Euphresco

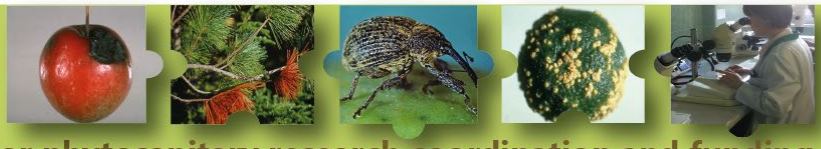
Final Report

Project title (Acronym)
Early detection of *Cryphonectria parasitica* in planting material (CPARA)

Project duration:

Start date:	01/04/2018
End date:	31/03/2020

Contents



1. Research consortium partners

Coordinator – Partner 1			
Organisation	Fera Science Ltd (Fera)		
Name of Contact (incl. Title)	Miss Ann Barnes	**Gender**:	F
Job Title	Senior Mycology Diagnostician		
Postal Address	Fera, Sand Hutton, York, YO41 1LZ		
E-mail	Ann.barnes@fera.co.uk		
Phone	+ 44 01904462494		

Partner 2			
Organisation	Austrian Agency for Health and Food Safety, Austria (AGES)		
Name of Contact (incl. Title)	Dr. Richard Gottsberger; Ulrike Persen; Thomas Leichtfried	**Gender**:	M; F; M
Postal Address	Institute for Sustainable Plant Production, Spargelfeldstrasse 191, 1220 Vienna, Austria		
E-mail	richard.gottsberger@ages.at; ulrike.persen@ages.at; thomas.leichtfried@ages.at		
Phone	+ 43 05055533353		

Partner 3			
Organisation	Institute for Agricultural and Fisheries Research (ILVO)		
Name of Contact (incl. Title)	Dr. Kurt Heugens	**Gender**:	M
Postal Address	Eenheid Plant – Gewasbescherming, Burg. Van Gansberghelaan 96 bus 2 9820 Merelbeke, Belgium		
E-mail	kurt.heungens@ilvo.vlaanderen.be		
Phone	+32 0092722487		

Partner 4			
Organisation	Federal Ministry of Food and Agriculture (JKI)		
Name of Contact (incl. Title)	Clovis Douanla-Meli	**Gender**:	M
Postal Address	Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Horticulture and Forests, Messeweg 11/12, D - 38104 Braunschweig		
E-mail	clovis.douanla-meli@julius-kuehn.de		
Phone	+49 05312994407		

Partner 5			
Organisation	Council for Agricultural Research and Economics (CREA-DC)		
Name of Contact (incl. Title)	Dr. Sauro Simoni	**Gender**:	M
Postal Address	via di Lanciola12/a 50125 Firenze, Italy		
E-mail	sauro.simoni@crea.gov.it		
Phone	+39 0552492229		

Partner 6			
Organisation	National Institute for Agricultural and Veterinarian Research (INIAV)		
Name of Contact (incl. Title)	Dr. Eugénia de Andrade	**Gender**:	F
Postal Address	Molecular Biology and GMO. Avenida da República, Quinta do Marquês, 2780-159 Oeiras, Portugal		
E-mail	eugenia.andrade@iniav.pt		
Phone	+ 351 214463760		

2. Short project report

2.1. Short executive summary

Cryphonectria parasitica is a devastating plant pathogen infecting chestnuts (*Castanea* spp.). The long-distance spread of *C. parasitica* is thought to occur through the movement of infected plants for planting, bark or wood material (Jeger *et al*., 2016). There is evidence of a long latent period on infected plants (Cunnington & Pascoe, 2003). Long latent periods are problematic as affected trees do not show symptoms for some time and are being shipped to previously non-affected areas. Plants for planting were emphasized as the highest risk pathway for entry of the pathogen into protected zones (Jeger *et al*., 2016), therefore a robust, sensitive detection method to screen for latent infections of planting material is required to support plant health inspection and potential certification regimes. This project was the result of a collaboration between specialists of *Cryphonectria parasitica* disease in *Castanea* spp.

The CPARA project aimed to support the diagnosis of *C. parasitica* in plants for planting, with a focus on latent infections. The project contributed to:

- Development of a diagnostic protocol for the detection of *C. parasitica* in planting material. This included development of an extraction protocol, and comparison of published real-time PCR tests. Two protocols for the detection of *C. parasitica* in planting material (Chandelier *et al*., (2019) test and a test optimised within this project) were evaluated through a test performance study (TPS) with eight participating laboratories across seven countries. Both tests performed comparatively. Therefore, the decision on which test to use may be based on the equipment and expertise available within the diagnostic laboratory. Both tests had reduced sensitivity in some participating laboratories. Consequently, verifying the chosen test within each diagnostic laboratory will be extremely important for the detection of latent infections.
- Validation of the published real-time PCR test by Chandelier *et al*., (2019) for use on planting material to detect early infections before symptom expression. This included validating the test on artificially infected plants which were assessed over a time course of disease development, and naturally infected samples taken from asymptomatic trees in areas where *C. parasitica* is known to be present. The test allowed the detection of *C. parasitica* in asymptomatic plant material of both artificially and naturally infected samples. The sampling method used in this project can form the basis of a protocol to support screening for latent infections in plants for planting. Results on how far away from the entry point the pathogen could be detected in latent infections were variable. Therefore, in the absence of symptoms, the trees should be screened for any cracks or wounds in the bark, which might have served as an entry point. Further work is needed to fully validate a sampling protocol which could be used on crop production sites or batches of plants for planting.

2.2. Project aims

The aim of the project was to develop an early, sensitive and fast diagnostic protocol for the detection of *Cryphonectria parasitica* in planting material. The availability of a diagnostic protocol that is rapid and sensitive will:

- Ensure that consignments of plants for planting can be tested

- Improve the quality of planting material (*Castanea* spp.) within possible certification schemes and avoid further spread of the pest.

The objectives of the project were:

- To optimise currently available real-time PCR tests for the detection of *C. parasitica* in planting material.
- To optimise currently available DNA extraction protocols that can be used with real-time PCR tests.
- To organise an inter-laboratory test performance study on the real-time PCR tests and DNA extraction protocols for the detection of *C. parasitica* on plant material.
- To test the real-time PCR test on symptomless plant material (artificially infected and naturally infected).
- To develop and validate a sampling protocol to support inspection of tree production sites or for laboratory analysis. This included improving knowledge on which part of the plant to sample, the optimum time period for sampling, the age of the tree and the amount of bark to be sampled.

2.3. Description of the main activities

2.3.1. Development of an optimised DNA extraction protocol and real-time PCR test

For the detection of *C. parasitica* there are two real-time PCR tests published: Rubio *et al*., (2017) and Chandelier *et al*., (2019). Both tests were compared by testing high and low concentrations (1 ng/reaction and 10 pg/reaction) of target DNA. The test by Chandelier *et al*., (2019) had higher sensitivity and was further optimised and verified for the detection of latent infections. For optimisation of the real-time test, four different master mixes were compared. After selection of the best performing master mix, different primer and probe concentrations were tested.

The DNA extraction methods from Chandelier *et al*., (2019) and Rubio *et al*., (2017) were compared and stepwise improved to obtain an optimised extraction method for infected *Castanea* bark. The method is a combination of the two extraction methods. First the grinding protocols for both methods were changed, the samples were placed in a plastic 5 mL screw-top tube and ground with a Geno/Grinder (SPEX, London, UK) as the tubes were much more robust when grinding after freezing in liquid nitrogen. The use of a CTAB extraction buffer (Rubio *et al*., 2017) was compared to extraction with TEX, RNase A and incubation (Chandelier *et al*., 2019). The semi-automated chloroform-based extraction using the Kingfisher mL (Rubio *et al*., 2017) was compared to extraction using the NucleoSpin kit (Chandelier *et al*., 2019). These options were crossed over to create four possible extraction methods. The final method was further optimised by testing different volumes of RNase A, incubation times and volume of sample to be extracted (0.05 -1.5 g). The efficiency of the extractions was determined by real-time PCR targeting the Cytochrome oxidase gene (COX) (Tomlinson *et al*. 2005) or the Chandelier *et al*., (2019) test.

The optimised DNA extraction method was then taken forward to compare to the original method in Chandelier *et al*., (2019) within a test performance study.

2.3.2. Verification of analytical sensitivity and specificity

The test by Chandelier *et al*., (2019) was tested for potential cross-reactions with non-target species closely related to *C. parasitica* or commonly found on *Castanea* spp. both *in silico* and *in vitro*.

To determine sensitivity of the test, dilutions of extracts from *C. parasitica* cultures of known concentration between 1.56 fg/µL and 800 fg/µL were prepared. All dilutions were tested on the optimised *C. parasitica* test in the presence of host DNA to account to the effect of potential inhibitors in bark material.

2.3.3. Ability to detect latent infections

The real-time PCR test by Chandelier *et al*., (2019) was assessed on naturally infected material asymptomatic material (or latent material) from both infected and supposed disease free areas in Germany. In twelve sites with confirmed infection in Baden-Württemberg, 41 samples were taken on healthy tissue of asymptomatic trees, healthy tissue of diseased trees, and diseased tissue of diseased trees. In four sites supposed to be disease free located in the Rhineland-Palatinate, 51 samples were taken on healthy tissue of asymptomatic trees. All samples were used for both culture isolation of *C. parasitica* and direct DNA extraction followed by real-time test as described in Chandelier *et al*., (2019).

In addition, 13 symptomless seedlings of *Castanea sativa* sourced from a nursery in Austria were analysed via real-time PCR. In the United Kingdom, 8 symptomless trees from high risk areas were extracted and tested via real-time PCR.

2.3.4. Development of a sampling protocol for detection of latent infections

To generate latent infected plant material, fifty 2-year-old chestnut trees (*Castanea sativa*) were inoculated with *C. parasitica*. In 25 trees, sterile pins were overgrown with mycelium and pushed into the bark of the stem at a height of approximately 15 cm. The other 25 trees were inoculated by pins dipped into a spore suspension. Five trees were used as negative controls, into which one sterile pin was pushed. From five weeks post inoculation the trees were sampled every two weeks until thirteen weeks post inoculation. Sections of bark (2 cm^2) were sampled from the 2 cm around the inoculation point, 2 cm below and above the initial sampling point, and a final sample which was 15 cm above the highest sampling point.

Artificial inoculation of chestnut trees was employed to detect the endophytic spatial distribution of infection and help inform any future sampling protocols. The 2-year-old chestnut trees (*Castanea sativa*), including two cultivars Bouche de Betizac and Südtiroler Gelbe were inoculated with *C. parasitica*. A T-shaped flap was cut into the bark with a sterile scalpel and an inoculum disc cut from the actively growing margin of a PDA culture was inserted under the bark flap. A wetted cotton ball was placed on top of the inoculation point and the wound was sealed with Parafilm to prevent desiccation of the mycelium. Non-inoculated agar plugs were used as controls. The progression of fungal infection was assessed 40, 77, 112, and 145 days post inoculation (dpi). Trees were cut into sections of 10 cm in length after 40, 77, 112 and 145 dpi. The distance away from the inoculation point at which *C. parasitica* could be detected by real-time PCR was assessed.

2.3.5. Test performance study

The aim of the test performance study was to provide more information on the robustness of the molecular method published by Chandelier *et al*., (2019) and compare it with the method

optimised during this project. Altogether, eight laboratories across seven counties participated in the TPS. The participants included project partners and additional participants were identified through a request issued within the European Mycological Network (EMN).
Samples in the TPS panel included ten samples of bark material with varying dilutions of infected bark for comparison of the extraction methods and five DNA extracts with varying dilutions of *C. parasitica* DNA for comparison of the real-time PCR methods. This allowed for extractions to be completed in each facility to allow comparison of methods between laboratories.
Results of the TPS were analysed by categorising the qualitative results provided by each participant into the number of positive agreements (PA), where both the expected result and the obtained result were positive; negative agreement (NA), where both the expected and obtained result were negative; positive deviations (PD), where the expected result is negative but the obtained result is positive, otherwise known as a false positive; finally, negative deviations (ND) where the expected result is positive but the obtained result is negative, otherwise known as a false negative.
The PA, NA, PD, ND values were then used to calculate various performance criteria. Accuracy (AC) signifies the level of agreement between the expected results and the results obtained using each procedure. Diagnostic specificity (SP) evaluates the capability of the procedure to not detect the pathogen when it is not present, for example in the healthy bark material. Diagnostic sensitivity (SE) evaluates the capability of the procedure to detect the pathogen when it is present, for example in the artificially infected bark samples or the *C. parasitica* pure culture extracts. Inconclusive results were removed from the data set used for the calculations. The performance criteria were calculated as follows: diagnostic sensitivity (PA/(PA + ND), diagnostic specificity (NA/ (NA + PD) and accuracy ((PA + NA)/(PA + NA + PD + ND)).

2.4. Main results (knowledge, tools, etc.)

2.4.1. Development of an optimised DNA extraction protocol and real-time PCR test

The extraction methods which used the TEX, RNase A and incubation steps (Chandelier *et al*., 2019) generally produced the best results, where all samples were found to be positive and Ct values were lowest. The method which combined the TEX, RNase A and incubation steps (Chandelier *et al*., 2019) followed by semi-automated chloroform-based extraction using the Kingfisher mL (Rubio *et al*., 2017) was chosen for further optimisation due to having fewer steps which lowers the risk of contamination and increases sample throughput.
To further optimise the chosen method, four different incubation times and volumes of RNase A were tested. A thirty-minute incubation time consistently produced the lowest Ct values, approximately one cycle lower. Overall, similar Ct values were obtained for 10, 25 and 40 µL RNase A; therefore, the lowest amount was chosen. Based on real-time PCR results and observations during extraction regarding grinding efficiency, a sample amount of 0.2 to 0.4 g is recommended.
Four master mixes were compared for their amplification efficiency and analytical sensitivity for the detection of *C. parasitica.* The *C. parasitica* test was found to be most sensitive when used with the iTaq Universal Probes Supermix (BioRad) master mix as the Ct values were approximately 4-6 cycles lower than the other master mixes for the same DNA concentration.

The highest amplification efficiency was achieved with 0.15 µM probe and 0.25 µM primers, therefore these concentrations were chosen for the optimised protocol tested in the TPS.

2.4.2. Verification of analytical sensitivity and specificity

In silico analysis showed a few species had a predicted binding site for all three oligos. These included *Cryphonectria radicalis, Holocryphia eucalypti* and *Pestalotiopsis guepinii*. Cultures of *C. radicalis* were also tested *in vitro* and no cross-reactions were observed. An amplification of *H. eucalypti* is expected to be delayed but cannot be excluded. However, this species is not expected to be found on any sample material as it is only reported on *Corymbia* spp., *Eucalyptus* spp., and *Tibouchina* spp. For *P. guepinii*, six mismatches were predicted for all three oligo binding sites. Therefore, no amplification is expected.

A range of different cultures were extracted and tested on the *C. parasitica* test to confirm *in vitro* specificity and by an internal control assay (FungiQuant) to confirm successful extraction. No cross-reactions were observed for any of the tested non-target species, whereas all target cultures of *C. parasitica* were detected reliably.

Dilution series from three different *C. parasitica* cultures were tested on the optimised test in the presence of host extract. The limit of detection was 50 fg DNA per reaction. The reproductivity of this result was confirmed on different machines on different days and by different users and found to be 100% reproducible.

2.4.3. Ability to detect latent infections

Asymptomatic material from both infected and supposed disease free areas in Germany were tested for the presence of *C. parasitica* through both culture isolation and real-time PCR. In sites with confirmed infection, healthy tissue of diseased trees and diseased tissue of diseased trees was also sampled. The results of culture isolation and real-time PCR varied among samples from infected sites. *C. parasitica* was isolated from almost all diseased tissue samples and also detected by real-time PCR. There was no culture growth of *C. parasitica* from samples taken on asymptomatic trees, but four of these samples were tested positive in real-time PCR analysis.

From samples of previously supposed disease-free sites taken on healthy tissue of healthy trees, there was no culture growth of *C. parasitica*. On the first site, slight infestation was noticed on scattered trees with limited cankered lesion, and two samples were tested positive with real-time PCR. On the third site distantly located and without sign of disease, one sample was tested positive. The other two sites show no disease symptom and all samples were tested negative.

Of the symptomless seedlings tested by real-time PCR in Austria, *C. parasitica* could be detected in one seedling. *Cryphonectria parasitica* could be detected in two out of the eight symptomless trees which were sampled from high risk areas in the United Kingdom. These results indicated that latent infection could be detected by real-time PCR.

2.4.4. Development of a sampling protocol for detection of latent infections

Of the artificially inoculated trees grown at Fera Science Ltd, most trees were free of symptoms when sampled (between 5 and 13 weeks after inoculation). From each tree, the inoculation point was sampled, which was positive in 47 out of 50 trees. Across all positive samples, the average Ct value on *C. parasitica* assay was 30.17 (+/- 4.03). In most trees, it was not possible

to detect the pathogen more than 2 cm away from the infection point. Overall, the fungus does not seem to extend far into the tissue in a latent infection.
Further trees were artificially inoculated at AGES, Austria. In all trees non-symptomatic sections tested positive for *C. parasitica*. Initially, the non-symptomatic spread of the pathogen was recorded mainly in the areas neighboring the inoculation point. From the third sampling date onwards (112 dpi) the pattern of infected sections was irregular. The uneven endophytic migration is illustrated by a schematic treelet diagram (Figure 1), and distance from the inoculation point at which the pathogen can be detected can be seen in Figure 2.

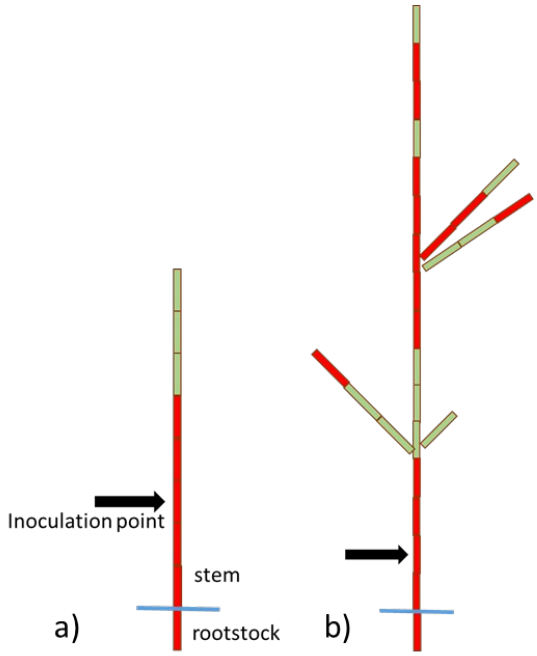


Figure 1: A depiction of the progress of infection on Bouche de Betizac cultivar of *Castanea sativa* at: a) 40 dpi, and b) 145 dpi. The red label sections were tested positive for *Cryphonectria parasitica* and the green labelled sections tested negative for *C. parasitica*.

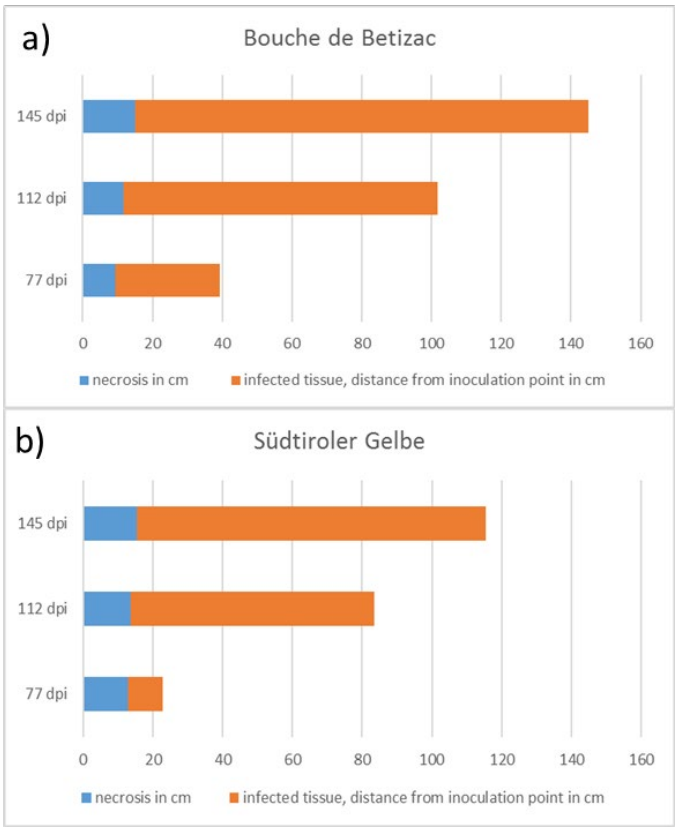


Figure 2: The relationship between visible symptoms (necrosis) on the bark material and the distance from the inoculation point at which *C. parasitica can be detected by* real-time PCR, for a) cv. Bouche de Betizac, and b) cv. Südtiroler Gelbe.

2.4.5. Test performance study

The DNA panel allowed comparison of the protocol for real-time PCR in Chandelier *et al*., (2019) (Test 1) and the optimised method developed in this study (Test 2). Negative DNA samples were correctly reported as negative with no false positives detected by any laboratory by either Test 1 or Test 2. Therefore, both tests achieved 100% specificity (Table 1). On the other hand, there was a much higher rate of false negative results. Test 1 had a diagnostic sensitivity of only 66.7%, with most of the false negative results from the highly diluted DNA extracts. Test 2 had a higher diagnostic sensitivity of 75%, all false negative results reported were for samples of highly diluted DNA extracts. Whether the low concentration DNA extracts were detected was highly laboratory dependent. Overall, the accuracy of both tests was equivalent at 83.0% and 83.3% respectively.

Table 1. Summary of TPS results for both the bark panel and the DNA panel

	Bark panel		DNA panel	
	Test 1	Test 2	Test 1	Test 2
	Chandelier *et al*. (2019)	Experimental procedure	Chandelier *et al*. (2019)	Experimental procedure
No. partners involved	8	3	8	3
No. samples analysed	96	36	48	18
No. samples included	90*	36	47*	18
Negative accord (NA)	27	12	16	6
Positive accord (PA)	61	23	23	9
Negative deviation (ND)	1	1	8	3
Positive deviation (PD)	1	0	0	0
Diagnostic sensitivity (SE)	98.4%	95.8%	66.7%	75.0%
Diagnostic specificity (SP)	96.4%	100.0%	100.0%	100.0%
Accuracy (AC)	97.8%	97.2%	83.0%	83.3%

*Seven results rated as undetermined were removed from the data set.

SE = 100% X PA/ (PA + ND)

SP = 100% X NA/ (NA + PD)

AC= 100% X (PA + NA)/ (NA + PA + PD + ND)

The bark panel allowed comparison of the extraction protocol in Chandelier *et al*., (2019) (Test 1) to the optimised extraction method developed in this study (Test 2).
The test performed equivalently in accuracy once the inconclusive results were excluded from the analysis, 97.8% (Test 1) and 97.2% (Test 2) accurate. However, Test 1 produced many more inconclusive results. The four inconclusive results from one participant included healthy bark samples and the negative isolation controls. The results were deemed inconclusive due to late Ct values around the laboratory cut off point which would have been repeated if additional sample material was available. Two infected bark samples were also reported as inconclusive, due to late amplification. Test 1 and 2 had specificity values of 96.4% and 100% respectively as Test 1 resulted in one false positive report in a healthy bark sample. However, sensitivity was slightly higher when using Test 1 (98.4%), compared to Test 2 (95.8%). Both procedures had false negative results in bark samples with the lowest ratio of infected material (1:5).

2.5. Conclusions and recommendations to policy makers

The test developed by Chandelier *et al*., (2019) was verified as the most sensitive published test for the detection of *C. parasitica* and therefore was taken forward to test symptomless plants for latent infections. Both the Chandelier *et al*., (2019) test and the Rubio *et al*., (2017) test are suitable for the detection of *C. parasitica* in symptomatic plant material. However, for laboratories undertaking latent testing, where a higher degree of sensitivity is required, it is recommended that the Chandelier *et al*., (2019) test is used. Young *Castanea* trees were artificially infected on two occasions, and the real-time PCR was able to detect the infection in symptomless material. However, the distance the pathogen could be detected from the inoculation point varied quite substantially. In the trees infected at Fera Science Ltd the fungus did not seem to extend far into the tissue in a latent infection and was not detected over 2 cm from the inoculation point. This is also supported by Chandelier *et al*., (2019), who could not detect the fungus more than 2 cm away from the lesion. Therefore, it is likely that the spore (or mycelium) after entering the bark through a wound remains dormant until environmental signals trigger outgrowth and active infection. When trying to sample latent infections in the field it is recommended that the physical point of entry of fungal tissue is sampled. In the absence of symptoms, the trees should be screened for any cracks or wounds in the bark, which might have served as an entry point. In young trees which were artificially infected at AGES positive detection of the pathogen by real-time PCR could be achieved at substantial distances from the inoculation point. Although, when mapping the spread of the pathogen the pattern of infection could be irregular. Therefore, alongside trying to sample potential pathogen entry points, it may be advisable to sample multiple areas along the stem. These finding can help inform future work to establish a sampling protocol for crop production sites or batches of plants for planting. More research is needed to provide a standardised sampling method that can be used on symptomless plant material. False negatives may be problematic in symptomless material if the correct areas are not sampled. This could be combatted via one or multiple strategies including sampling a larger number of trees in an area / consignment, sampling a larger area on the individual trees, or sampling an area repeatedly over a period of time. Additional research is needed to decipher the best sampling approach and generate data on the accuracy of the method when diagnosing latent plant material for *C. parasitica*.

Trees were sampled which had the potential to be naturally infected with *C. parasitica* and multiple samples which exhibited no symptoms were detected as positive for *C. parasitica* on the real-time PCR test. This suggests the proposed sampling protocol and test can be successful at detecting latent infections. *C. parasitica* was also detected in symptomless trees from areas which were supposed to be free from the pathogen, for example the Rhineland-Palatinate in Germany. These results raise the importance of latent testing in the appraisal of the current disease distribution. By enabling direct detection of *C. parasitica* in plant material, the real-time PCR also allows accurate assessment of disease progression. It is recommended to include latent testing in the symptom-based annual monitoring of chestnut blight in order to confirm the pest free area status and enhance efficacy of control measures.
Both extraction methods selected for the TPS performed comparatively with accuracy levels of 97.8% and 97.2%. Therefore, the decision on which method to use may be based on the equipment and expertise available within the diagnostic laboratory.

Sensitivity was an issue for both procedures, with many false negative results reported for highly diluted DNA extracts even though these concentrations were above the limit of detection (LOD) for these methods. The ability to detect the low concentration DNA samples was highly laboratory dependent. Therefore, both tests perform very well in some cases. However, the test may have reproducibility and robustness issues and be less sensitive in laboratories which differ in their equipment or have different levels of experience with the method. The reduced level of detection for the low concentration DNA may be problematic in the early detection of *C. parasitica* and shows the importance of optimisation and verification of the LOD within individual laboratories. The results of this project will provide valuable validation data to diagnostic laboratories involved in the detection of *C. parasitica* in planting material.

References

- Chandelier, A; Massot, M; Fabreguettes, O; Gischer, F; Teng, F; Robin, C (2019). Early detection of *Cryphonectria parasitica* by real-time PCR. European Journal of Plant Pathology, DOI: 10.1007/s10658-018-1538-0.
- Cunnington JH, Pascoe IG (2003). Post entry quarantine interception of chestnut blight in Victoria. *Australasian Plant Pathology* 32: 569-570
- Jeger, M; *et al* (2016). Risk assessment and reduction options for *Cryphonectria parasitica* in the EU. EFSA Journal 14(12).
- Rubio, S., Barnes, A., Webb, K. and Hodgetts, J. (2017). A real-time PCR assay for improved rapid, specific detection of *Cryphonectria parasitica*. Annals of Applied Biology, 171: 52–61. DOI:10.1111/aab.12354.
- Tomlinson, J. A., Boonham, N., Hughes, K. J. D., Griffin, R. L. & Barker, I. (2005). On-Site DNA Extraction and Real-Time PCR for Detection of *Phytophthora ramorum* in the Field. *Applied and Environmental Microbiology,* 71**,** 6702-6710.

2.6. Benefits from trans-national cooperation

The overarching benefit of trans-national cooperation was to build stronger relationships between international laboratories. These relationships enabled transfer of experience and knowledge in the detection of *C. parasitica* in the respective countries involved. The project has led to the successful validation of a protocol, which could be used to detect *C. parasitica* in planting material and help control the spread of the disease.

The collaboration allowed the opportunity to test developed protocols within the framework of a collaborative test performance study and demonstrates the usefulness of broad comparative laboratory testing for method validation, which is recommended by the European Plant Protection Organization (EPPO). The validation data from the TPS can be added to the EPPO validation database and included in a new version of the EPPO diagnostic protocol.

The partners hope to continue collaborations into the future with an aim to submit results of the project to a peer reviewed scientific journal. As the laboratories have successfully collaborated on this project, there are strong links which could benefit future collaborations.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

Manuscript in preparation: Interlaboratory comparison of diagnostic protocols for the molecular detection of *Cryphonectria parasitica* in bark.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

None.

4. Open Euphresco data

Relevant data will be published in the EPPO Database for diagnostic expertise.