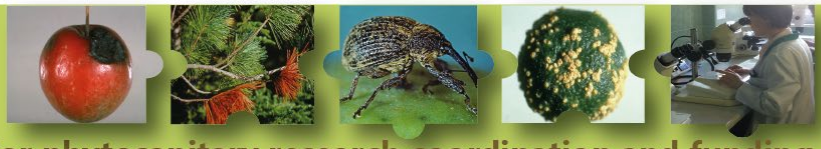

Final Report

Project title (Acronym)
Early detection of fungal storage pathogens on pome fruits (EARLDETEC)

Project duration:

Start date:	01-07-2018
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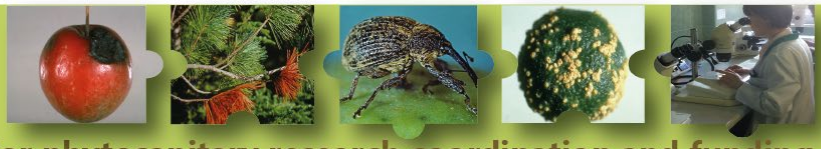
Contents



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2. Short project report

2.1. Short executive summary

Fungal storage pathogens of pome fruits are responsible for major losses worldwide. The fruits get infected in the field, but symptoms start to be visible during the storage period, which is problematic for their marketing and trading. Detecting the latent infections is a challenge but provides data on the pathogen colonisation during fruit development or at harvest time. Knowing this, measures for disease control could be improved.
Several activities were undertaken in the partner countries, including the monitoring of storage diseases and the development of new diagnostic tests. A range of tools for managing these pathogens is now available and can help the deployment of a pre storage monitoring for storage diseases in the future.

2.2. Main activities

The incidence of postharvest diseases of pome fruits and their causative agents were assessed in all participating countries. Fungal pathogens were isolated and identified from cultures. The occurrence and frequency of the observed pathogens seem to be dependent on climate conditions, cultivar and localisation of the orchards.
In Poland, the occurrence of fungal storage diseases was investigated on four apple cultivars grown in the central part of the country. The fruits were selected from IPM - managed orchards and stored in a cold room for 5-7 months. The occurrence and frequency of fungal storage pathogens was observed. Further studies concentrated on the development of fast and sensitive detection methods for *Monilinia* sp. and *Neofabraea* spp. For this purpose, apples (cv. Topaz) were inoculated in the orchards with fungal spore suspensions one month before harvest. The same fruits were sampled three times at 30 days intervals after harvest. The dry peel of asymptomatic fruits was subjected to total DNA extraction. Several protocols for DNA extraction were tested and the DNA was checked for purity and absence of PCR inhibitors. Several primer sets (for LAMP method) were validated for the diagnosis of *Neofabraea* and *Monilinia* spp: The LAMP test from Michalecka, 2020 (unpublished data) for the detection of *N. vagabunda*, the LAMP test from Michalecka, 2020 (unpublished data) for the detection of *N. perennans* and *N. kienholzii* and the LAMP test from Poniatowska, 2020 (unpublished data) for the detection of *M. fructicola*, *M. fructigena, M. laxa* and *M. polystroma*.
In Russia, research focused on *Monilinia* spp. Asymptomatic apples were kept at 4°C. After 10 days brown rot symptoms appeared, and the causal agent was identified as *M. fructigena* by morphological characteristics and molecular methods. To determine the distribution of *M. fructigena* in the fruits, different parts of the fruits were analyzed: The wash water of asymptomatic apples (0,1%Tween + 0,1% pepton solution) was centrifuged (5 000 rpm for 30 min) and 100 µl of the solution was placed on Potato Dextrose Agar (PDA). Colonies of the fungus were observed after incubating for 7 days at 24°C. Two techniques were evaluated for the DNA extraction from ground peels: a grinding with extraction buffer from a GMO-MagnoSorb – commercial kit (SYNTOL) followed by DNA extraction with the same kit and grinding with liquid nitrogen before DNA extraction with GMO-MagnoSorb – commercial kit (SYNTOL). A real-time PCR test (Brouwershaven *et al*., 2010) was performed on CFX96 Touch real-time PCR Detection System (BioRad) using the 'Phytoscreen' commercial kit for *Monilinia* detection (SYNTOL).

The time between (artificial) inoculation and the earliest positive detection of *M. fructicola* by real-time PCR was also investigated. Therefore, apple tissues were prepared as follows: small pieces (0,5 x 0,5 cm) of apple tissues were cut out using a sterile scalpel (20 mg per apple), 20 ml of phosphate buffer (PB) were added to each sample, followed by shaking for 5 min at 120 rpm. After that, the liquid was filtered and centrifuged for 10 min at 5 000 rpm at 5°C. 1 ml of phosphate buffer saline (PBS) was added to the sediment and an automated DNA extraction from the obtained solution was performed followed by real-time PCR.

Until recently the main causal agents of postharvest decay of pome fruit in the Netherlands were unknown. 400 samples of decayed apple and pear fruits from various production areas were collected from commercial controlled atmosphere (CA) storage facilities and analysed in the laboratory. Three methods for the detection of latent infections (with the aim of enhanced symptom expression) were evaluated at harvest. The real-time PCR test (Köhl *et al*., 2018) was used for the quantification of selected pathogens (*Neofabraea vagabunda* and *Cadophora luteo-olivacea*) on apples and pears. Furthermore, a method for sample processing was successfully developed.

For the assessment of the incidence of postharvest pathogens in Romania, fruits with visible symptoms were sampled from storage facilities in several counties at different dates. Pathogens causing rot symptoms were isolated and identified. For the establishment of detection protocols, two of the most important target pathogens were selected (*Neofabraea* spp. and *Monilinia* spp.). Fungi were grown on two different culture media (Potato Dextrose Agar and Tomato Juice Agar) and isolates were preserved by freezing at -80°C on glycerol. DNA extraction protocols and the multiplex PCR test (Köhl *et al*., 2018) were evaluated, and fungi could be confirmed.

In Austria symptomatic apples were sampled from 4 storage facilities after short (1 – 4 months) and longer (5 – 8 months) storage including fruits from areas in 4 federal states to survey the incidence of storage rot pathogens. Fungi were isolated and identified by morphological characteristics and by molecular tests. The position of the rots on the fruits was also monitored.

To observe the fungal colonization of apples before harvest, fruit spurs and asymptomatic fruits were collected during the growing season from June to August over 3 vegetation periods from two sites (integrated and organic production) with a *Neofabraea* – record in the past. The fruits and spurs were surface washed with deionized sterile water; this water was then filtered, and the filters were used for DNA extraction. For the identification of the fungal pathogens DNA barcoding (Sanger Sequencing) was performed, using primer pairs for internal transcribed spacers ITS 1 and ITS 4 and ITS 5 and ITS 4 (White *et al*.,1990). For the identification of *Fusarium*, the test from Raja *et al*. (2017) was used. For the identification of *Neofabraea* spp. the test from Michalecka *et al*. (2016) was used. Additionally, the fruit spurs were investigated morphologically. After surface disinfection the fruit spurs were placed onto Potato Dextrose Agar amended with Streptomycin sulphate and incubated. In 2020, apples from the above described orchards were sampled at harvest time. To determine the presence of *N. vagabunda* on the fruit surface, the samples were washed and the wash water was analysed. The washed apples were also used for direct diagnosis.

Two methods of recovering *Neofabraea* spp. from the fruit surface were compared. Method 1: samples were washed with 50 ml of distilled water, method 2: samples were washed with 50 ml of a solution of 0.1% soy peptone and 0.01% Tween 80 according to Martins *et al.* (2013). The wash solution was centrifuged at 5 000 rpm for 30 minutes. 70 µl of the pellet was used

for DNA Extraction. The real-time PCR test from Köhl, 2018 was performed. Stem and peel of the apples were examined separately. They were homogenized in BIOREBA extraction bags with CTAB buffer with a BIOREBA Homex 6. 70 µl of the sample was used for DNA extraction and real-time PCR (Köhl, 2018) was carried out. The symptom occurrence of *N. vagabunda* was monitored at the end of the storage period.

2.3. Main results

2.3.1. Monitoring storage diseases

A survey of storage diseases that started 2012 in Poland led to the following results:
On apple cvs 'Gala', 'Ligol' and 'Golden Delicious' bull's eye rot caused by *Neofabraea* spp. was the most frequent observed disease while on apples cv. 'Gloster gray' mold caused by *Botrytis cinerea* predominated. The blue mold caused by *Penicillium expansum*, brown rot caused by *Monilinia* spp. and fungi of *Alternaria* spp. occurred at significantly lower intensity. New storage diseases caused by *Colletotrichum* spp., *Neonectria galligena* and *Diaporthe eres* were also detected. For the first time in Poland it was shown that *D. eres* can be pathogenic to apples during cold storage. In September 2020, the incidental presence of *Stemphylium vesicarium*, causing brown spot of pears, was detected during harvest period. This disease was also observed in Poland for the first time.
In Russian apple samples collected from Ryazan area, the fungi causing brown rot symptoms were identified as *M. fructigena* by morphological culture characteristics. The results were confirmed by Sanger sequencing of the ITS region. *Penicillium spp*., *Aspergillus spp*., and *Trichothecium* spp. were isolated from the washout of asymptomatic apples from the same area, but no *Monilinia* spores could be detected by morphological analyses. Samples of asymptomatic apples were stored at 4 °C and showed brown rot symptoms after 10 days. This confirmed that also symptomless fruits may contain latent infections of *M. fructigena*. Two methods of sample preparation and DNA extraction followed by real time PCR were conducted from asymptomatic apple peels. No *Monilinia* spp. could be detected in these samples. As a proof of principle, the detection of *M. fructicola* from apple tissue previously inoculated was successful after a prolonged incubation period (7 days) and when highly inoculated (3 injections, spore concentration 2×10^5 spores/ml).
In the Netherlands the surveys of storage pathogens revealed the presence of common postharvest pathogens, such as *Botrytis cinerea* and *Neofabraea vagabunda*, but also a number of new and emerging postharvest pathogens, such as *Fusarium avenaceum* on pear and apple, *Neonectria candida* and *Neofabraea kienholzii* on pear, and *Colletotrichum godetiae* and *Truncatella angustata* on apple. In most cases these newly described postharvest pathogens were isolated at low incidences only. In contrast, two latent postharvest pathogens more frequently appeared: *Cadophora luteo-olivacea* causing side rot on pears, and *Fibulorhizoctonia psychrophila* as the causal agent of lenticel spot on apples and pears. For both diseases incidences ranged from very low to nearly 100% of stored fruits. Thus, these latter two fungal species are presently considered as the most important latent postharvest pathogens on pome fruit in the Netherlands.
On apples sampled in Romania in 2019, four different species of *Penicillium*, *Botrytis* spp. *Stemphylium* spp., *Fusarium* spp., *Alternaria* spp., *Monilinia fructigena*, *Gloeosporium* spp., *N. vagabunda* and *Trichoderma harzianum* were found. Also, one entomopathogenic fungus, *Akanthomyces muscarius*, was isolated. The fungus identity was confirmed by DNA barcoding

using ITS1 - ITS4 primers and blast in BOLD systems, GenBank (NCBI) and EPPO Q-bank. The same protocol was used to confirm three isolates of *M. fructigena,* one isolate of *N. vagabunda* and one of *T. harzianum.* In 2020, *Penicillium* spp., *Botrytis* spp., *M. fructigena*, *N. vagabunda* and *T. harzianum* were identified. On apples sampled in 2021, six isolates of *Penicillium spp.*, *Botrytis* spp., *Monilinia* spp. and *Trichoderma harzianum* were detected.
In 2019, 99 samples were analysed from Romanian fruits (60 organic apples and 41 conventional apples, belonging to 10 different varieties - Rubinola, Topaz, Renoir, Gemini, Red Prince, Golden Delicious, Starkrimson, Generos, Idared and Gala) and 2 from Poland. 14 isolates of *Neofabraea* spp. were obtained from Romanian apples (Fig. 1) and 2 from imported apples (Ciceoi & Iacomi, 2019), In 2020, 5 isolates of *Neofabraea* spp. were obtained from of 24 organic apples, belonging to 8 different varieties cv. ‘Stark prim’, ‘Dalinette’, ‘Gala’, ‘Rubinola’, ‘Topaz’, ‚Produkta’, ‘Renoir’ and ‘Gala King’.

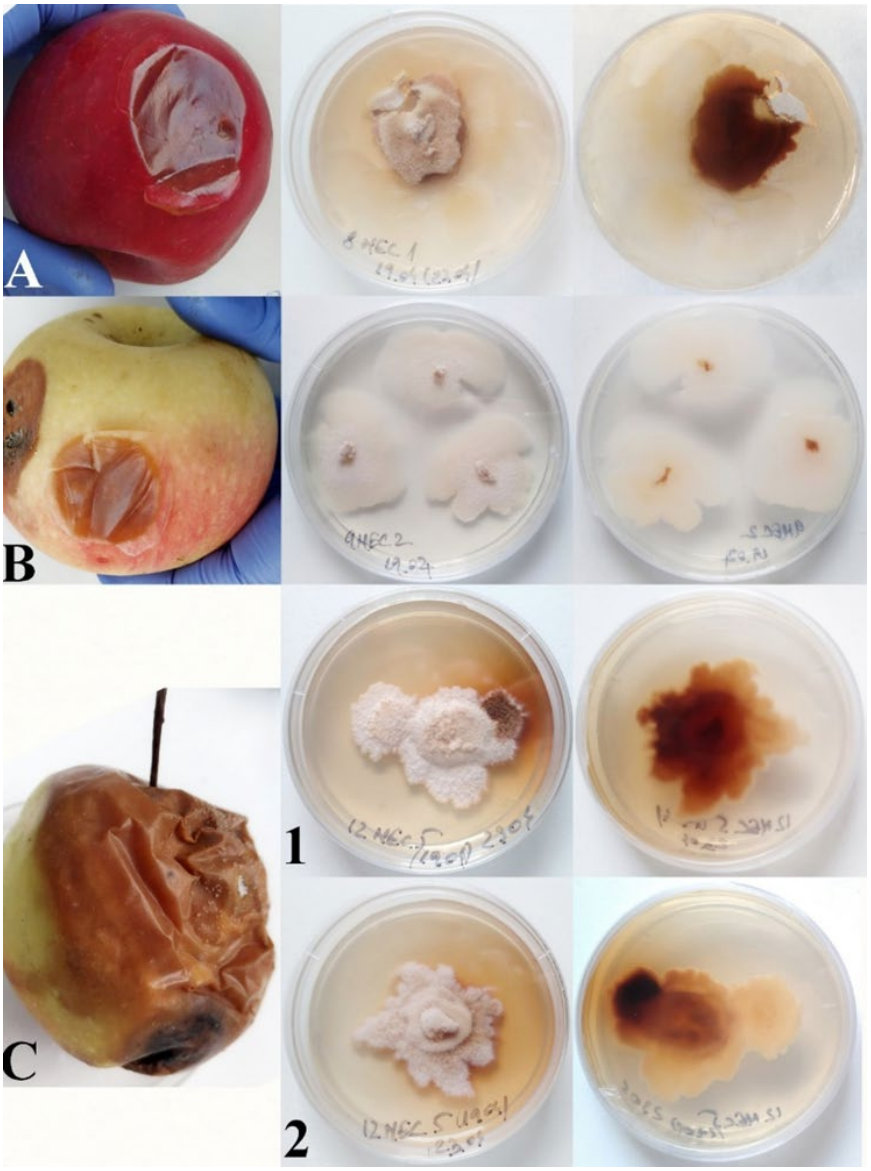

Fig. 1: *Neofabraea* isolated in 2019. Courtesy of Moara Domneasca, ILFOV County (RO), A: cv. Idared, B, C: cv. Jonathan

In 2021, no *Neofabraea* could be isolated from 21 samples of organic apples. Generally, the position of the rots was monitored and the necrotic area was situated on one side of the fruit, not at the stalk or calyx. Sampling the tissue underneath the skin from diseased apples proved to be the best area for isolation.

To obtain a spore suspension for artificial inoculation, variations of Tomato Juice Agar were tested. Three samples of *N. vagabunda* from different origins were compared: a control sample DabLig3 from Poland and two Romanian isolates Gol11-19 and Gol15-19.

Three Tomato Agar Media, based on: 1) Italian tomato sauce (Freshona), 2) fresh homemade tomato sauce, and 3) boiled tomato & sweet pepper mix sauce, all incubated at 16°C and 14°C, were tested. Different colony morphologies were observed (Fig. 2).

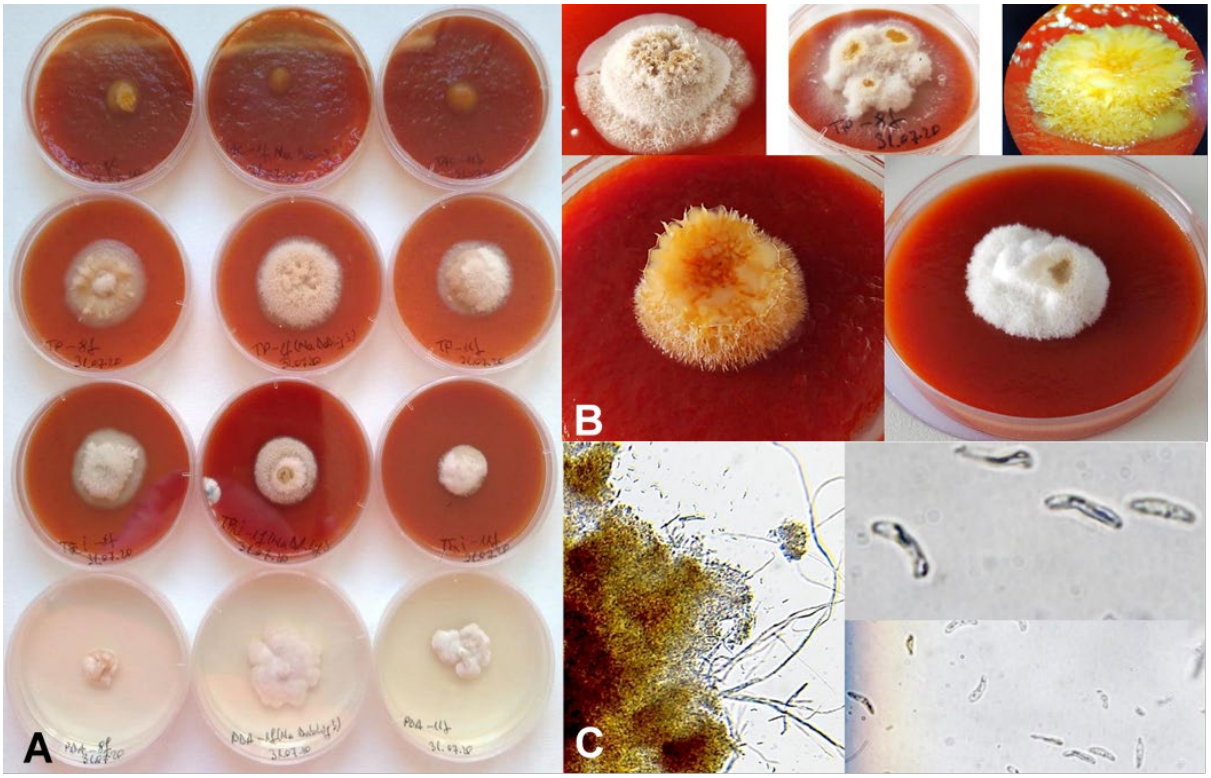

Fig. 2: Testing different agar media for optimum spore production. A: *N. vagabunda* on 3 different Tomato Juice Agars and PDA. B: Different colony shapes, C: macroconidia of *N. vagabunda*

A long-term preservation of fungal isolates was tested (-20 °C on glycerol). All isolates were able to regenerate viable cultures even two years after storage, with the exception of two Romanian isolates.

On symptomatic apples from Austrian storage facilities the pathogenic fungi *Fusarium avenaceum, F. latericium, N. vagabunda, N. kienholzii, N. perennans* and *Botrytis* spp. were detected frequently. To a lesser extend *Penicillium carneum, Alternaria* spp., *Diplodia seriata, Monilinia fructigena* and *Neonectria ditissima* were isolated and identified. Morphological identification was carried out through macroscopic and microscopic studies. During the first 3 months after harvest *Penicillium* spp., *Monilinia* spp. and *Fusarium* spp. prevailed, after longer storage *Neofabraea* spp., *Penicillium* spp., *Fusarium* spp., *Monilinia* spp., *Alternaria* spp.,

Mucor spp., *Botrytis cinerea* and *Neonectria* spp. were detected and identified. For molecular in-depth identification of the isolated fungi to species level the extracted fungal DNA was amplified by PCR using specific internal transcribed spacer primers (ITS 1 / ITS 4 and ITS 5 / ITS 4), the PCR products were sequenced and compared with the other related sequences in GenBank (NCBI).

In 2019, the pathogen load of Styrian apples was investigated during the growing season. Both previously described washing methods were successfully tested for findings of *Neofabraea* spp. Primers for real-time PCR (Köhl, 2018) showed to be not specific, except for *N. vagabunda.* With progressing vegetation period according to the sample dates, the pathogen load of *N. vagabunda* increased in both management types (organic and conventional). A slight tendency of increasing amount of DNA towards harvest could also be determined for *N. perennans / kienholzii / malicorticis*. *Neofabraea* spp. was detected in higher quantities from samples from organic than conventional production. Fruits and spurs were examined separately. In 89 % of the samples, the spurs showed higher quantities of *N. vagabunda* than the fruits. In contrast, in 67 % of the samples, the fruits showed higher DNA quantities of other *Neofabraeas* than the spurs. No growth of *Neofabraea* spp. could be observed by morphological analyses of the fruit spurs. Instead, other fungi as *Alternaria* spp., *Epicoccum nigrum* or spp. were isolated.

2020 apples from the same orchards were sampled at harvest time. A higher amount of *N. vagabunda* was found in the wash water than in squashed (and previously washed) peels and stems. This shows that at this point the pathogen seems to live predominantly on the fruit surface and has invaded the apple skin to a lower amount. Other *Neofabraea* spp. were excluded from the study in this year due to problems with their identification and because of previous results about their lower occurrence. From peels, the detection of *N. vagabunda* was only possible in samples from the organically managed farm where it was close to the limit of detection. The apples were also surveyed at the end of the storage period. No symptoms of *Neofabraea* spp. could be detected on the conventionally produced fruits. Fruits from the organic orchard showed *Neofabraea*-symptoms on 6.3 % of the stored fruits after 6 months of storage. Further studies would be necessary to be able to correlate the amount of fungus spores at harvest to the incidence of rots after storage.

2.3.2. Development of detection protocols

Two sets of LAMP primers were successfully tested in Poland: one specific to the aspartyl protease gene of *N. vagabuda* and one specific to a GTP-binding protein fragment gene of *N. perennans* and *N. kienholzii*. For *Monilinia* spp. one LAMP primer set was proposed that targets the heat shock protein 60 (HSP60) gene of *M. fructicola*, *M. fructigena*, *M. laxa* and *M. polystroma.* The newly developed and simply validated LAMP tests enabled fast (around 35 minutes), specific (no reaction was observed with the DNA of other post-harvest fungi) and sensitive detection. The use of these LAMP tests allowed to detect 1-2 pg/µl of target DNA in fungal pure cultures (*Neofabraea* spp. or Monilinia spp. according to the LAMP test used), but in apple skin the sensitivity was around 10 pg/µl. Pre-amplification of targeted gene fragments in conventional PCR prior to LAMP test raised the sensitivity of the protocol up to 10 times.

The multiplex PCR protocol (Michalecka *et al*., 2016) was adapted for the Hotstar Taq Master Mix kit – Qiagen. The primers were also successfully used in simplex PCR and adapted for the 5 x HOT FIREPol® Blend Master Mix – Solis BioDyne.

Annealing temperatures had to be raised for *N. vagabunda* and *N. perennans* to increase specifity:

Pathogen	Primers	Annealing temperature
N. perennans	Neo uni Neo_spnov-loTub-319	66°C
N. vagabunda	Neo uni Neo_alba3	64°C

ONFIT (overnight freezing incubation technique), ethephon treatment and chemodiagnostic tests appeared to be useful to detect *Alternaria* spp., *Colletotrichum* spp., and *Cladosporium* spp. For the detection of *Neofabraea* spp. and *Cadophora* spp. these tests turned out to be less appropriate. The overnight freezing-incubation technique (ONFIT) is a well-established method for detecting e.g. latent *Monilinia* infections (Luo and Michailides, 2003). However, one of ONFIT's disadvantages is its duration: it takes 7 to 9 days to detect the latent infection, which is activated by causing senescence in epidermal cells by freezing the fruit at –20°C for 48 h (Luo and Michailides, 2003). Once the epidermis is damaged, the fruit is first incubated for 5 to 7 days at 25°C and a high relative humidity (RH) and then examined for signs of brown rot or other pathogens that are present. An ethephon (ethylene) treatment acts as a trigger for fruit senescence, which has the effect of accelerating the development of postharvest pathogens (Prusky *et al*., 1996).

After evaluating different protocols, the most promising one is the DNA extraction followed by specific real-time PCR. This approach proved to be more reliable than ONFIT, and turnover time is shorter. Still, to the rather low level of detection of this procedure the infection level needs to be rather pronounced.

Real-time PCR tests were developed for the quantification of *N. vagabunda*, *N. perennans*, *C. luteo-olivacea*, and *F. psychrophila* in fruit and in environmental samples. In another related project these tests were used for the evaluation of inoculum status in orchards during the growing season (Köhl *et al*., 2018). The real-time PCR tests were used to detect latent infections of *N. vagabunda* and *Cadophora luteo-olivacea* on apples and pears. A method for sample processing was developed that allows sampling, peeling, freeze dry, grinding, processing for analyses. The sampling strategy, i.e. sample size and sample locations were discussed. Validation data show that the method is sensitive and specific. However, developing an adequate sampling protocol; i.e. how many fruits and what parts should be sampled, is still a challenge and needs further refinement.

A new method for analysing the presence of fungal pathogens on fruits was developed. This approach is based on washing fruits and fruit spurs during the growing season and analysing the wash water via real-time PCR according to Köhl *et al*., 2018 for the presence of pathogens. The experiments showed that this is a relatively simple and accurate method. The exact correlation between the presence of the pathogen, latent infections and disease expression needs further exploitation.

2.4. Conclusions and recommendations to policy makers

One of the aims of the project was to determine the main fungal pathogens causing storage decays in the participating countries. The activities in this transnational project enabled a deep

insight in the range of problematic pathogens responsible for storage diseases in apples and pears. Different approaches to detect and identify the pathogens involved were systematically developed and validated. A range of tools for managing these pathogens is now available to support pre-storage monitoring for these diseases in the future. Still, further research is needed to complete the knowledge on the relationship between pathogen detection and disease incidence. This also includes e.g. data on the sample size or cultivar susceptibility.
Currently, no single method has emerged to robustly and reliably control postharvest diseases of pome fruit in practice.
It is recommended to test the fruits for the presence of the pathogens, which are causing problems in storage. On the basis of these data further measures can be taken: measures can either be applied before harvest, or storage conditions can be adapted (storage time and/or storage conditions) to minimize the expected losses.
Moreover, latent postharvest diseases should be regarded as complex problems that require multiple actions at different stages of the disease process in a sustainable system intervention approach for their control. Such approach requires a deep understanding of the epidemiology of the causal agents in the orchard, fruit defence mechanisms against pathogens, and the molecular biology of host-pathogen interactions in order to develop novel disease control methods; such as the deployment of resistant cultivars and early detection of the presence of pathogens on fruit and in the orchard. This integrated approach requires collaboration between specialists in storage conditions, pre/postharvest pathologists and molecular biologists.

2.5. Benefits from trans-national cooperation

International collaboration allowed to obtain valuable information on the methods used in the various countries and unpublished information on the research activities on storage diseases were shared. Insights into the presence and recurrence of different pathogens in the various countries were provided. Discussions about experimental approaches improved the knowledge and understanding of the detection and identification of (latent) fungal storage diseases. The exchange of samples, protocols and experience was not only useful within the project, but also for a good international cooperation in general. An European network of storage disease experts could be further developed and intensified.

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Related publications

- Giraud M, Coureau C, Perrin J and Westercamp P (2021). Apple lenticel rots: state of knowledge on the epidemiology of *Neofabraea vagabunda*. Acta Horticulturae 1325, 59-66

3. Publications

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

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

- Ciceoi R, Zugravu MM, Iacomi BM (2019). First report of entomopathogenic fungus *Akanthomyces muscarius* on stored apples in Romania. Journal of Horticulture, Forestry and Biotechnology, 23(4): 14-17. https://journal-hfb.usab-tm.ro/2019/Volum%2023 (4)%20-%20PDF/4Ciceoi%20Roxana.pdf
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- Harteveld DOC, Pham KTK, Wenneker M (2020). Confirmation of black rot on different apple cultivars caused by *Diplodia seriata* in the Netherlands. Plant Disease: https://doi.org/10.1094/PDIS-10-19-2103-PDN
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4. Open Euphresco data

None.