bulletin 52:434 – 445).



Another outcome of the project was a session that was proposed on ‘The biosecurity risks of international movement of tree seeds’ at the 26th IUFRO World Congress that will be celebrated in 2024 in Stockholm (Sweden). The session was accepted and will be integrated within the theme ‘Strengthening Forest resilience and adaptation to stress’. Researchers from this project will be coordinating this session and the work carried out in this project has been submitted as an oral presentation.

Finally, a review paper entitled “The biosecurity risks of international tree seed movements” (Fracic et al.) has been submitted to Current Forestry Reports. This article addresses the issues of seed transport (e.g., what are the pathways for the introduction of infected seeds into a new environment), and the risks of introduction, establishment and spread of seed borne fungal pathogens in new environments. It also discusses management options.

2.2. Project aims

The introduction and spread of harmful organisms have important economic and ecological consequences. Traditionally tree seed material has been considered a relatively low risk pathway for the movement of exotic pests and pathogens, although there is a growing awareness that the risks are only poorly understood. Internet trade in seeds and the ongoing search for new tree species as part of adaptation measures to counter climate change impacts may also lead to unregulated trade in seed or the collection of seed from areas where little is known about potential seed-borne pests. In addition, spread through the movement of infected seed may be one of the drivers for the emergence of diseases such as pitch canker caused by *Fusarium circinatum* on pine and Douglas-fir, Neonectria canker caused by *Neonectria neomacrospora* on fir or *Sirococcus* species on a wide range of conifers.

Seeds can carry fungi both externally and internally. These fungi can be pathogenic and lead to germination failure, poor germination, seedling diseases and can reduce the commercial value of a crop. In contrast, other fungi present on seeds might be saprophytic and have no effect on seed quality, and in some cases, these might be beneficial. The role of some of these fungi on seeds is not always understood. Different methodologies have been used to determine the microbiome associated with seeds. Digital X-rays provides evidence of full seeds and are effective at detecting insect or mechanical damage. Detection of pathogens has mainly come from culturing seed material onto agar media, which is time consuming, requires physical space to incubate agar plates, often has only a low level of success and requires professional expertise for fungal identification. Recently, more advanced techniques such as high-throughput sequencing have been used and showed that the fungal communities on seeds can be highly diverse and a potential pathway for common or emerging pathogens. It is likely therefore, that pathogen frequency associated with tree seeds and the risk of pest movement via this pathway is significantly underestimated. Therefore, better detection and surveillance methods are required to improve understanding and management of known and emerging risks

The overall aim of this project is to state current knowledge and collectively work towards diminishing the impact of seed trade as a pathway for pests and pathogens.

The specific objectives of this project are:

- To carry out an exhaustive review of the literature on seed borne pathogens in conifers in order to give a picture of the current knowledge on the topic.
- To map over time the volumes, origins, and destinations of the trans-boundary movement of conifer seed. The focus will be on the movement of larger quantities of seed for industrial purposes (e.g. planting for breeding or bulking), however estimates will also be provided on the movement of smaller quantities for private purposes (e.g. brought by passengers, ordered via internet or private shipments).

- To investigate the correlations between changes in seed movement patterns and changes in the geopolitical (regulatory and/or investment) environment.
- To compare and improve culture-based and molecular methods for detection of fungi associated with conifer seeds currently used in different laboratories.
- To enhance the dialogue between the project and the stakeholders, as well as among the project partners themselves.
- To disseminate the work done throughout the project with emphasis on stakeholders and other target audiences.

2.3. Description of the main activities

WP1. Project management and coordination

The main objectives were:

- Lead the project coordination providing a central point for communication for all project partners as well as being the link between the project and the EUPHRESCO network coordinator.
- Collate all the information for the full research proposal and submit it on behalf of the research consortium.
- Find a platform for communication and to share working documents for all partners.
- Organise meetings and chair the meetings during the duration of the project and update the EUPHRESCO coordinator.
- Monitor the progress for the different working packages during the project and produce interim reports with the contributions from working package leaders. The coordinator will be responsible for the monitoring the project progress against the deliverables. In case of any event that might affect the implementation of the project, the coordinator will inform the EUPHRESCO coordinator.

WP2. Literature review of seed borne pathogens of conifers, their impact in tree health and control measures Project management and co-ordination

There were three objectives in WP2:

- To carry out an exhaustive review of the literature on seed borne pathogens of conifers in order to provide an updated database of bibliographic references available for the partners of the project.
- To provide data on detection of seed borne pathogens of conifers in Europe, and especially of regulated fungal pathogens or pathogens on EPPO alert lists.
- To prepare a review paper dealing with seed borne pathogens in tree seeds.

For the first objective the bibliographic management tool Zotero (www.zotero.org) was chosen for the collection, organization and sharing of bibliographic references among project partners. This software offers many advantages compared to other tools; it is free and allows collaboration with large groups of researchers, as is the case of the Cosepath project (14 partners), at no extra costs. A Zotero group named "COSEPATH" was created at the beginning of the project. In June 2021 each partner was invited to be member of the group. Keywords related to the subject of the project (seed-borne pathogens, forest trees, invasion,...) were defined and used to search for bibliographic data (journal articles, book or book chapter) in different search tools (Google Scholar, PubMed, Scopus, Web of Sciences, Cabi Direct). All references and their corresponding pdf files (when available) were entered in the Zotero database.

The second objective, the web-based European notification system for Plant Health interceptions (Europhyt/Trace databases;



https://ec.europa.eu/food/plant/plant_health_biosecurity/europhyt/interceptions_en) has been consulted and the interceptions concerning conifer seeds were extracted for the period 2012-2022.

For the third objective, a group of researchers from the different participating partners on this EUPHRESCO project discussed the writing of a review article on seed-borne pathogens.

WP3. Seed movement and seed sources – mapping pathways

The objectives were:

- To map over time the volumes, origins, and destinations of the trans-boundary movement of conifer seed. The focus was on the movement of seed for industrial purposes (e.g. planting for breeding or bulking), however estimates were provided on the movement for other purposes (e.g. passengers, private shipments).
- Correlations between changes in seed movement patterns and changes in the geopolitical (regulatory and/or investment) environment were also investigated.

The international trade in conifer seed for propagation has the potential to move invasive alien species contaminating seed into new environments. While a large proportion of the international trade in forest tree seed is within biogeographical regions such as Europe or North America, a significant proportion is also traded between biogeographical regions. It is this trade between biogeographical regions that presents the greatest risks of invasive species impacts (Dalmazzone & Giaccaria 2014).

Knowing the movement in international trade of conifer seed is therefore important to understanding the risk of this pathway for invasive alien species. Mapping these trade patterns over time will help understand geopolitical environments that may lead to changes in the risk profile of the transboundary movement of conifer seed.

To enable an analysis of trade patterns of conifer seed for planting over time, a database was created of the export and import volumes of tree seed internationally. The data was extracted from Trade Map[®] which has been developed by the International Trade Centre UNCTAD/WTO (ITC) (Trade Map 2023).

Data in Trade Map[®] is sourced primarily from The United Nations Comtrade database which “*aggregates detailed global annual and monthly trade statistics by product and trading partner for use by governments, academia, research institutes, and enterprises. Data compiled by the United Nations Statistics Division covers approximately 200 countries and represents more than 99% of the world’s merchandise trade*” (Comtrade 2023).

Other national sources of information are also used where available. Data within Trade Map[®] is referenced via a “Harmonized Commodity Description and Coding System”, referred to as the “Harmonized System” or HS codes. The International Convention on the Harmonized Commodity Description and Coding System entered into force on 1 January 1988.

The stated objectives of this convention are “(i) to facilitate international trade and the collection, comparison and analysis of statistics by harmonizing the description, classification and coding of goods in international trade; (ii) to reduce the expenses related to international trade and (iii) to facilitate the standardization of trade documentation and the transmission of data” (World Customs Organisation 2023).

The HS codes provided by the World Customs Organisation contain 6-digits. The 6-digit code relevant to the conifer seed for planting is 120999 - *Seeds, fruits and spores, for sowing (excluding leguminous vegetables and sweetcorn, coffee, tea, maté and spices, cereals, oil seeds and oleaginous fruits, beets, forage plants, vegetable seeds, and seeds of herbaceous plants cultivated mainly for flowers or used primarily in perfumery, medicaments or for*

insecticidal, fungicidal or similar purposes). A code classification at this level would include many different types of non-tree seeds traded for planting. Fortunately, many countries or regions add further digits to their HS coding system to better define the commodity.

Immediately apparent from the HS code descriptions that data on only conifer forest tree species was not available, with the available codes including conifer and non-conifer tree or forest tree species, or in the case of the Russian Federation only one species of conifer tree (*Abies nordmanniana*). According to Jansen et al. (2019) the main tree species traded in Europe as seed for sowing are: *Fagus sylvatica* (cca 90,000 kg annually), *Quercus robur* (cca 160,000 kg annually), *Quercus petraea* (cca 150,000 kg annually) and *Quercus rubra* (cca 18,000 kg annually). For comparison, in Europe, only around 1,200 kg of seeds of the most traded conifer species (*Picea abies*) seeds is traded annually. Jansen et al. (2019) also report comparatively little volumes of imports to EU from non-EU countries. **Table 1** provides a list of the extended HS codes and classifications that enabled more targeted data extraction from the listed countries.

Data for a number of other countries not using sufficiently targeted HS codes were also derived from the extracted data. These countries included China, Greece, Hong Kong, Ireland, Israel, Japan, Mexico, Norway, Portugal, Republic of Korea, Romania, Slovakia, and Switzerland. A full list of countries used in the analysis is provided in **Table 2**.

Table 1. List of more specific HS codes for use in the trade of forest tree seed for sowing

Extended HS Code	Classification	Country(s) using code
12099910	Forest-tree seed for sowing	European Union countries (Austria, Belgium, Croatia, Czechia, Denmark, Finland, France, Germany, Hungary, Italy, Luxembourg, Netherlands, Poland, Slovenia, Spain, Sweden), Georgia, Türkiye, United Kingdom.
12099910	Tree and flower seeds, of a kind used for sowing	Australia
1209991000	Seeds, fruit and spores for sowing, nes: forest-tree seeds	Serbia
1209990051	Seed; tree, of a kind used for sowing	New Zealand
1209991010	Caucasian fir seeds (<i>Abies nordmanniana</i> (steud.) spach)	Russian Federation
1209991010	Tree seeds, for sowing, other than nut trees of chapter 8	Canada
1209992000	Tree and shrub seeds, fruits and spores, of a kind used for sowing	United States of America (USA)

WP4. Methodology used in different laboratories for the identification of pathogens on seeds, practical cases study, interlaboratory tests

The objective was:

- To compare and improve culture-based and molecular methods for detection of fungi associated with conifer seeds currently used in different laboratories, two approaches were followed, cultural methods and molecular methods using Illumina MiSeq sequencing and MinION nanopore sequencing.



Cultural Methods

To optimise the isolation and culturing protocols for detection of conifer seed fungi, seed lots from 8 different conifer species (*Pinus sylvestris*, *P. nigra*, *P. pinea*, *Abies alba*, *A. nordmanniana*, *Pseudotsuga menziesii*, *Picea abies*, and *P. sitchensis*; one seed lot each) were exchanged among project partners so that each partner analysed seeds of two or more species.

For isolation and cultivation of seed-borne fungi the following four protocols were applied (all of them are regularly used in the laboratory of project partners):

- Protocol 1: Disinfection in 1% NaOCl for 10 min; Isolation on PDA (Potato Dextrose Agar); Incubation at 20 °C +/- 2 °C for 5–7 days alternating 12 h light and 12 h darkness; Subculturing on PDA;
- Protocol 2: Disinfection in 2% NaOCl for 10 min and rinsing with sterile water; Isolation on PDA amended with streptomycin sulphate (80 mg/L); Incubation at 20 °C +/- 2 °C for 5–10 days in the dark; Subculturing on Spezieller Nährstoffarmer Agar' (SNA) medium for *Fusarium* and on PDA medium for other fungi;
- Protocol 3: Disinfection in 1% NaOCl for 30 sec. and rinsing with sterile water 3 times; Isolation on 1.6% H₂O-agar pH 6.7 containing 50 µg/ml streptomycin; Incubation at 25 °C; Subculturing on 0.5x PDA pH 6.7; Incubation at 20 °C +/- 2 °C for 5–10 days in the dark.
- Protocol 4: Disinfection in 2–4% NaOCl for 10 min; Isolation on PDA amended with streptomycin sulphate (80 mg/L); Incubation at 20 °C +/- 2 °C for 5–10 days in the dark; Subculturing on PDA.

With each protocol, a total of 200 healthy looking seeds per analysed seed lot were used for fungal assessment. After subculturing, isolates were grouped according to the colony characteristics and assigned to genera/species based on their morphology.

Illumina MiSeq

For selected seed lots, seed-borne fungi were also assessed using a metabarcoding approach which allowed characterising fungal communities directly from DNA extracted from the seeds. Since some fungi (e.g. biotrophs) are particularly difficult or impossible to grow on nutrient media, a DNA-based method should allow to detect a higher species diversity than traditional plating. Moreover, such method is less laborious and cheaper when large numbers of seeds are used. For this, DNA was extracted from surface sterilised seeds using a commercially available kit, the ITS region was amplified by PCR and PCR products were sequenced on the Illumina MiSeq platform. Quality filtering and assignment of sequences to species was performed using established bioinformatics tools.

MinION nanopore sequencing

Instead of the short-read sequencing employed for example in the Illumina platforms, long-read sequencing using Oxford Nanopore Technologies uses a different sequencing approach in which single DNA molecules are sequenced individually in real time, resulting in lengths up to 100 kB. From selected seed lots, DNA was extracted from surface sterilised seeds using a commercially available kit, the ITS region was amplified by PCR and PCR products were sequenced on a MiniOn sequencer.

Quality filtering and assignment of sequences to species was performed following a self-made pipeline (still no completely standardized pipeline available).

2.4. 4. Main results

WP1. Project management and coordination

Prior to the starting of the project several meetings took place to form the consortium of researchers and to collate all the information for the full research proposal to be submitted to EUPHRESCO. Three main project meetings were organised during the duration of the project: a kick-off (May 2021), a mid-term (June 22) and a final meeting (May 2023). Satellite meetings for the different working packages were also organised. All the meetings were online on Teams.

A webpage for the project was created within Forest Research website:

<https://www.forestresearch.gov.uk/research/euphresco-2020-a-339-seed-borne-pathogens-of-conifers-cosepath/>

A collaboration with ISTA was established through Nicolas Denacé from the Groupe d'Étude et de contrôle des Variétés Et des Semences in France who gave us the possibility to collaborate with ISTA Reference Pest List project by reviewing some scientific articles on seed-borne/transmitted pests.

COSEPATH proposed a session "The biosecurity risks of international movement of tree seeds" at the 26th World Congress IUFRO – Forest & Society towards 2050 in Stockholm (2024). The session was accepted and the work carried out in this project was presented at this session: EUPHRESCO project COSEPATH, are seeds a potential pathway for pests and pathogens and a risk for future forestry?

Interim reports were submitted to EUPHRESCO.

WP2. Literature review of seed borne pathogens of conifers, their impact in tree health and control measures Project management and co-ordination

A total of 153 bibliographic references were collected during the project. These are listed in **Appendix 1** of this report. This number is relatively low. It reflects the few studies carried out to date on the subject. Topics include seed-borne organisms (fungi, bacteria, including endophytes), diagnostic methods for seed borne fungi (classical and molecular methods, including high-throughput sequencing), control measures, risks of introduction/establishment/spread of invasive pathogens via the international seed trade, pathogenicity testing, seed production and storage.

Interception data on conifer seeds were extracted from the Europhyt/Trace database. The results must be interpreted with caution as they only concern interceptions for non-compliance with European legislation on Plant health. However, they do allow establishing trends in terms of diversity and origin of conifer species imported into Europe. A total of 87 "non-compliant" conifer seed interceptions were identified over the period 2012-2022, mostly for administrative problems (incomplete documents). Only one case of interception concerned a fungal problem: *Pinus taeda* seeds originating from USA intercepted for the presence of *Gibberella circinata* in 2017.

As shown in **Figure 1A**, more than 60% of the interceptions concerned *Pinus* seeds. Regarding the origin of disputed conifer seeds, USA was the country with the highest number of conifer genera intercepted (**Figure 1B**). An Excel file with all the extracted data was created and is available in **Appendix 2**.

A review paper entitled “The biosecurity risks of international tree seed movements” (Francic *et al.* 2023) was prepared for Current Forestry Reports. This document addresses the issues of seed transport (e.g., what are the pathways for the introduction of infected seeds into a new environment?), and the risks of introduction, establishment and spread of seed borne fungal pathogens in new environments. It also discusses management options. The paper was submitted in June 2023 and a revision after reviewer’s comments was submitted in September 2023. The paper was published on the 29th of December 2023.

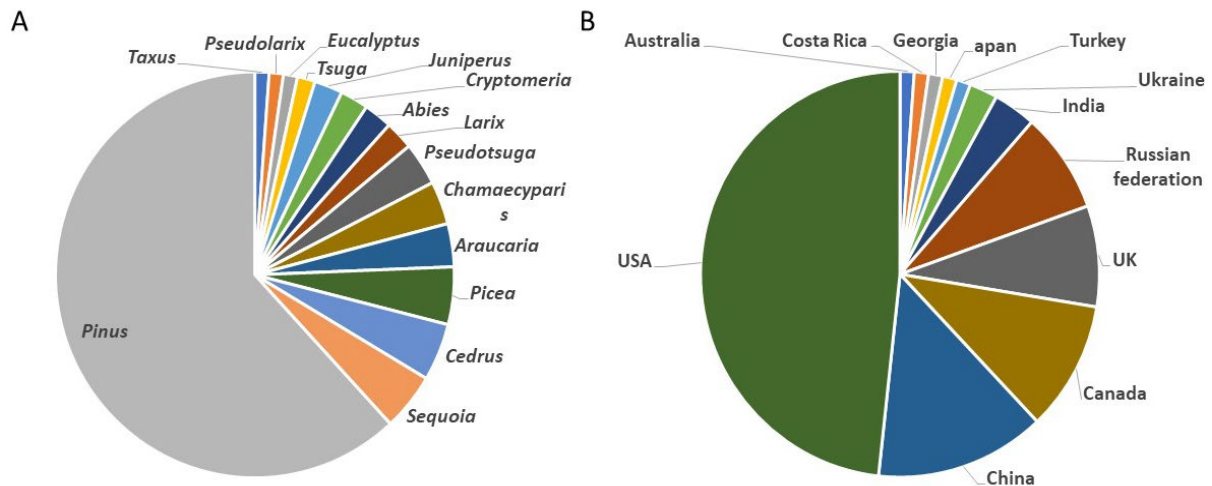


Figure 1. Distribution of interceptions of conifer seed lots over the period 2012-2022 in the Europhyt/Trace database. **A:** by conifer genus, **B:** by country of seed origin.

WP3. Seed movement and seed sources – mapping pathways

Data was extracted on annual volumes by weight (kilograms) for each of the countries listed in **Table 1** based on the HS code for years 2003 through to 2022. Available data for years 2003 to 2004 and 2022 were incomplete and therefore discarded, leaving data for a 16-year period from 2005 to 2021. It was also apparent on analysing the data that the reported export and import volumes for each combination of countries did not always equate. In a small number of cases the differences were substantial. Some of the variation is likely due to the different codes or code definitions used, and some to differing accuracy levels in data collection. In all cases to ensure import and export data matched for each country combination, the maximum recorded volume was taken as the value.

The final database then contained over 13,000 datapoints, corresponding to the annual trade in tree seed between 38 countries over 16 years. To simplify the presentation of the analyses on trading patterns, the country data was grouped based on a representation of biogeographical regions (**Table 2**).

Table 2. Groupings of the country seed trade data based on Biogeographical Region

Biogeographical Region	Country data included*
Maritime Europe (North, West and Central Europe)	Austria, Belgium, Czechia, Denmark, France, Germany, Ireland, Luxembourg, Netherlands, Norway, Sweden, Switzerland, United Kingdom
Mediterranean Europe (South Europe)	Spain, Greece, Italy, Portugal
Northeast Europe	Finland, Poland, Russian Federation
Southeast Europe	Croatia, Hungary, Romania, Serbia, Slovenia, Slovakia
Eastern Asia	China, Hong Kong, Japan, Republic of Korea
Western Asia	Georgia, Israel, Türkiye
North America	Canada, Mexico, United States of America
Oceania	Australia, New Zealand

* Countries in bold provided the source data

The first analysis of the data looked at the variation across all countries in annual volumes of traded seed (**Figure 2**).

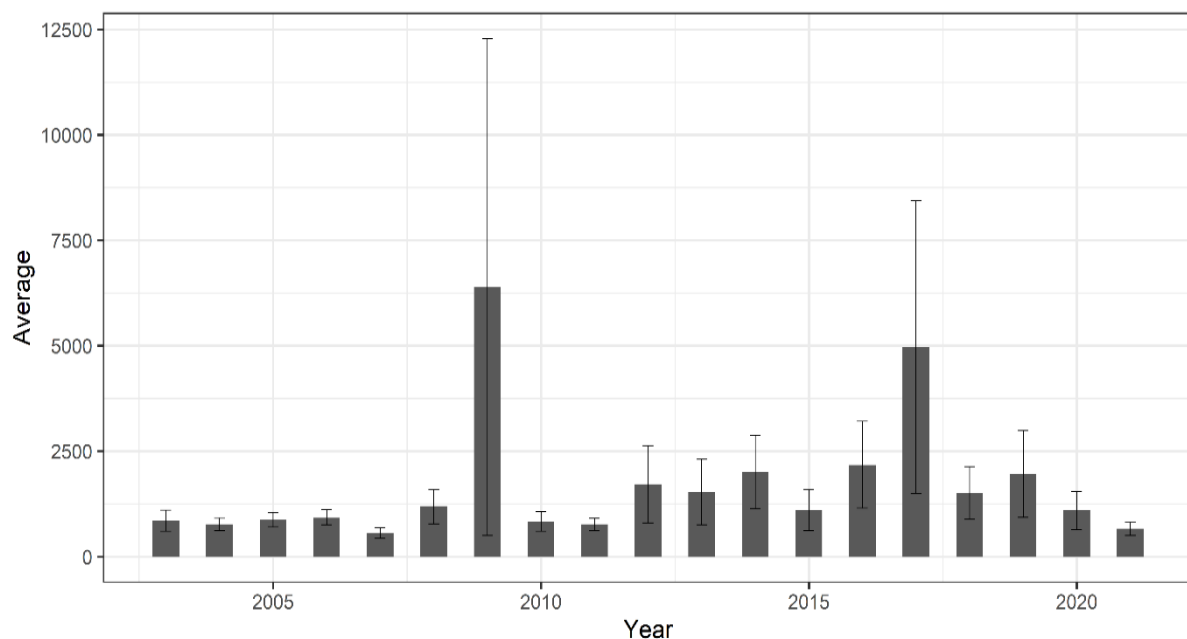


Figure 2. Volumes (in kg) of traded tree seed from 2003-2021 across all countries for which records were included.

The largest trading years apparent in the data are 2009 and 2017 when large volumes of seed were exported from Spain to Portugal (2009) and from Portugal to Türkiye (2017). It is unclear what these spikes in seed volume are related to. Possible explanations could be improper coding of seed traded for consumption rather than sowing (e.g., *Pinus pinea* and almond seeds), or seed traded to recover from forest fires or changes in government policies (e.g., ecological restoration, carbon sequestration etc.).

The second analysis of the trade data is provided in a Sankey plot which was generated using sankey Network function from sankeyD3 package in R. (Breitwieser et al. 2019). **Figure 3** provides a visual indication of the seed trade flows between regions during 3 time periods: 2005-2009, 2010-2014 and 2015-2019. Sums of exchanged seeds are shown in brackets.

Around 14×10^6 kg of seeds were traded between 2005 and 2009, mostly within south Europe ($\sim 9 \times 10^6$ kg). This is mainly due to the big amount of seed imported to Portugal from Spain in 2009. The second biggest exchange of seeds was during that time period was within north, west and central Europe ($\sim 1 \times 10^6$ kg). In addition, around 7×10^5 kg of seeds were exported from the same region to south Europe, and around 5×10^5 kg of seeds were imported to the region from Oceania and southeast Europe. Around 3×10^5 kg of seeds were exchanged between North American countries, and the same amount of seeds was exported from the North America to north, west and central Europe.

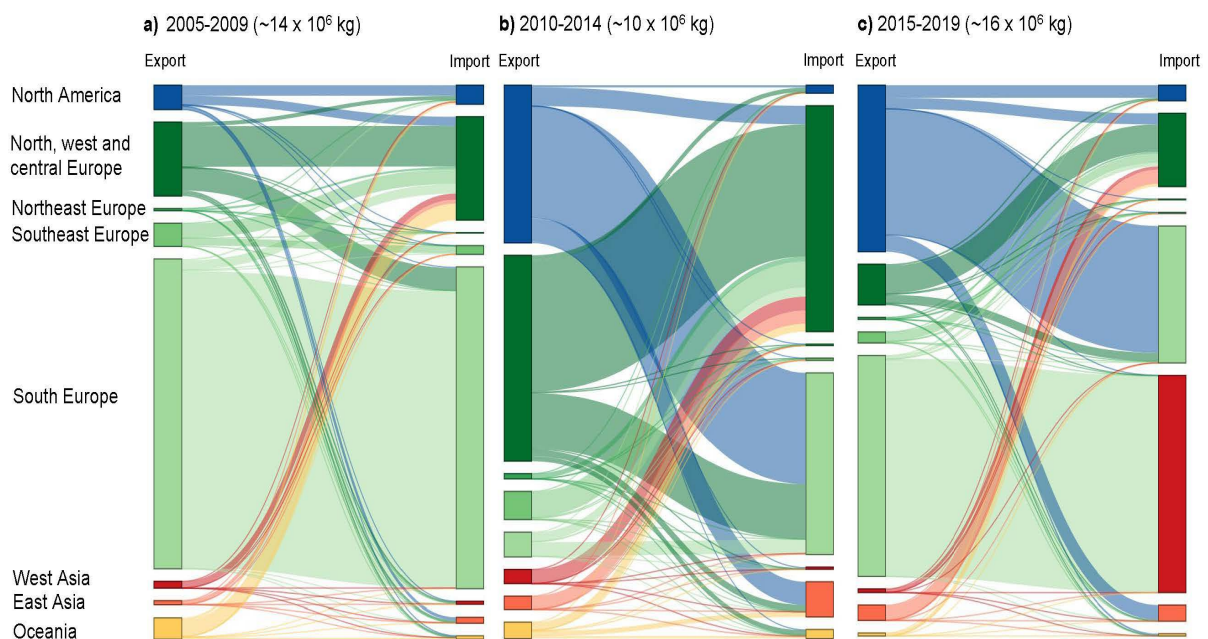


Figure 3. Sankey plot showing seed trade flows between regions during 3 time periods: 2005-2009, 2010-2014 and 2015-2019. North America – blue, Europe – green, Asia – red/orange, Oceania – yellow.

During 2010-2014 around 10×10^6 kg of seed were exchanged again mainly within north, west and central Europe ($\sim 3 \times 10^6$ kg). In addition, south Europe imported around 2.3×10^6 kg and around 1×10^6 kg of seeds from North America and north, west and central Europe, respectively. North, west and central Europe imported seeds from many regions including southeast and south Europe ($\sim 7 \times 10^5$ kg), as well as from North America ($\sim 4 \times 10^5$ kg), west and east Asia ($\sim 3 \times 10^5$ kg each) and Oceania ($\sim 1.6 \times 10^5$ kg). East Asia imported 5×10^5 kg seeds from North America.

Half of the total seed exchange during 2015-2019 were the seeds exported from south Europe to west Asia (i.e., around 8×10^6 kg from Portugal to Turkey in 2017). The second biggest exchange was between North America and south Europe (i.e., around 4.5×10^6 kg from the USA to Spain). Around 1×10^6 kg were exchanged within north, west and central Europe. The

same region also imported around 3×10^5 kg seeds from southeast Europe, and 4×10^5 kg and 5×10^5 kg of seeds from North America and East Asia, respectively.

The third analysis compared the change in total trade volumes between the biogeographical regions of a five-year period from 2007 to 2011 with a second five-year period from 2017 to 2021. The results are displayed as a Chord Plot using circulize package version 0.4.15 in R (Gu 2014) in **Figure 4**.

The analysis indicates that overall North America is a growing exporter of forest tree seed, especially for south Europe and east Asia. The imports to Europe grew from 37,318 during 2005-2009, to 2.3×10^6 kg in 2010-2014 which doubled during 2015-2019. East Asia imported 94,665 during 2005-2009, and around 5×10^5 kg in each of next two time periods. Trade from Mediterranean Europe to Western Asia also increased significantly, while trade within Mediterranean Europe declined significantly over these time periods.

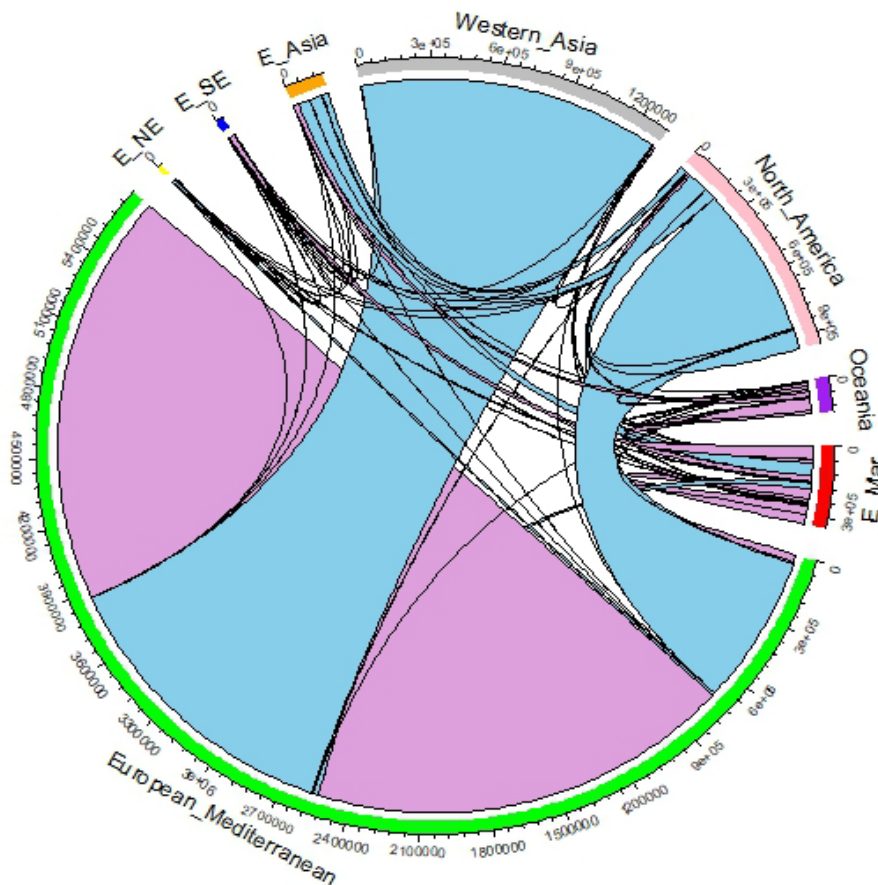


Figure 4. A Chord plot comparing the differences in the volume of tree seed trade between biogeographical regions between years 2007-2011 and 2017-2021. The magnitude of the change is indicated by the width of the lines between regions, while decreases are in purple and increases are in blue. Abbreviated annotations are E_Mar (Maritime Europe), E_NE (Northeast Europe), E_SE (Southeast Europe), and E_Asia (East Asia).



WP4. Methodology used in different laboratories for the identification of pathogens on seeds, practical cases study, interlaboratory tests

Confirming results from previous studies, seed-associated fungi were recovered from all tree species analysed and with each isolation protocol applied. However, important differences in the incidence of seed-associated fungi as well as in the diversity and composition of the fungal communities were observed among tree species and isolation protocols.

For example, using protocol 3 fungi were isolated from 116 seeds (58%) of *Pinus pinea*, but only from 24 seeds (12%) of *Pinus nigra*. Not only the number of isolates obtained varied between the two tree species, but also the genera to which the isolates belonged (**Figure 5**). In *P. nigra*, fungal isolates of the genus *Penicillium* were recovered from 62.5% of the positive seeds, followed by the genera *Chaetomium*, *Trichoderma*, *Aspergillus* and *Rhizopus*. The high presence of *Penicillium* might be an indication of poor-quality seeds caused by unsuitable storage conditions (high moisture content), handling (damaged seed coat), insect damages, etc. Noteworthy, all these genera are quite frequent in the environment (air, soil, dead organic matter) and are rather contaminants than true seed-borne fungi. In *P. pinea*, most fungal isolates (81%) belonged to the genus *Alternaria* (**Figure 5**) whose species are often common saprophytes; however, a few are plant pathogens, some of them producing a variety of toxic compounds. The known seed-borne genus *Fusarium* was detected on four seeds (3.4%), while the other genera isolated from the seeds (*Trichoderma*, *Chaetomium*, *Aspergillus*) were rather environmental contaminants that were not killed by the surface disinfection of the seeds.

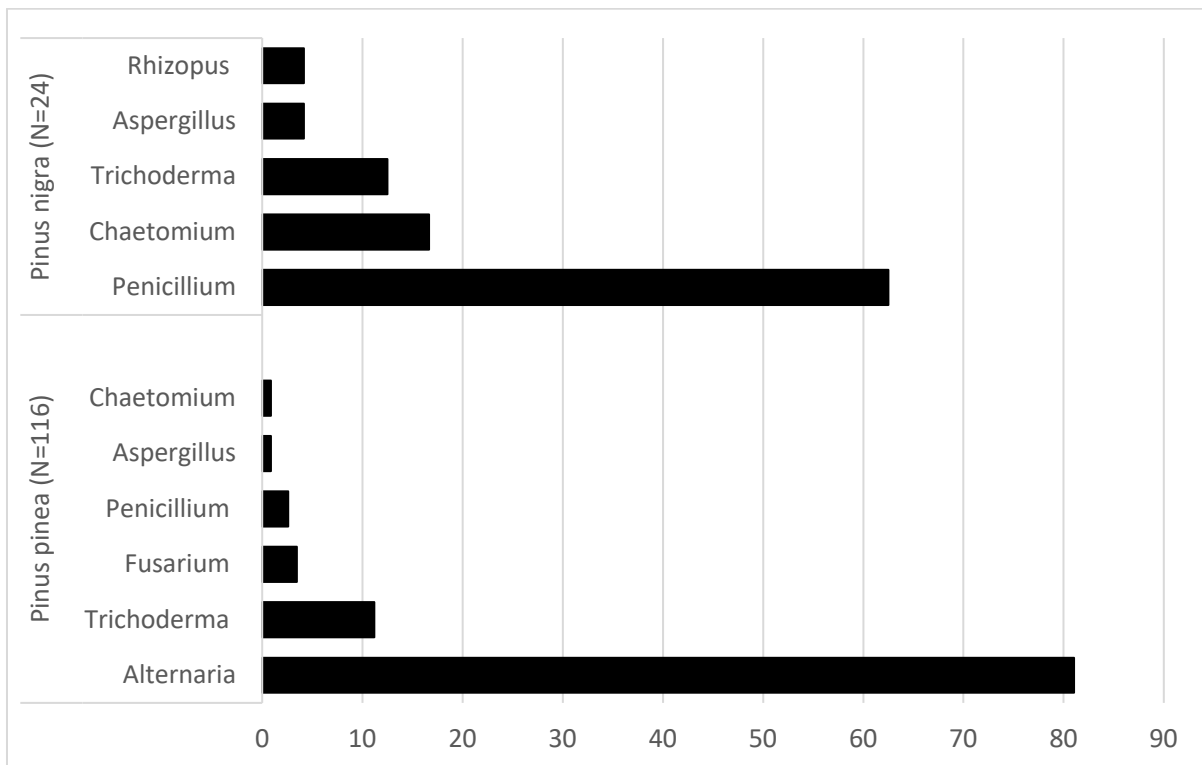


Figure 5. Diversity and incidence of fungal genera recovered from healthy looking seeds of *Pinus nigra* and *P. pinea* using the isolation protocol 3. In brackets the number of positive seeds (from which fungi could be isolated) per species is given.

As shown in **Figure 6** for Douglas fir (*Pseudotsuga menziesii*), the composition of the fungal community detected in the seeds was also affected, at least partially, by the applied isolation protocol.

While some species/genera were detected at similar incidences with both isolation protocols, other species/genera were identified only using one of the two protocols or their incidence was higher with one protocol than with the other. Nevertheless, both protocols allowed to detect the known seed-borne fungi *Alternaria*, *Diplodia sapinea* and *Sydowia polyspora*, even if at different incidences (e.g. *S. polyspora*: 0.4% protocol 3 and 10.5% protocol 4).

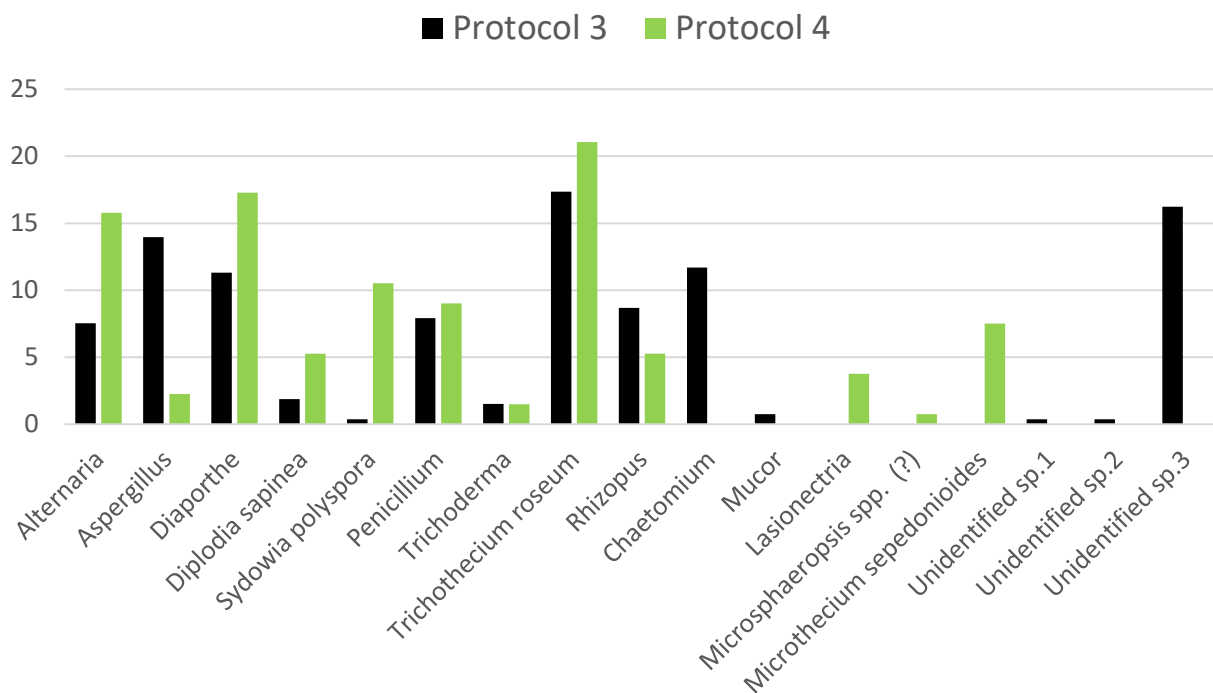


Figure 6. Diversity and incidence of fungal genera recovered from healthy looking seeds of Douglas fir using the isolation protocols 3 and 4.

In the comparison of culturing-based and HTS methods for detection of conifer seed fungi, our analyses showed that the diversity of seed-borne fungal taxa as revealed by HTS (in our case Illumina MiSeq sequencing) is considerably higher than when assessed by plating the seeds on agar media. Thus, for studies aiming at investigating the diversity of seed-borne fungi a high-throughput amplicon sequencing approach should be applied. However, it should not be forgotten that such an approach does not allow to assess whether the fungi detected are alive or not. On the contrary, traditional plating only reveals fungi that are alive and culturable. When opting for this approach, it would be recommended to use more than one agar medium, as some differences in fungal incidence and diversity among agar media were observed.

Unfortunately, sequences obtained with the MinION nanopore method contained several errors (unclear nucleotides). This combined with the non-standardized downstream data processing (with large data losses) made a comparison with Illumina MiSeq data not possible. In the meantime, nanopore technologies for sequencing long DNA molecules have substantially improved (error rate about 1%) and such a comparison would make more sense and be feasible.



2.5. Conclusions and recommendations to policy makers

Plant health regulations and phytosanitary measures aim to reduce the risks of spreading harmful organisms that could represent a threat for agriculture, horticulture and forestry. In this context, seeds have been considered one of the safest ways to introduce plant species and germplasm into new areas and consequently their trade is regulated less strictly. The high demand of seeds for forest regeneration purposes results in huge volumes of seeds being moved across countries and continents, with variable patterns over time. As shown by recent studies, tree seeds can harbour taxonomically and functionally diverse fungal communities, including potentially pathogenic species. Hence, the global seed trade could theoretically represent a high risk of introduction of potential plant pathogens between biogeographical regions.

Measures that aim to reduce the risk of pathogen introduction from traded forest tree seed starts with the requirement to have phytosanitary certificates for all seed imports in Europe and can be complemented by the implementation of improved technologies such as HTS methods for the detection of pathogens when inspecting plant material. However, the implementation of such technologies still faces some challenges (e.g. requirements for laboratory infrastructure, bioinformatics, data sharing and validation, standardised protocols).

Measures that aim to mitigate the risk of pathogen introduction from traded forest tree seed take two forms: preventing seed exposure before harvest or treating the traded seeds to kill potential pathogens. The measure for preventing seed exposure before harvest is “area freedom”, i.e. seeds have to be harvested from production areas free of a pathogen. This specific measure effectively mitigates phytosanitary risk when the pathogen of concern is known. For example, in 2007, the Commission Decision 2007/433/EC (EC433 2007) specified that plants of the genus *Pinus* and the species *Pseudotsuga menziesii* intended for planting, including seeds and cones for propagation purposes, might be moved within the EU only if they are accompanied by a plant passport prepared and issued in accordance with the provisions of Commission Directive 92/105/EEC (EEC105 1992) and they have been grown throughout their life or since their introduction into the EU in a) a place of production of a Member State where the organism is not known to occur, or b) a pest-free area, established by the responsible official authority in a Member State, or c) where no signs of the pathogen have been observed during official surveys carried out within 2 years before the movement and have been tested immediately before the transfer. As previously mentioned, while the risk of seeds carrying *Fusarium circinatum* has been recognised and seeds of pines and Douglas-fir have consequently been regulated for trade, many unknown and unregulated potential pathogens can be moved to new areas with traded seeds

The second form of risk mitigation measures involves the use of treatments to kill the pathogens. Currently available treatment options include the application of chemicals as liquids, powders or as a fumigant. Chemical treatment of seeds can be used to eliminate surface contaminations by pathogens and in some cases prevent or remove infection of any resulting seedling. Common treatments against pathogens living on the seed surface include immersion in a 1% sodium hypochlorite solution for 10 min, whereas treatments against internally borne pathogens include the application of slurries of Captan (active ingredient is penconazole (triazole)) or Thiram (active ingredient is thiram (thiocarbamate)). While chemicals may be effective against some specific pathogens, they cannot target all fungal species found in association with tree seeds. Moreover, chemical methods are not always environmentally friendly. For this reason, they have been increasingly replaced by nonchemical methods, such as treatment with biopesticides and application of irradiation or heat.



The use of non-chemical treatments is limited by their effectiveness and their impact on seed viability. For example, irradiation is effective at killing fungal pathogens at doses of 2–5 kGy, but such rates will significantly reduce seed viability, thereby limiting the practical use of irradiation as a treatment option. Plant pathogens are more susceptible to heat requiring treatments of 60–70 °C for around 2 h. While tree seed tolerance to heat varies, seed from genera such as *Pinus* are relatively heat tolerant. A particular study showed that the use of hot water treatment at 51–52 °C for 30 min was able to reduce *F. circinatum* contamination on *P. radiata* seeds. A combination of chemical and heat treatments reduced inoculum levels of *F. circinatum* on pine seeds. Hot water-treated seeds can be a better alternative to hydrogen peroxide and fungicides and could easily be implemented as standard practice in commercial nurseries to minimise the spread of the pathogen. Australia for example provides heat treatment options for internally borne pathogens of coniferous tree seeds at 54 °C for 86 h, 60 °C for 24 h or 66 °C for 8 h. Similarly, it has been shown that heat treatment at 55 °C for 8, 9, 10 and 11 h can completely eliminate pathogenic fungi such as *Diplodia sapinea* and *F. circinatum* from *P. radiata* seed coat, embryo and gametophyte, without adversely affecting seed germination. Heat treatment could thus be a promising technique to be applied during seed production or pre/post transport to reduce the infestation rate of traded seed lots.

There are still some knowledge gaps and further studies should aim to investigate seeds as a vector for fungal pathogens; to date seeds have been proven to be a pathway for only five tree pathogens. In addition, detailed studies are needed to assess if and how frequently seed-borne fungi are transmitted to developing seedlings and if from there they can establish in the new environment and cause damage to the local tree communities. Data on seed movements is also scarce and there is a need to understand the biological significance of the detection of pathogens on seeds. Improvement in detection technologies is needed together with the validation of new approved methods.

2.6. Benefits from trans-national cooperation

In this project 14 partners from 13 countries have been collaborating to gather information about the risk of pathogens on conifer seeds that could be spread and introduced into new biogeographical areas through trade. One of the main benefits of this collaboration has been the access to the expertise and knowledge of the different participants. Therefore, networking is one of the main benefits. Participants had a common interest, and this project has facilitated the collaboration between scientists, regulatory bodies, and institutions with diverse specializations and experiences. This project provided a platform to the partners to share different views, technical skills, test different approaches, find new solutions, review papers for other organisations and to disseminate our research at an international congress and to publish a review in a peer reviewed scientific journal.

The project has allowed discussion, and we have joined together to gather information and to improve and harmonise methodology used for pathogen detection on seeds. One of our objectives was to compare and improve culture-based and molecular methods for detection of fungi associated with conifer seeds currently used in different laboratories. This encouraged the discussion of the diverse methods used in the laboratories and to compare our results when testing same seed lots. Working together has avoided unnecessary duplication of efforts, helping us to fill knowledge gaps more efficiently and harmonise procedures.

As a fruit of our joined efforts, we worked together to propose a session “The biosecurity risks of international movement of tree seeds” at the 26th World Congress IUFRO – Forest & Society towards 2050 in Stockholm (2024). The session was accepted, and we contacted with other researchers worldwide to contribute to our session. This also provided a way to add new members to our network. The work carried out in this project was also presented at this

session: EUPHRESKO project COSEPATH, are seeds a potential pathway for pests and pathogens and a risk for future forestry?

During the project, researchers from different partners reviewed scientific articles on seed-borne/transmitted pests on conifers to collaborate with an ISTA Reference Pest List project. This work was coordinated with Nicolas Denancé from the Groupe d'Étude et de Contrôle des Variétés et des Semences in France. The information was part of a publication in which researchers who contributed were acknowledged: Denancé N & Grimault V (2022). Seed pathway for pest dissemination: The ISTA Reference Pest List, a bibliographic resource in non-vegetable crops. EPPO Bulletin 52:434 – 445).

Finally, a review paper entitled “The biosecurity risks of international tree seed movements” (Francic et al. 2024) was published in Current Forestry Reports (Current Forestry Reports (2024) 10:89–102 <https://doi.org/10.1007/s40725-023-00211-3>). This article addresses the issues of seed transport (e.g., what are the pathways for the introduction of infected seeds into a new environment), and the risks of introduction, establishment and spread of seed borne fungal pathogens in new environments. It also discusses management options.

In summary, the collaboration had enabled shared learning, efficient resource use, and stronger, more resilient partnerships that are essential for addressing global challenges.



3. Outreach

3.1. Article(s) for publication in journals

Franić, I., Cleary, M., Aday Kaya, A.G., Bragança H., Brodal, G., Cech, T.L., Chandelier, A., Doğmuş-Lehtijärvi, T., Eschen, R., Lehtijärvi, A., Ormsby, M., Prospero, S., Schwanda, K., Sikora, K., Szmidla, H., Talgø, V., Tkaczyk, M., Vettraino A.M., Pérez-Sierra, A. (2024). The biosecurity risks of international forest tree seed movements. *Current Forestry Reports* 10, 89–102. <https://doi.org/10.1007/s40725-023-00211-3>

3.2. Grey literature

None.

3.3. Events

Congress: 26th World Congress IUFRO – Forest & Society towards 2050 in Stockholm (2024)

Session T1.32 The biosecurity risks of international movement of tree seeds

Talk: EUPHRESCO project COSEPATH, are seeds a potential pathway for pests and pathogens and a risk for future forestry?

Authors: Ana Maria Perez Sierra, Anne Chandelier, Guro Brodal, Venche Talgo, Thomas Cech, Katharina Schwanda, Michael Ormsby, Rene Eschen, Michelle Cleary, Simone Prospero, Anna Maria Vettraino, Helena Braganca, Gulden Aday, Clovis Douanla-Meli, Tugba Dogmus Lehtijarvi, Carolyn Pike, Katarzyna Sikora, Hanna Szmidla, Miłosz Tkaczyk, Fuskay Oskay, Steve Woodward, Iva Franić



4. Open Euphresco data

None.