EUPHRESCO Final Report

For more information and guidance on completion and submission of the report contact the EUPHRESCO Call Secretariat (bgiovani@euphresco.net).

Strawberry Pathogens Assessment and Testing **(SPAT)**

Project Duration:

Start date:	***15/07/2013***
End date:	***15/07/2015***

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2. Executive Summary

Strawberry Pathogens Assessment and Testing *(SPAT)*

Strawberry (*Fragaria* × *ananassa*) is an important food crop that is susceptible to a range of pests, including fungi, bacteria, viruses and nematodes. The main fugal pathogens in Europe are *Phytophthora cactorum* (crown rot)*, Colletotrichun acutatum, Verticillium dahliae, Botrytis cinerea* (grey mould)*, Mycosphaerella fragariae* (purple stain), and *Sphaerotheca macularis*, while the main bacterial disease is *Xanthomonas fragariae* (angular leaf spot). The objectives of the project were to make an overview of literature on strawberry diseases in partner countries and on all of the testing methods that are applicable in testing for *X. fragariae* and *P. fragariae.* Another objective was to develop and update diagnostic protocols and evaluate their strength and weaknesses by a ring test.

Literature on strawberry diseases at national level is relatively poor, with the exception of Russia. During the duration of the project, a questionnaire was circulated amongst strawberry growers and producers and field samples were collected and tested. These provided information on the occurrence of strawberry diseases in the partner countries. The status of virus diseases was only surveyed in Russia, Austria and Ireland. Their importance has not been evaluated in this project. Leaf diseases were evaluated over a period of 5 years (2008-2013). In Estonia leaf diseases only occur in fields of small producers. In Ireland, Spain and Lithuania (mainly *Podosphaera aphanis*) leaf diseases in strawberries were estimated important for the crop. Grey mold was deemed to be the most important disease for 60 % of Lithuanian respondents; grey mold is also a main disease in Austria and Ireland. 25% of producers in Spain would consider grey mold to be the second most important disease for strawberry crops. *Macrophomona phaseolina* was only mentioned by 20% of the Spanish producers. *Fusarium spp.* symptoms were only observed in Spain. In 2013 *Verticillium*-diseases occurred mainly in Austria and Lithuania; between 2008 and 2013 *Verticillium*-diseases were also observed in Spain and Lithuania. Crown rot occurred frequently in Spain, Lithuania, Austria and Ireland. Peer review records suggest that *P. fragariae* is of major commercial concern in Ireland. *X. fragariae* only occurred in Austrian fields; however there are records of its occurrence also in Ireland and Spain. More field inspections would be necessary to gain a representative picture of the occurrence and relevance of strawberry diseases.

Available diagnostic methods to diagnose strawberry diseases were reviewed and analysed during the project. A systematic review of PCR-based methods used for detection or quantification of the most important strawberry pathogens was put together. The systematic review concentrated on *Fusarium* spp., *P. fragaria*, *C. acutatum*, *V. dahliae*, *B. cinerea*, *M. phaseolina* and *X. fragariae*. Using appropriate subject headings, all scientific databases were searched from their inception up to April 2014. A total of 259 titles and abstracts were reviewed. 23 scientific publications met all the inclusion criteria. The accuracy and sensitivity of PCR diagnostic methods was the focus of most studies included in this review. The systematic review revealed that real-time PCR (rtPCR) is a particularly promising technique for diagnosing and quantifying pathogen populations in strawberry. This technique allows accurate, reliable and high throughput detection of target DNA in symptomless strawberry leaves and various environmental samples.

An example of rtPCR applications for the detection of strawberry pathogens is the detection *V. dahliae* microsclerotia in soil samples. Microsclerotia can be quantified by real-time PCR. This would save a considerable amount of time compared to time consuming traditional procedures used to detect *V. dahliae* microsclerotia (wet sieving followed by a classical plating and counting of grown microsclerotia). Unfortunately, experiments conducted during the project, showed that the wet sieving technique is to date the only suitable method for quantification of *V. dahliae* microsclerotia from soil. Real-time PCR is much faster, but sensitivity of a practicable procedure is too low to give reliable recommendations on the choice of strawberry varieties concerning susceptibility to *V. dahliae*.

One main objective was to optimize the use of molecular methods to detect and/or quantify the most important quarantine and emerging pathogens of strawberry in Europe. In this work we have developed and optimized protocols for specific detection of the following pathogens: *Fusarium oxysporum f. sp. fragariae, F. solani, Macrophomina phaseolina, Phytophthora cactorum, Botrytis cinerea, Verticillium dahliae* and *V. albo-atrum* by conventional PCR, and *Phytophthora fragariae* by real-time PCR in symptomatic/ asymptomatic samples of strawberry.

The protocols were validated in labs of participating countries and there was a pronounced variation in the percentage of correctly detected samples (56-96%) among the participating labs and between the assays tested. False negative results

could be attributed to a reduced sensitivity due to processes of lyophilization or vacuum concentration of primers and/or extracted DNA from samples, which were decided on to simplify transportation of the material tested. Contamination during the rehydration of samples and/or primers or handling with the PCR mix may have led to false positive results. However, these assumptions would have to be examined in more detail. Under optimal conditions, using freshly extracted DNA and primers, all tested assays should be suitable to detect the selected diseases directly from diseased strawberry plants. However, the ring test pointed out that, for implementation of these molecular methods in different laboratory conditions, some optimization processes is necessary in order to obtain robust diagnostic assays, capable to provide reproducible results using different equipment, reagents and laboratory set up. The data generated in this ring test can be used for validation processes.

More research is still needed to get the adequate overview of the pathogens occurrence. Also, although RT-PCR is the best suitable detection method, the pre-analytical criteria still need further investigations. New diagnostic protocols need some additional optimization to yield same results in different laboratory conditions. However, since it often happens that several diseases and conditions form a complex, the need for a universal diagnostic approach was recognised.

In addition to this, nematodes were not included in our study. The role of nematodes in strawberry diseases and quantification of soil-borne pathogens need to be further investigated.

3. RESULTS

Full scientific report of the SPAT project

WP 1: Project Management and Coordination

Lead: Evelin Loit, EE-EMU; Co-lead: *Ulrike Persen, AGES-AT*

The objective of WP1 was the coordination of the research consortium:

- To be a contact point for the EUPHRESCO Call Secretariat and all of the partners regarding project issues
- To organize and assemble a midterm progress report and the final project report
- Organize collaboration and steering activities and exchange of scientists
- Coordinate publications and PR related issues (more details on page 18)

A kick off meeting was held on September 12th, 2013 in Tartu, Estonia (EMU).
A midway project meeting was held on September 23, 2014 in Vienna, Austria.
A final meeting was held on June 11th 2015 in Madrid, Spain (INIA).
A final scientific report including validated diagnostic protocols has been assembled and delivered to the EUPHRESCO Call Secretariat.

WP 2: Mapping and evaluation of the current status of quarantine, emerging and major strawberry pathogens

Lead: *Ulrike Persen,* *AGES-AT*
Main Partners: *Evelin Loit, EE-EMU, Rytis Rugienius LT-LRCAF*
Contributing Partners: all

- **Objectives and tasks of the project**

The objective of WP 2 was to assess the level of strawberry diseases in different European countries by different means and the incidence and distribution of soil-borne pathogens emerging in strawberry fields.

- **Methods used and results obtained**

To gain information on the status of strawberry diseases in the partner countries the following methods were used:

Questionnaire

A questionnaire (Appendix I) was developed and translated by the partners into their national languages.

A total of 219 questionnaires were sent out to producers and advisors with a total response of 21 %.

Table 1. Emission and response of questionnaire

	No of questionnaires issued	responses	responses in %

Ireland	42	12	29
Spain	20	4	20
Lithuania	26	5	19
Austria	124	23	19
Estonia	7	1	14
total	219	45	21

Answers were provided to the following subjects:

General information:

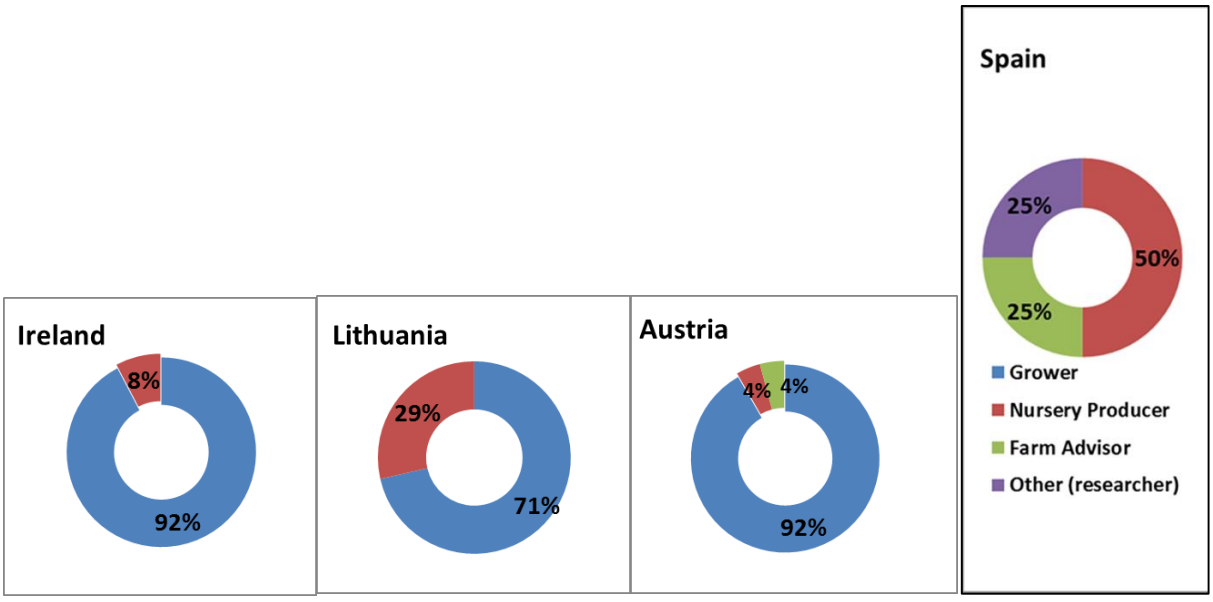

Fig. 1: Information about people that filled out questionnaires

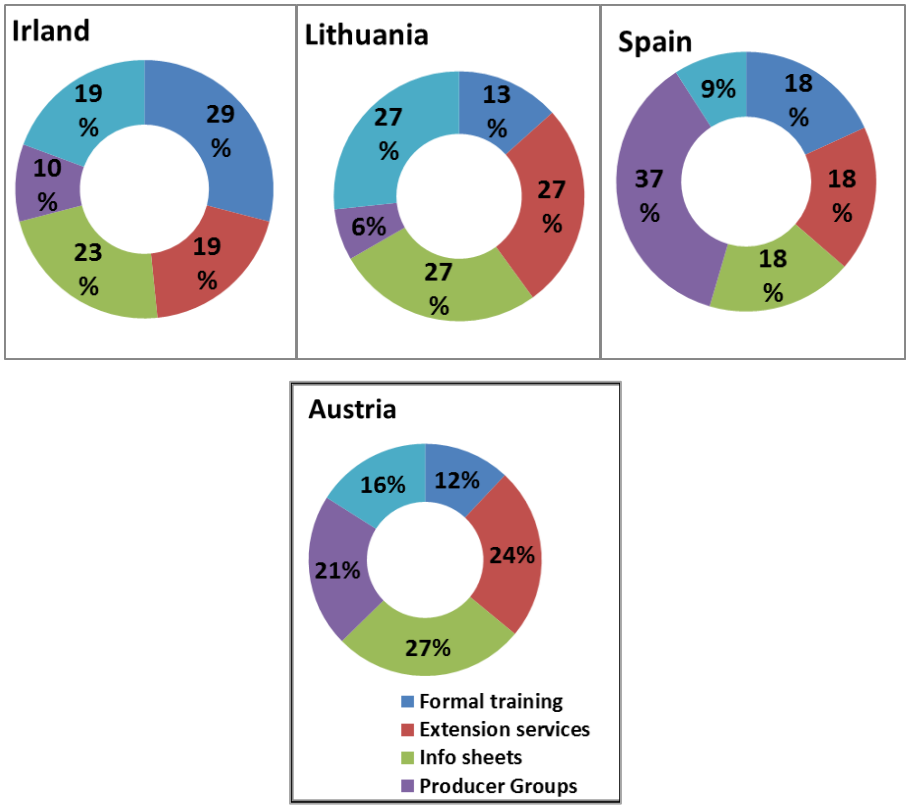

Fig. 2: Source of information / education about strawberry production

According to the answers of the questionnaire the source of knowledge on strawberry production is rather uniform in the participating countries (multiple answers possible). The information from Spain was obtained mainly within producer groups (no farmers were involved in questionnaires).

Production System

"*Please indicate the production system(s) that best represents that which you work with*"

In Lithuania and Austria the system "Standard with straw" is dominant. In Spain and Ireland all strawberry plants were cultivated in greenhouses or plastic tunnels whereas the main production in Austria and Lithuania is not protected.

Table 2: strawbery production systems, data in % of respondents

	standard with straw	standard without straw	ridges, mulch film/ fleece	Coverage with plastic foil	Coverage with plastic foil and fleece	Greenhouse or plastic tunnel

Spain	0	0	25	0	0	100
Ireland	0	0	8	8	17	100
Lithuania	60	40	0	40	0	20
Austria	61	0	39	0	22	26

One major factor for disease incidence is the choice of strawberry varieties. The tabels below show that only few cultivars were grown in more than one country: Elsanta (A, IE), Darselect (A, LT), Asia (A, LT), Sonata (A, LT, IE, EE) and Rumba (A, LT, EE) In addition we surveyed the susceptibility of the most prevalent cultivars.

Table 3: susceptibility of the main cultivars in Ireland

	Nr 1	Nr 2	Nr 3	Nr 4	Nr 5	Nr 6
Ireland	Elsanta	Sonata	Korona	Capri & Morano	Red Glory	Vibrant
Botrytis cinerea	vs	vs	not applicable	vs	vs	s
Podosphaera aphanis	vs	s		vs	vs	r
Phytophthora cactorum	vs	vs		s	s	t
Phytophthora fragariae	s	s		s ?	s	t ?
Verticillium dahliae	s	s		s ?	s	vs

vs = very susceptible, s = susceptible, t = tolerant, r = resistant

Table 4: susceptibility of the main cultivars in Lithuania

	Nr 1	**Nr 2**	**Nr 3**	**Nr 4**	**Nr 5**	**Nr 6**

Lithuania	Elkat	Darselect	Venta	Pegasus	Senga Sengana	Sonata
Botrytis cinerea	r		s	r	s	
Podosphaera aphanis	r			s		
Phytophthora cactorum	r		r	r		
Phytophthora fragariae	r					
Verticillium dahliae	r			r	r	
Mycosphaerella fragariae	r	vs	r	r		
Diplocarpon earliana	r	vs	r	r		
Xanthomonas fragariae						t

Table 5: Susceptibility of the main cultivars in Austria.

	Nr 1	Nr 2	Nr 3	Nr 4	Nr 5
Austria	Elsanta	Darselect	Asia	Clery	Sonata
Podosphaera aphanis		s	t		
Phytophthora cactorum	vs	t	t	t	vs
Phytophthora fragariae	vs	t	t	t	vs
Verticillium dahliae	vs	t	t	t	s
Mycosphaerella fragariae	s		t	s	
Diplocarpon earliana	s		t	s	

In Austria and Ireland the main cultivar Elsanta is (very) susceptible to root diseases, whereas in Lithuania the most common cultivar is resistant to most diseases.

Table 6: Strawberry varieties cultivated according to questionnaire

	Elsanta	Darselect	Daroyal	EliannY	Clery	Madeleine	Symphony	Asia	Sonata	Salsa	Rumba	Malwina	Jolly	Sabrina	Fortuna	Camarosa	Esplendor	Sabrosa	Primoris	Festival	Aguedilla	Caprie	Avo	Red Glory	Korona	Polka	Vilkat	Venta	Kent	Honeyoye	Elkat	Syria	Roxana	Figaro	Flair	Florentina	Evie-2	Florin	Samba
AT	X	X	X	X	X	X	X	X	X	X	X	X	X																										
E														X	X	X	x	X	X	X	X	X																	
IE	X								X														X	X	X														
LT		X		X				X	X		X															X	X	X	X	X	X	X	X	X	X	X	X		
EE									X		X															X													X

Diseases affecting strawberry production

"Which are the three most important strawberry diseases, in order, from your point of view, in 2013 and during the last 5 years?"

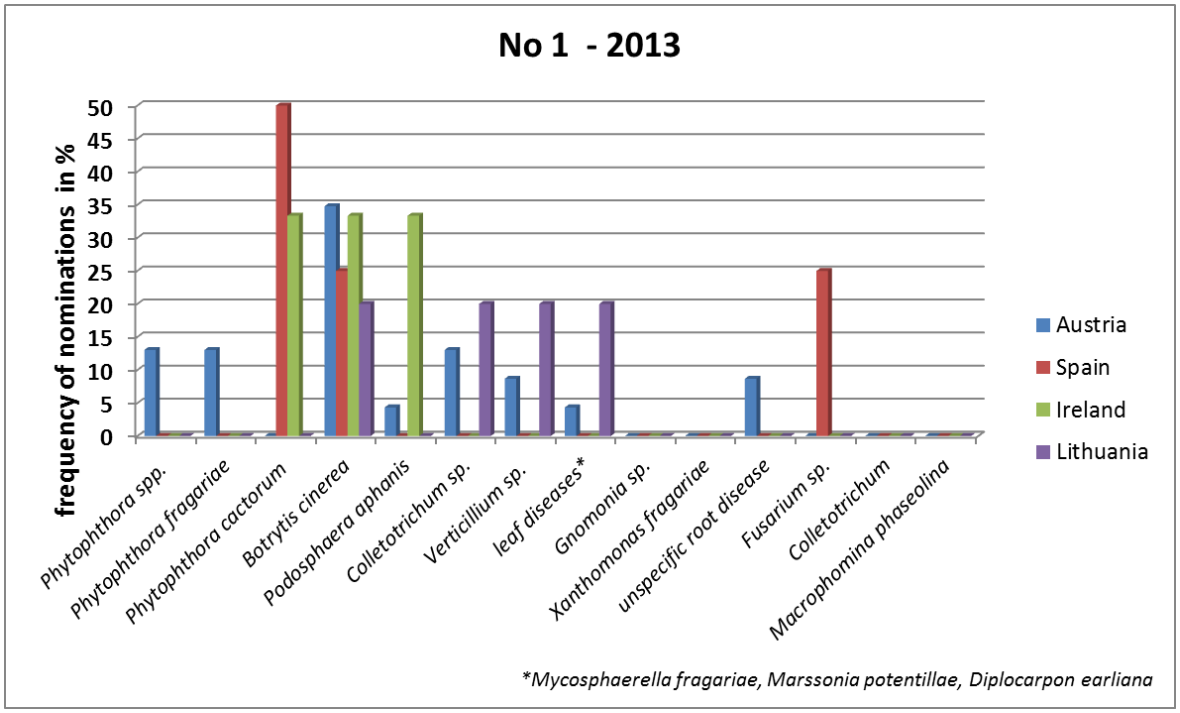

Fig. 3

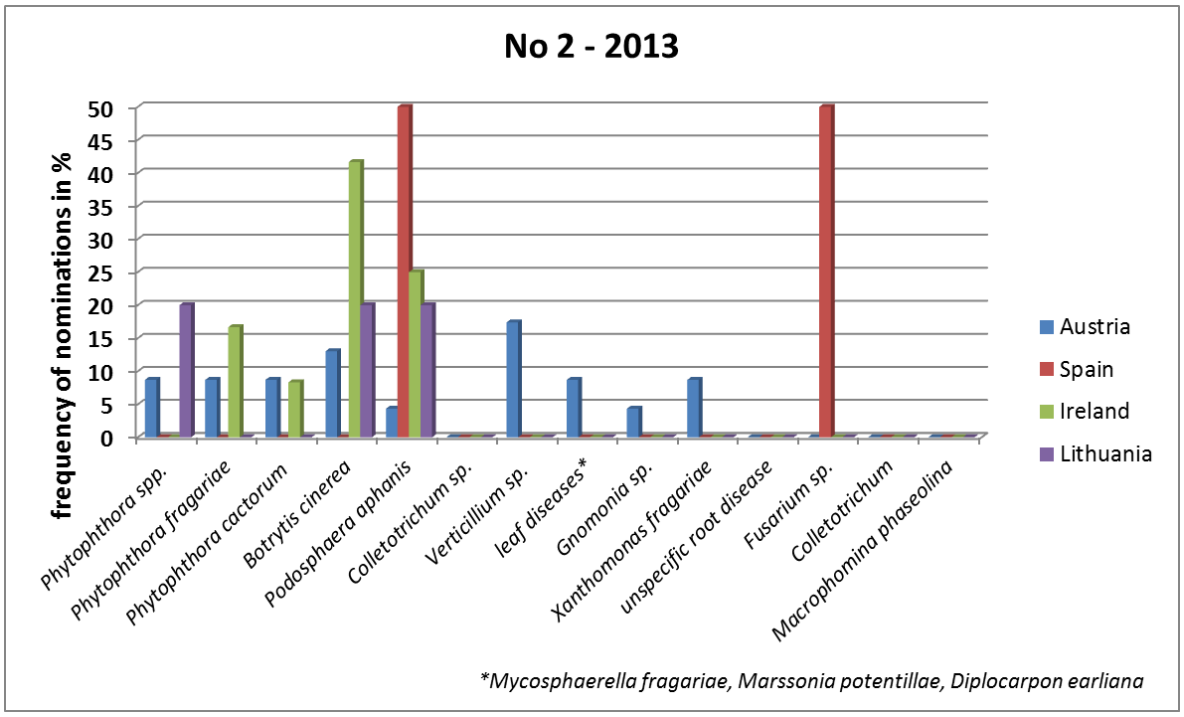

Fig. 4

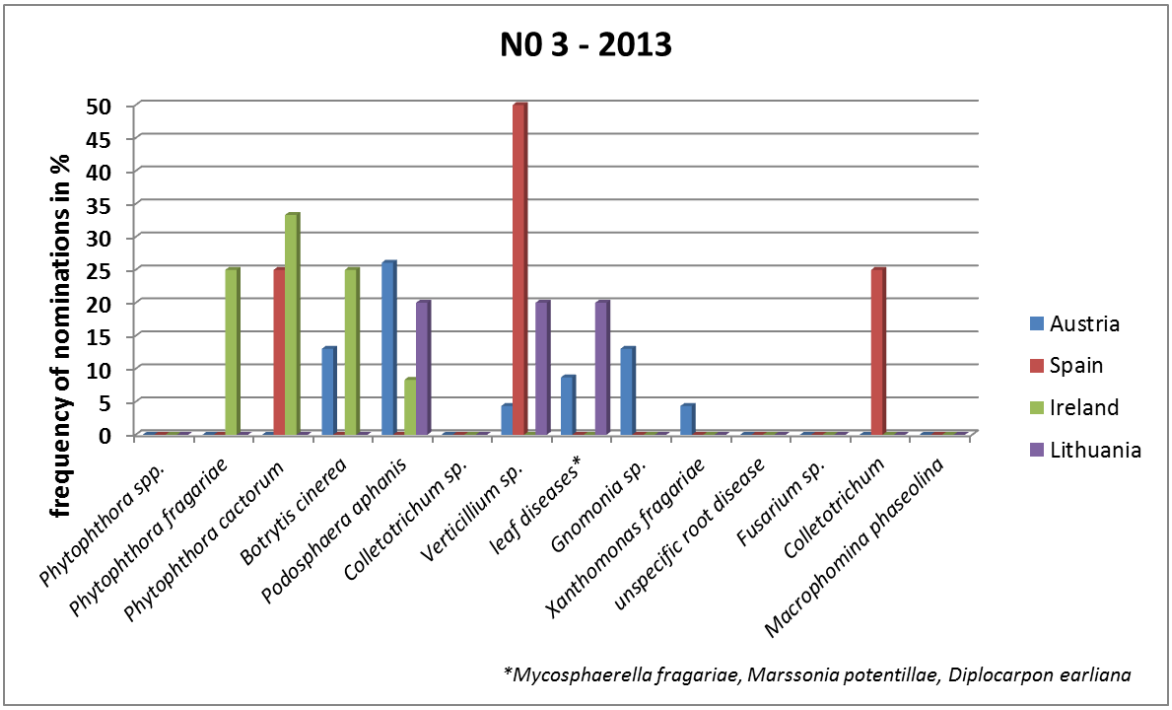
N0 3 - 2013
frequency of nominations in %
50
45
40
35
30
25
20
15
10
5
0
Phytophthora spp.
Phytophthora fragariae
Phytophthora cactorum
Botrytis cinerea
Podosphaera aphanis
Colletotrichum sp.
Verticillium sp.
leaf diseases*
Gnomonia sp.
Xanthomonas fragariae
unspecific root disease
Fusarium sp.
Colletotrichum
Macrophomina phaseolina
Austria
Spain
Ireland
Lithuania
*Mycosphaerella fragariae, Marssonia potentillae, Diplocarpon earliana

Fig. 5

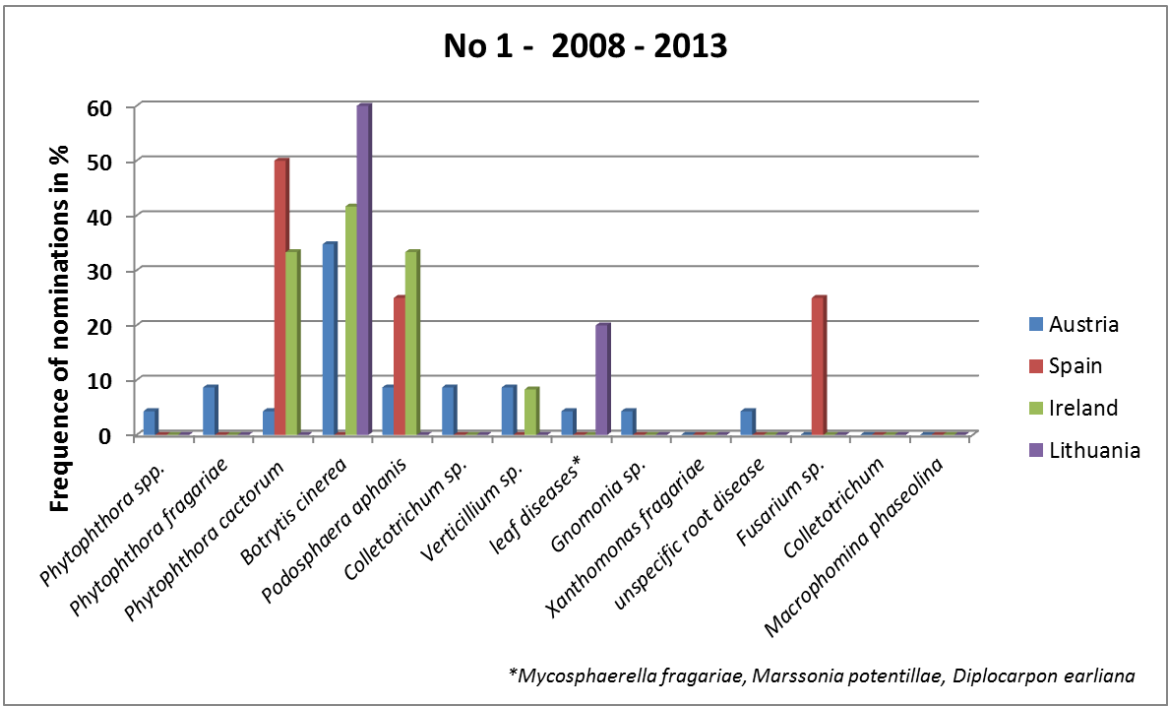
No 1 - 2008 - 2013
Frequence of nominations in %
60
50
40
30
20
10
0
Phytophthora spp.
Phytophthora fragariae
Phytophthora cactorum
Botrytis cinerea
Podosphaera aphanis
Colletotrichum sp.
Verticillium sp.
leaf diseases*
Gnomonia sp.
Xanthomonas fragariae
unspecific root disease
Fusarium sp.
Colletotrichum
Macrophomina phaseolina
Austria
Spain
Ireland
Lithuania
*Mycosphaerella fragariae, Marssonia potentillae, Diplocarpon earliana

Fig. 6

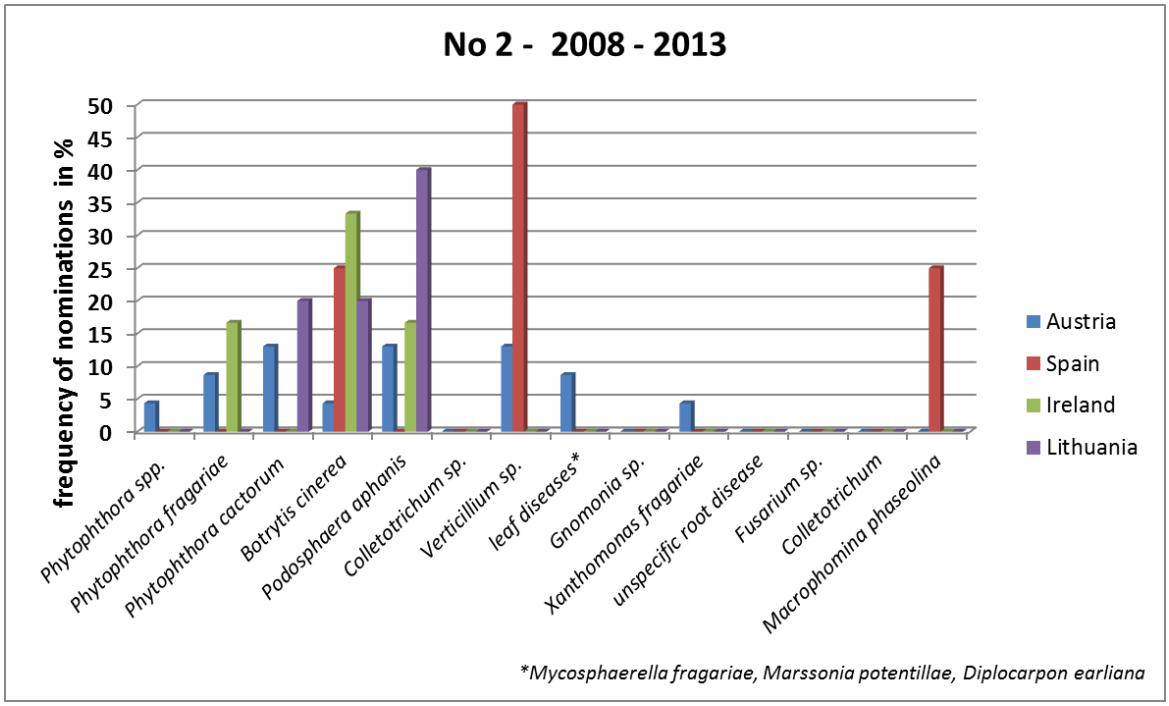

Fig. 7

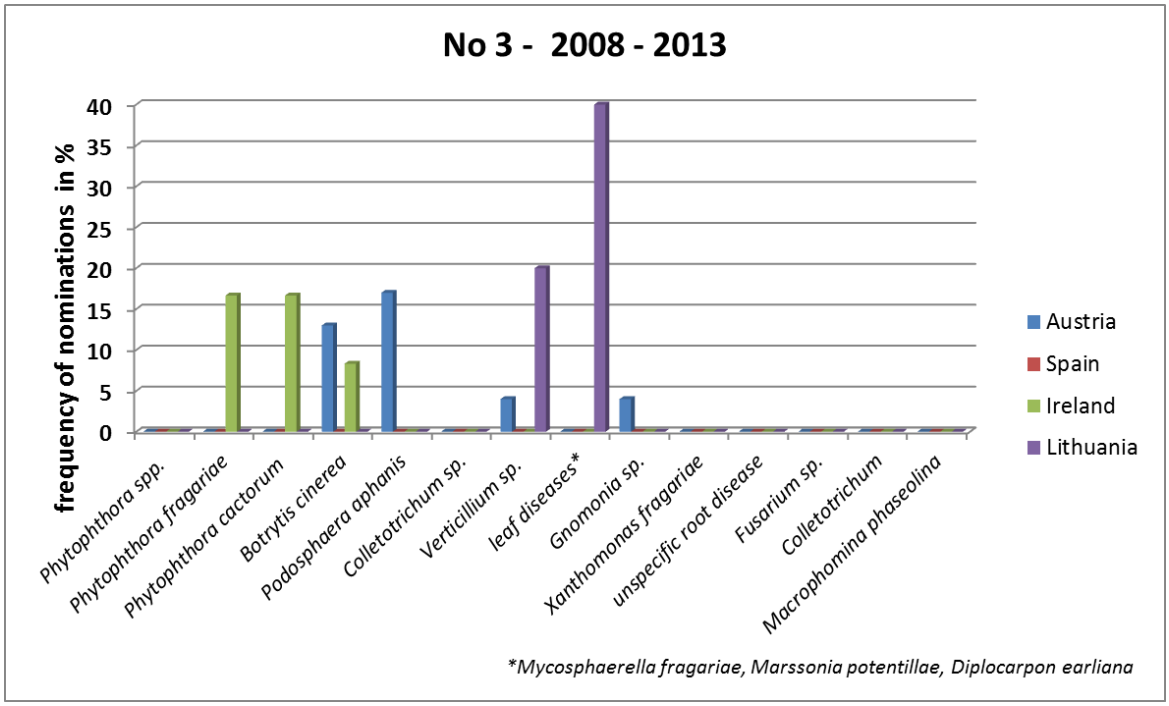

Fig. 8

In Ireland the most important diseases are caused by *P cactorum* (effected plants= 10%) *B. cinerea* (13%) and *P. aphanis (*50%*)*. In Austria the prevalent causal organisms for strawberry diseases is *B. cinerea* (effected plants = 15%). Root diseases

that are also important are *Colletotrichum sp.*, *Verticillium sp*. and *Podosphaera aphanis*. However, the questionnaire might not be representative where the number of respondents was low.

Fig. 9 shows that due to different meteorological conditions the disease severity can vary between farms (Lithuania 2013).

In farm 2 there were more infections compared with other farms. The least amount of pathogens was found in farm 5. The main diseases in all farms were *Botrytis cinerea (42%), Podospaera aphanis* (38%) and leaf diseases.

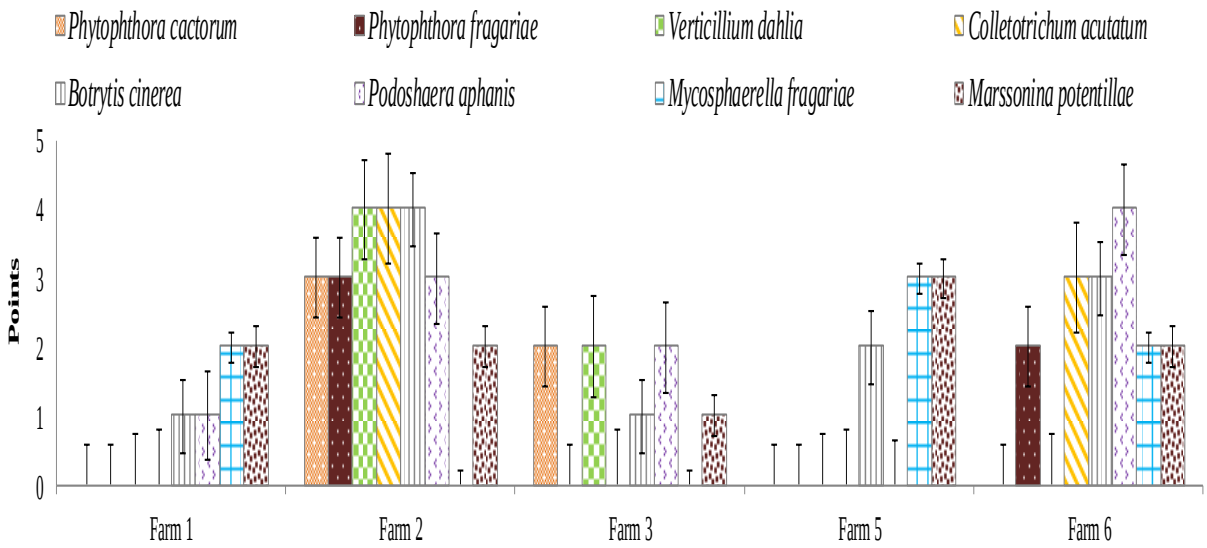

Fig. 9. The diseases severity presented in points. Point scale percentage plant tissue affected: 1=0-5 %, 2 =5-25 %, 3= 25-50 %, 4= > 50%. Note. Standard error (SE) of pathogens is listed as vertical bars.

The evaluation of the Lithuanian questionnaire data revealed that *B. cinerea, and leaf diseases* were caused most damages.

Interestingly, Spanish producers had most frequently problems with *P. cactorum* but on a very low level of infection (affected plants 1-2%). *Fusarium sp*. and *P. aphanis* were also often observed.

Major diseases in Estonia were caused by: *Botrytis cinerea (*30% yield loss); the main level of damage was observed in the cultivar Sonata; all varieties are susceptible to *Podosphaera aphanis*; leaf spots and unidentified viruses were also reported.

Testing and diagnosis of symptomatic field samples

LITHUANIA

Isolate collection

One of the most important strawberry diseases in the world is grey mould, caused by *Botrytis cinerea*. *Botrytis* spp. comprises 22 species and a large number of host-specific pathogens. The fungus has capability to develop infection at the temperature from 2°C to 28°C (optimal 20 °C) and leaf wetness periods above 80 %for more than 4 hours. *B. cinerea* infects leaves, fruits, flowers, petioles, stems and often starts as blossom blight. The pathogen usually remains invisible until ripening; affected fruits may rot before or after they are ripe. Grey mould seriously reduces yield (from 15% up to 50%) and post-harvest quality.

During 2013-2014 project experiments were collected 273 isolates of *Botrytis* spp. from different cultivars of strawberries. 82 isolates were collected from cultivars DarSelect, Venta and Elkat located in Babtai throughout this investigation. A totally of 273 isolates were collected from 14 different areas of Lithuania.

Isolates were first identified with BC108 and BC563 primer sets and then classified according transposable elements and only after that analysed with Simple Sequence Repeats.

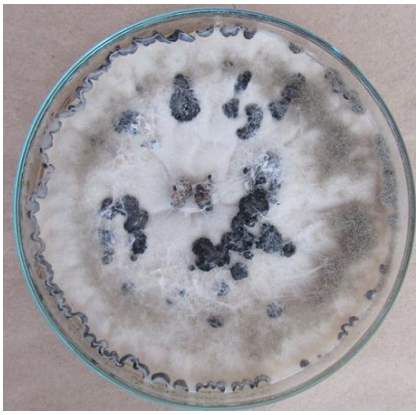

Fig. 10: Botrytis spp. isolate grown on PDA media

DNA extraction

DNA was extracted from the 273 isolates collection. All isolates were grown on PDA at 22±°C and purified to a single spore (Fig. 10). Fungal genomic DNA was

extracted from 200 mg of mycelium material collected from Petri dish with spatula. Mycelia were grounded in liquid nitrogen using a mortar and pestle. DNA was extracted according to Genomic DNA Purification Kit K0512 (ThermoScientific) (Genomic DNA Purification Kit). Samples were incubated in Grant Bio PHMT Thermoshaker (Grant). DNA were dissolved in 100 µl of 1x TE buffer and stored at -20°C. DNA concentration measured with NanoDrop 1000 spectometer (ThermoScientific).

Tab. 7. *Botrytis* spp. isolates collection 2012-2014

Nr	District	Area	Year	Variety	Number of isolates
1.	Prienai	Unknown	2014	Marmolada, Sonata, Felicita	17
2.	Prienai	Klebiškis	2014	Unknown	6
3.	Šiauliai	Adomiškių	2014	Malvina	20
4.	Kaunas	Kauno	2012	Unknown	1
5.	Kaunas	Kaunas	2012	Elkat	4
6.	Kaunas	Kaunas	2013	Elkat	5
7.	Kaunas	Babtai	2012	DarSelect, Elkat	7
8.	Kaunas	Babtai	2013	DarSelect, Venta, Elkat	82
9.	Kaunas	Babtai	2014	DarSelect, Elkat	10
10.	Šiauliai	Kantminių	2014	DarSelect, Elene, Syria	22
11.	Šiauliai	Maniušių	2014	Sonata, Syria	30
12.	Radviliškis	Vežlys	2014	Pandora, Sonata	19
13.	Panevėžys	Sodeliškių	2014	Selvik, Rumba, Elkat, Felicita, Filut	19
14.	Kėdainiai	Akademija	2014	Senga Sengana	7
15.	Kėdainiai	Labūnava	2014	Syria, Vikat, Pegasus, Pandora	24
Total					**273**

Identification

Botrytis spp. isolates were identified with BC108 and BC563 primers. PCR amplification performed in a 20 µl reaction volume containing: 2.5µl 10x Tag Buffer, 2µl dNTP Mix 2mM each, 0.1µl of each primer (100pM/ µl) (Bc108+ and Bc563–), 1.5µl 25mM $MgCl_2$, 1µl of DNA, 0.1µl Taq DNA Polymerase (recombinant) (5U/µL) (ThermoScientific), 12.7µl DNase/Rnase-free Water. Primer design is provided in the table 8. PCR reactions were performed in a Mastercycler (Eppendorf, Germany). Amplification: 1 cycle of 2 min at 94 ^{o}C, 35 cycles of 45 s at 94 ^{o}C, 50 s at 50 ^{o}C, 50 s at 72 ^{o}C; 1 cycle of final extension for 5 min at 72 ^{o}C. The PCR product was separated

by electrophoresis on a 1.5 % agarose gel in 1x TAE buffer and visualized by staining with Ethidium bromide (CarlRoth). Size marker used GeneRuler mix 1 kb DNA Ladder (ThermoScientific). Primers Bc563 and Bc108 amplify 0.48 kb and 0.36 kb (fig. 11). We sampled *B. cinerea* population obtained from different cultivars or strawberries. Altogether DNA extracted from 273 isloates, but only 158 were identified with BC108 and BC563 primers as *B. cinerea.*

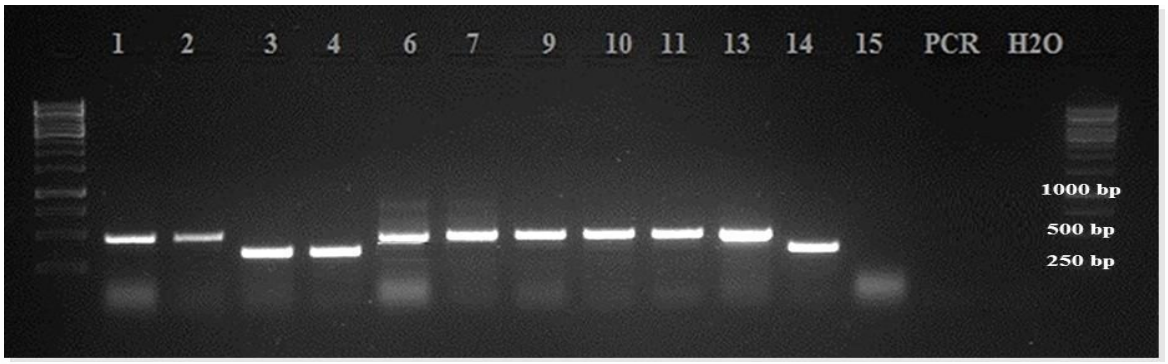

Fig. 11: Example of gel electrophoretic profile of the DNA. The identification fragment is 360 bp and 480 bp.

Transposon detection

PCR 25 µl reaction volume containing 1.5 µl of DNA, 8 µl DNase/Rnase-free Water, 12.5 µl REDTaq® ready mix (Sigma) , 1.5 µl of each F300 and F1500 primer for Flipper and 1.5 µl of each LTR98 and LTR728 primer for Boty. Primer design is provided in the table 8. PCR reactions were performed in a Mastercycler epgradient (Eppendorf, Germany). The program applied for amplification was as: 1 cycle of 5 min at 94 ^{o}C, 40 cycles of 1 min at 94 ^{o}C, 1 min at for Boty – 60.5 ^{o}C and for Flipper - 58 ^{o}C, 2 min at 72 ^{o}C; 1 cycle of final extension for 5 min at 72 ^{o}C (Fournier et al., 2003). The PCR product was separated by electrophoresis on a 1.5 percent agarose gel in 1x TBE buffer and visualized by staining with Ethidium Bromide (CarlRoth) with AlphaDigiDoc^{TM}RT (SYNGENE). The DNA fragments amplify at 1250bp for Flipper and 648bp for Boty. Size marker used GeneRuler 100bp plus DNA Ladder.

According to the transposable elements *Boty* and *Flipper* presence or the absence two sibling cryptic populations *transposa* and *vacuma* have been described (Table 8). Initially, two sympatric sibling species or transposon types were described: 1) *transposa* that contained two transposons *Boty* and *Flipper* and 2) *vacuma* which contained no transposons.

The frequency and distribution of transposon types varied between different locations (Fig. 11). Among the 62 isolates from Kaunas district, the frequencies of transposon types ranked from highest to lowest were: 33.01% *vacuma,* 26.21% *boty*-only, 21.36% *transposa* and 19.42 % *flipper*-only. In Babtai were collected the most isolates, the frequencies of transposon types were: 29.13 % *vacuma*, 17.48% *transposa*, 18.45% *boty*-only and 14.56% *flipper*-only.

Table 8: *Botrytis cinerea* frequency of transposons type of three locations in Lithuania

Orign	Number of isolates	Types transposons			
		Flipper	***Boty***	***Vacuma***	***Transposa***
Babtai	49	15	19	30	18
Kaunas	9	3	4	4	2
Kaunas dist.	4	2	4	0	2
Total	62	20	27	34	22

Cryptic species

Molecular studies revealed that *B. cinerea* population are grouped into two different cryptic species (group I and II), which also coincide with resistance to the fungicide fenhexamid. Group I is resistant and II – sensitive to fenhexamid. 59 *B. cinerea* isolates were selected and analysed to identify the cryptic species.

PCR in a 20 µl reaction volume containing 1.5 µl of DNA, 2 µl 10x PCR buffer (Sigma), 12.1 µl DNase/Rnase-free Water, 1 µl $MgCl_2$ (Fermentas), 0.4 µl 10 mM dNTP (Fermentas), 1 µl REDTag Genomic DNA Polymerase, 1µl of each 262 and 520L primer. PCR reactions performed in a Mastercycler (Eppendorf, Germany). The program applied for amplification was as: 1 cycle of 5 min at 94 ^{o}C, 35 cycles of 30 s at 94 ^{o}C , 1 min 30 s at 55 ^{o}C, 1 min at 72 ^{o}C; 1 cycle of final extension for 5 min at 72 ^{o}C (Fournier et al., 2003). The 262 and 520L primers (Fournier et al., 2003), amplified a DNA fragment of 1172bp. Primer design are provided inTable 10. Digestion was made directly after PCR amplification. Digestion was made in water-thermostat (Biosan). The reaction volume was 31 µl containing: 10 µl of PCR reaction mixture, 18 µl DNase/Rnase-free Water (Fermentas), 2 µl 10x Tango Buffer (Fermentas), 1 µl hHaI (Fermentas). The digestion conditions were 2 hours at 37 ^{o}C and directly after digestion reaction was inactivated with 1.24 µl 0.5 M EDTA (20 nM final concentration). Fragments were resolved on 1.5% agarose gels stained with ethidium bromide

(CarlRoth). The restriction fragment amplified at 601 bp (I group) and 517 bp (II group) (Fournier et al., 2003; Isenegger et al., 2008). Size marker used GeneRuler 100bp plus DNA Ladder (Fermentas). Primer design is provided in the table 10.

Among the 59 isolates the prevailing was sensitive *B. cinerea* group II – 83.05 % and group I only 16.95 % (Table 9).

Table 9: *Botrytis cinerea* group I and II strains

Origin	Number of isolates	Group	
		I (resistant)	II (sensitive)
Babtai	46	9	37
Kaunas	9	0	9
Kaunas distr.	4	1	3
Total	59	10	49

Microsatellite SSR

Five of the nine microsatellite markers developed by Fournier *et al.* (2002) were used for genotyping. PCR amplifications were multiplexed. Microsatellite PCR amplification was performed in a 10 µl reaction volume containing 1 µl of DNA, 1 µl PVP, 0.1 µl DDT, 1 µl 10x Tag Buffer, 1µl dNTP Mix 2mM each, 0.7 µl 25mM $MgCl_2$, 0.05 µl Taq DNA Polymerase (recombinant) (5U/µL) (ThermoScientific), 4.65 µl DNase/Rnase-free Water, 0.5 µl of Primer mix 1 or 2. Primer mix 1: BC2, BC6. Primer mix 2: BC3, BC7 and BC10 (table 8). PCR reactions were performed in a Mastercycler epgradient (Eppendorf, Germany). The program applied for amplification was as: 1 cycle of 5 min at 95 ^oC, 28 cycles of 30 s at 95 ^oC, 90 s at 59 ^oC, 30 s at 72 ^oC; 1 cycle of final extension for 30 min at 60 ^oC (Fournier et al. 2003).

Fragments were separated in an automated single capillary genetic analyser sequencer. Fragment analysis was performed using 3130x Genetic Analyser" (Applied Biosystems Ltd.) using 36 cm capillary array and POP-7 polymer. Data was analysed using GeneMapper software v.4.0 (Applied Biosystems Ltd.) The DNA fragments amplifies: 1) BC2 - 200bp, 2) BC3 - 200bp, 380bp, 3) BC6 - 100bp, 150bp, 300bp 4) BC7 - 150bp 5) BC10 - 160bp, 200bp.

For the genetic analysis of *B. cinerea* observed (*Ho*) and expected (*He*) heterozigosity were calculated according to the Nei's genetic diversity method. Cluster analysis was performed using Bootstrap within PowerMarker V3.25 software.

Table 10: Primers sequences

Name	Nucleotide sequence (5' - 3')
Bc108+	5'-ACCCGCACCTAATTCGTCAAC-3'
Bc563–	5'-GGGTCTTCGATACGGGAGAA-3'
F300	5' GCACAAAACCTACAGAAGA 3'
F1500	5' ATTCGTTTCTTGGACT 3'
LTR98	5'AGCCTGTAGAATCACCAACG 3'
LTR728	5'CGGTATTTCTGGTTGGCA 3'
Bc2-F	FAM-5'CATACACGTATTTCTTCCAA 3'
Bc2-R	5'TTTACGAGTGTTTTTGTTAG 3'
Bc3-F	NED-5'GGATGAATCAGTTGTTTGTG 3'
Bc3-R	5'CACCTAGGTATTTCCTGGTA 3'
Bc6-F	HEX-5'ACTAGATTCGAGATTCAGTT 3'
Bc6-R	5'AAGGTGGTATGAGCGGTTTA 3'
Bc7-F	TAMRA-5'CCAGTTTCGAGGAGGTCCAC 3'
Bc7-R	5'GCCTTAGCGGATGTGAGGTA 3'
Bc10-F	ROX-5'TCCTCTTCCCTCCCATCAAC 3'
Bc10-R	5'GGATCTGCGTGGTTATGACG 3'

SSR

A group of 67 *B. cinerea* isolates from different agroecological region were analyzed. Four polymorphic microsatellite markers were surveyed for the 67 *B. cinerea* strains. The number of alleles varied from 7 to 23. The most polymorphic microsatellite marker was BC6 (PIC value 0.88) and the least was BC7 (PIC value 0.79). The observed heterozygosity varied from 0.02 to 0.61 with an average value of 0.24. Expected heterozygosity value varied from 0.81 to 0.89 with an average of 0.84. Observed heterozygosity values were lower than expected heterozygosity in all investigated locus (Table 11).

Table 11: Estimated allele size range, number of alleles from different agroecological regions of Lithuania

No.	Marker	Number of alleles	Allele size range, bp	H_0[1]	H_e[2]	PIC[3]
1	BC2	9	144-174	0.83	0.02	0.81
3	BC6	23	84-268	0.89	0.61	0.88
4	BC7	12	109-133	0.81	0.21	0.79

5	BC10	9	162-191	0.82	0.12	0.80
Mean		13.25		0.84	0.24	0.82

[1]-expected heterozygosity, [2]-observed heterozygosity, [3]-polymorphic information

The dendrogram (SSR) was created using 67 fragments generated with 4 microsatellite primer pairs (Fig. 12). The *B. cinerea* strains clustered into three main groups. One isolate that was from Babtai (collected in 2013) from DarSelect cultivar strawberry, claded separately from the rest. The main group consisted of 45 stains, second of 15 and third of 4 isolates (Fig. 12).

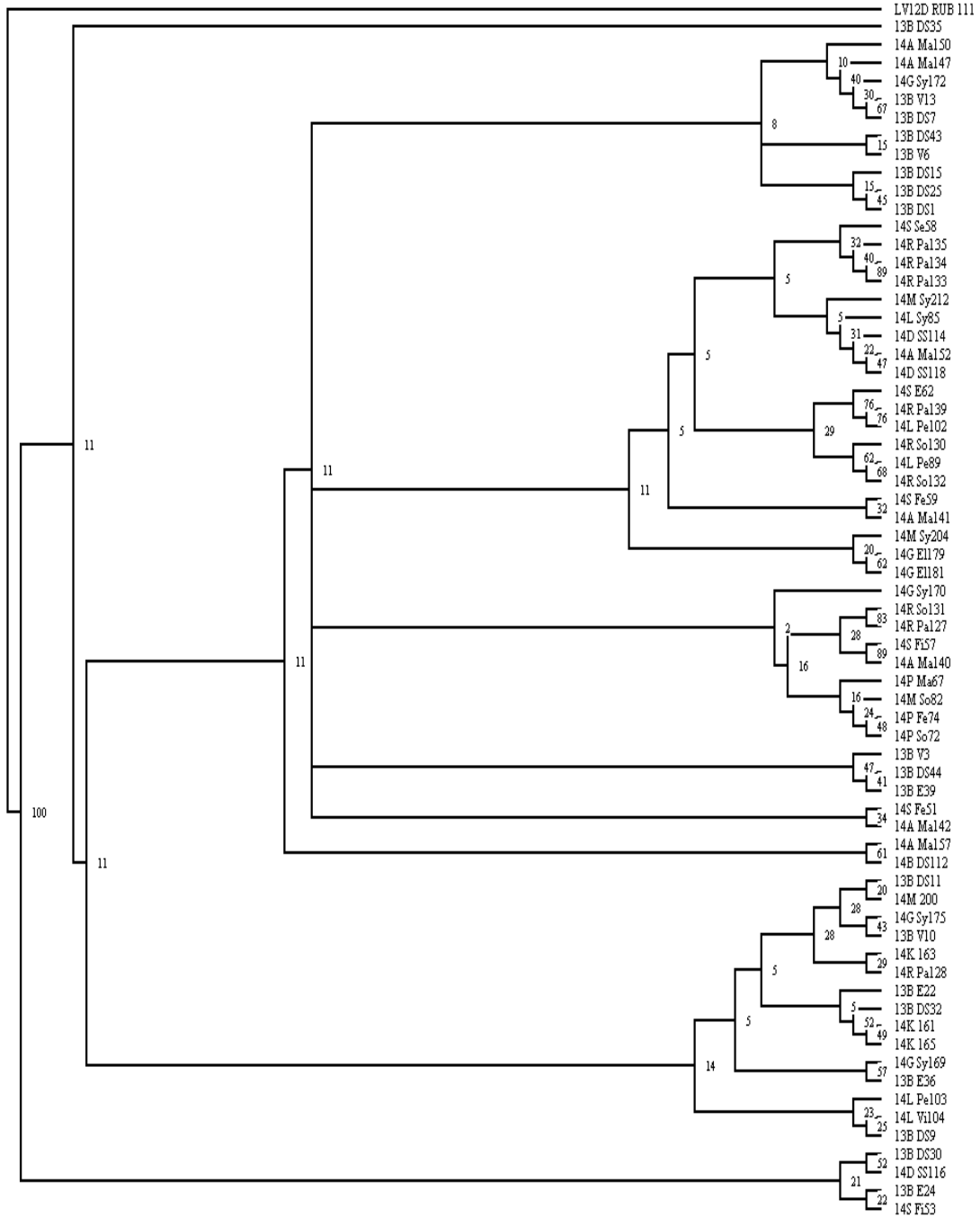

Fig. 12: *Botrytis cinerea* phylogenetic tree from different agroecological regions

A group of 55 *B. cinerea* isolates from Kaunas district was analyzed. Four polymorphic microsatellite markers were surveyed for the 55 *B. cinerea* isolates. The number of alleles varied from 7 to 9. The most polymorphic microsatellite marker was BC7 (PIC value 0.72) and the least was BC6 (PIC value 0.61). The observed heterozygosity varied from 0 to 0.89 with an average value of 0.29. Expected

heterozygosity value varied from 0.64 to 0.75 with an average of 0.71. Observed heterozygosity values were lower than expected (Table 12).

Table 12: Estimated allele size range, number of alleles from Kaunas district

Marker	Number of alleles	Allele size range, bp	H_0[1]	H_e[2]	PIC[3]
1 BC2	8	144-195	0.71	0.17	0.66
3 BC6	8	85-136	0.64	0.89	0.61
4 BC7	9	115-139	0.75	0.09	0.72
5 BC10	7	164-187	0.73	0.00	0.69
Mean	8		0.71	0.29	0.67

[1]-expected heterozygosity, [2]-observed heterozygosity, [3]-polymorphic information

The dendrogram was created using 55 sequences generated with 4 microsatellite primer pairs (Fig. 13). The *B. cinerea* strains clustered into three main groups, but 2 isolates claded separately. The separated isolates were from Babtai (collected in 2013) from DarSelect cultivar strawberries. The main group consisted from 29 stains, second from 22 and least from 2 isolates (Fig. 13).

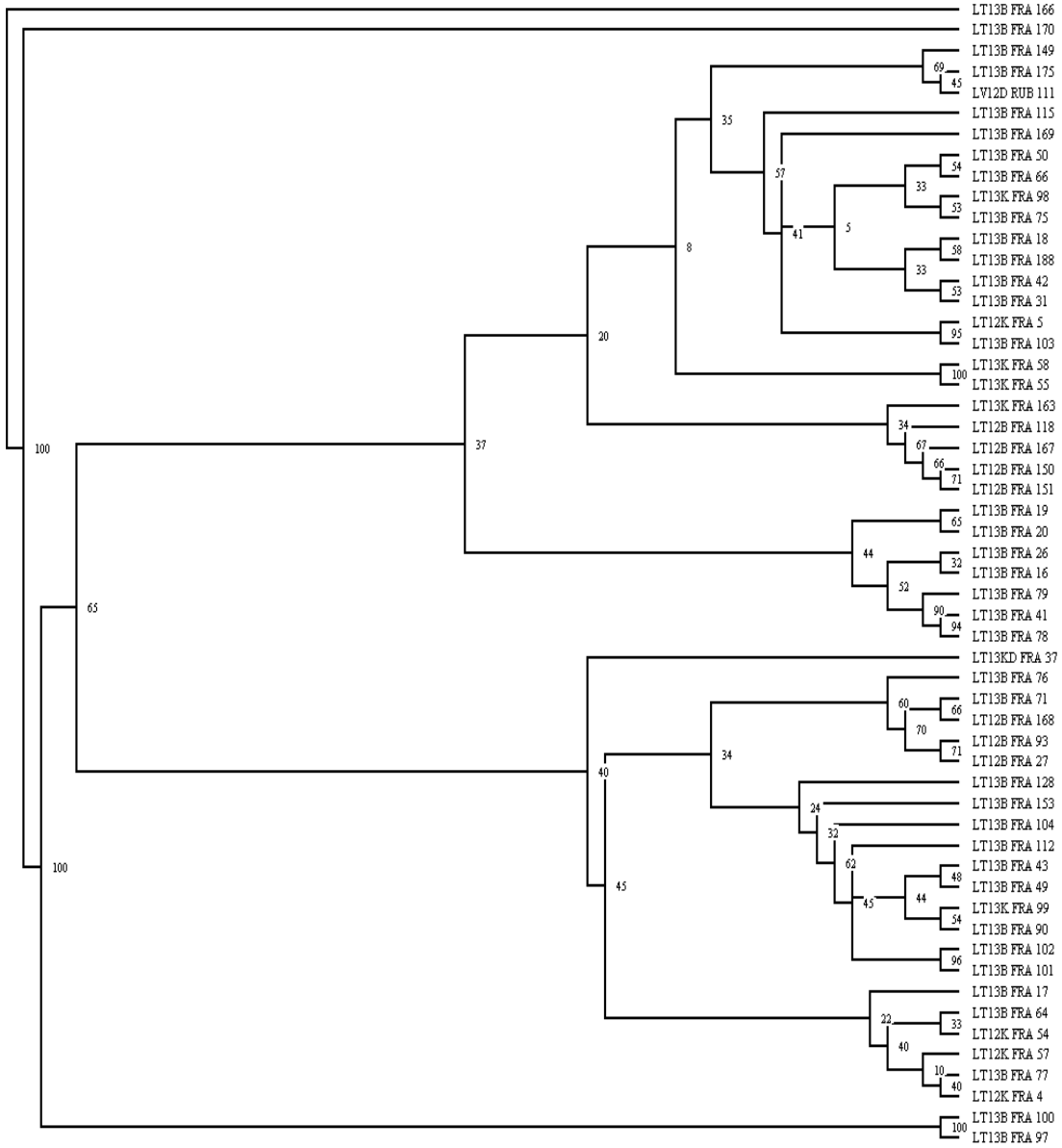

Fig. 13: *Botrytis cinerea* phylogenetic tree from Kaunas distr.

Material and methods

Fungal pathogens from plants:

Symptomatic strawberry plants were thoroughly washed and vertically cut in two pieces. One half was used for morphological determination of pathogenic fungi. Suitable tissue samples were used for further analysis. They were placed on different media and incubated at 15, 18 and 20°C. Diagnosis of isolated fungi was performed by morphological analysis

The other half samples was taken from different tissue parts and DNA was extracted with Qiagen Plant DNeasy Kit (Qiagen, Hilden Germany) according to the manufacturer. The extracted DNA was diluted 1:20 and both concentrations were used as DNA templates for the specific PCR detection of the pathogens listed below.

- Verticillium dahliae: PCR method by Carder et al. (1994) in EPPO Diagnostic Standard PM 7/78 (1)
- Verticillium albo-atrum: PCR method by Carder et al. (1994) in EPPO Diagnostic Standard PM 7/78 (1)
- Phytophthora cactorum: PCR method by Causin et al. (2005)
- Phytophthora fragariae: 2 PCR methods by Ioos et al. (2006)

Fungal pathogens from soil:

- Samples were prepared for morphological analysis by wet sieving technique. According to a standardized method (Harris and Yang 1993, 1996; modified by Steffek et al. 2006) the number of existing microsclerotia per gram soil was determined
- Phytophthora spp. were trapped by baiting technique (Werres, 2001) before microscopic examination

Phytoplasma:

- Realtime PCR method by Christensen et al (2004)

Virus pathogens:

Investigations for Strawberry latent ringspot virus (SLRSV), Arabis mosaic virus (ArMV) and Tomato ringspot virus (ToRSV) with specific rt-PCRs. The RNA extraction was carried with the Rneasy Kit from Qiagen *according* to the *manufacturer's* protocol.

- Strawberry latent ringspot virus (SLRSV) method by Olmos et al. (2002)
- Arabis mosaic virus (ArMV) method by MacKenzie et al. (1997)
- Tomato ringspot virus (ToRSV) method by Griesbach (1995)

Bacteria:

The most important bacterial disease on strawberries is *Xanthomonas fragariae*. This pathogen usually produces typical symptoms (angular leaf spots). According to EPPO PQR *X. fragariae* has not been reported in Austria. However according to the literature review performed in this project the pathogen was occasionally reported. During the field surveys (2014-2015) no symptoms resembling this disease could be observed. Therefore the samples were not tested for *X. fragariae.*

Results:

2014:

In May and June 2014 filed trips were made to the Austrian provinces Styria, Upper Austria, Lower Austria and Burgenland. 48 symptomatic plants and 12 soil samples were taken. Microsclerotia of *V. dahliae* were detected in 9 out of 10 soil samples that were taken around symptomatic plants. The results of the survey showed that 3 samples presented a low risk for susceptible strawberry cultivars, 4 samples high risk and 4 samples a very high risk. *V. dahliae* or *V. albo atrum* were also detected in samples from 3 farms. Samples from 4 farms tested positive for *P. cactorum* or *P. fragariae*. No causal agents for phytoplasma diseases could be detected. In 2014 two symptomatic strawberry plants were investigated for Strawberry latent ringspot virus (SLRSV), Arabis mosaic virus (ArMV) and Tomato ringspot virus (ToRSV), no viruses could be detected.

Tabel 13: Results of analysis of plant and soil samples 2014

			soil	plant				
			wet sieving method	PCR				
farm	field	cultivar	number micro sclerotia V. dahliae	*V. dahliae*	*V. albo atrum*	*P. fragariae*	*P. cactorum*	*phyto-plasma generic*
A	A 1	Sonata	0,4	negativ	negativ	negativ	negativ	negativ
	A 2	Sonata		negativ	negativ	negativ	positiv	negativ
	A 3	Sonata	1,6	negativ	negativ	negativ	positiv	negativ
	A 6	Sonata	2,4	negativ	negativ	negativ	positiv	negativ
	A 7	Sonata		negativ	negativ	negativ	negativ	negativ
	A 8	Sonata		negativ	negativ	negativ	negativ	negativ
	A 9	Sonata		negativ	negativ	negativ	positiv	negativ
B	B 1	Elsanta	1,6	negativ	negativ	negativ	negativ	negativ
	B 2	Eliane	2,4 2,6	negativ	negativ	negativ	negativ	negativ
	B 3/1	Elsanta	0,6	negativ	negativ	positiv	(positiv)	negativ
	B 1A	Fenella		negativ	negativ	negativ	negativ	negativ
	B 2A	Fenella		negativ	negativ	positiv	negativ	negativ
	B 4 / 2		0,4					
	B 5 / 2							
C	C 1	Elsanta	0,4	negativ	negativ	negativ	negativ	negativ
	C 2	Elsanta		negativ	negativ	negativ	negativ	negativ
	C 3	Elsanta		negativ	negativ	negativ	negativ	negativ
D	D 3	Elsanta		negativ	negativ	negativ	negativ	negativ
	D 4	Elsanta		negativ	negativ	negativ	negativ	negativ
	D 5	Elsanta		negativ	negativ	negativ	negativ	negativ
	D 8	Elsanta		negativ	negativ	negativ	negativ	negativ
	D 9	Elsanta		negativ	positiv	negativ	negativ	negativ
	D 10	Elsanta	0	negativ	positiv	negativ	negativ	negativ
	D 11	Elsanta		negativ	(positiv)	negativ	negativ	negativ
E	E 2	Elsanta	4,2	negativ	negativ	negativ	negativ	negativ
	E 3	Elsanta		negativ	negativ	negativ	negativ	negativ
	E 6	Sonata		negativ	negativ	negativ	negativ	negativ
F	F 1, Reihe 4	Darselect		negativ	negativ	negativ	negativ	negativ
	F 2, Reihe 4	Darselect		negativ	negativ	negativ	negativ	negativ
	F 3, Reihe 4	Darselect	2,6	negativ	negativ	negativ	negativ	negativ
	F 5, Reihe 1	Darselect		negativ	(positiv)	negativ	negativ	negativ
	F 6, Reihe 1	Darselect		negativ	(positiv)	negativ	negativ	negativ
G	G 1	Elsanta		negativ	negativ	positiv	negativ	negativ
	G 2	Elsanta		negativ	negativ	negativ	negativ	negativ
	G 3	Elsanta		negativ	negativ	negativ	negativ	negativ
	G 4	Elsanta		negativ	negativ	negativ	negativ	negativ
H	H 1		1,4	positiv	negativ	negativ	negativ	negativ
	H 2			positiv	negativ	negativ	negativ	negativ
	H 3			positiv	negativ	negativ	negativ	negativ
I	I 1	Daroyal		negativ	negativ	negativ	negativ	negativ
	I 1		1,8	negativ				
J	J 2	Elsanta		negativ	negativ	negativ	negativ	negativ
	J 2 gesund	Elsanta		negativ	negativ	negativ	negativ	negativ
	J 3, Feld 1	Elsanta		negativ	negativ	negativ	negativ	negativ
	J 4	Elsanta		negativ	negativ	positiv	negativ	negativ
	J Feld 2 ges	Elsanta		negativ	negativ	negativ	negativ	negativ
	J 1	Elsanta		negativ	negativ	negativ	positiv	negativ
	J 2	Elsanta		negativ	negativ	negativ	positiv	negativ

2015:

18 samples from six farms were collected 2015. In contrast to the previous year only one sample tested positive for *Phytophthora sp*. (by PCR method and morphological). No *Verticillium sp*. could be detected in any plant sample (neither by PCR nor through morphological investigation). In two soil samples ca. 3 microsclerotia per gram of soil were detected meaning a very high risk for susceptible strawberry cultivars. *Phytophthora sp*. were isolated from 2 soil samples by baiting method (Werres et al. 2001). No symptomatic plants with typical virus symptoms were observed, all samples tested negative for phytoplasma. Several pathogens causing black root rot were isolated from roots (*Fusarium sp., Pythium sp. Rhizoctonia fragariae, Pyrenochaeta sp.*)

Table 2: Results of analysis of plant and soil samples 2015

				soil		plant				
				wet sieving method	morphological	PCR				
Betrieb	farm	field	cultivar	microsclerotia V. dahliae / g soil	Phytophthora sp.	*V. dahliae*	*V. albo atrum*	*P. fragariae*	*P. cactorum*	*phyto-plasma generic*
Aschauer	K	K 1	Alba			negativ	negativ	negativ	negativ	negativ
		K 2	Alba			negativ	negativ	negativ	negativ	negativ
Zachalmel	L	L 1	Elsanta			negativ	negativ	negativ	negativ	negativ
		L 2	Malvina			negativ	negativ	negativ	negativ	negativ
		L 3	Primy			negativ	negativ	negativ	negativ	negativ
Seifried		M 1	Clery	2,8		negativ	negativ	negativ	negativ	negativ
		M 2	Alba			negativ	negativ	negativ	negativ	negativ
		M 3	Alba			negativ	negativ	negativ	negativ	negativ
		M 4	Asia			negativ	negativ	negativ	negativ	negativ
Schlögl	N					negativ	negativ	negativ	positiv	negativ
Scharnböck	O	O 1				negativ	negativ	negativ	negativ	negativ
		O 2				negativ	negativ	negativ	negativ	negativ
		O 3		0	positiv	negativ	negativ	negativ	negativ	negativ
		O 4		3,2	positiv	negativ	negativ	negativ	negativ	negativ
Holzer	P	P 1	Elsanta			negativ	negativ	negativ	negativ	negativ
		P 2	Elsanta			negativ	negativ	negativ	negativ	negativ
		P 3	Elsanta			negativ	negativ	negativ	negativ	negativ
		P 4	Elsanta			negativ	negativ	negativ	negativ	negativ

Literature review at national levels

LITHUANIA

In Lithuania there are only few strawberry pathogens recorded according to the EPPO database. The review of EPPO database listed only 5 strawberry pathogens in Lithuania.

In Lithuania reaseach on strawberry pathogens is rarely carried out. 13 peer reviewed publications about strawberry diseases in Lithuania are available. The most publications are about *B. cinerea (*Rasiukevičiūtė et al. 2013; Raudonis 2003; Valiuškaitė 2003; Valiuškaitė et al. 2008; Valiuškaitė et al. 2010a, 2010b.), leaf diseases (Lanauskas et al. 2006; Raudonis 2003; Valiuškaitė 2003; Uselis et al. 2009, 2006), *P. fragariae (*Rugienius et al. 2006; Sasnauskas et al. 2007) suggesting that these pathogens are the most relevant. The pathogens *V. albo atrum, Colletotrichum acutatum* and *P. cactorum* were cited only once. There are no references on other pathogens occuring in strawberry crops.

IRELAND

Despite the existence of a traditional strawberry industry in Ireland there is relatively little information available on the diseases affecting this crop. The main source of information relating to Ireland are:

- Notifications in relation to regulated pathogens
- Scientific reports relating principally to husbandry aspects of production.

Relatively little research has been done in Ireland on strawberry pathology. However of the publications available, the majority concern *Botrytis cinerea* (Kavanagh et al. 1984; Kavanagh 1986; Anon 1977, 1980, 1982,1983) and *P. fragariae* (Kavanagh et al. 1984; Anon 1982,1983), suggesting that these two pathogens were of most commercial concern. In addition, there are occasional mentions of mildews (Anon 2001) and other *Phytophthora* spp. (Waterhouse, et al.1964; Anon 1982,1983).
There are no references to *V. dahlia* or *Gnomonia comari*, in relation to strawberry crops or as occurring in Ireland.

SPAIN

1. Phytosanitary and related records for Spain according to book entitled “Patogenos de plantas descritos en España”. (2010). 2end Edition. Ministerio de Medio Ambiente y Medio Rural y Marino (now Magrama) y Sociedad Española de Fitopatología (Spanish Society of Phytopathology).

RUSSIA (RU)

Basic Information about phytopathogens of strawberry in Russia available in the following source:

- Notifications in relation to regulated pathogens (European Plant Protection Organisation, EPPO)
- Scientific articles on plant protection, collection of scientific papers from the conferences on agriculture (1958 – present)

AUSTRIA

Basic Information about phytopathogens of strawberry in Austria available in the following sources:

- Notifications in relation to regulated pathogens (European Plant Protection Organisation,EPPO)
- Electronic databases OVID including AGRICOLA, AGRIS CAB-abstracts

Table 15: Results of literature reviews on strawberry diseases

	Ireland	Spain	Russia	Lithuania	Austria
Virus					
Arabis Mosaic Virus	IE 1		RU 1, 26, 27, 28	EPPO 1998	CABI/EPPO 1997
Raspberry ringspot virus	IE 1		RU 7, 8,9,10,11, 28		EPPO 2014, NPPO 2014-06,
Strawberry crinkle virus	IE 1		RU 26, 28		EPPO 2014
Strawberry latent c virus	IE 1				EPPO 2014

Strawberry latent ringspot virus (SLRSV)	IE 1	ES 21	RU 26, 27, 28		EPPO 2014, NPPO 2014-06
Strawberry mild yellow edge virus	IE 1		RU 26		EPPO 2014
Strawberry mottle virus			RU 26, 28		
Strawberry pseudo mild yellow edge virus			RU 26, 27		
Strawberry vein banding virus	IE 1		RU 12		EPPO 2014
Strawberry witches broom	IE 1				
Tobacco ringspot virus			RU 13, 14, 15,		
Tomato black ring virus	IE 1		EPPO 1994 RU 26, 27, 28		EPPO 2014, NPPO 2014-06
Tomato ringspot virus			RU 16, 17, 18, 27		
Tomato spotted wilt			RU 19, 20, 21		
Fungi					
Alternaria tenuissima			RU 35		
Aureobasidium pullulans			RU 35		
Botrytis cinerea (*Botryotinia fuckeliana*)	IE 4,5,6,7,8,9, 10	ES 21	RU 24, 29, 35	LT 4, 5, 11, 12, 13	AT 17, 19
Cladosporium herbarum			RU 35		
Colletotrichum accutatum	IE 1	ES 3	RU 32, 33, 35	EPPO 2001 LT 1	AT 3, 10
Colletotrichum gloeosporioides		ES 21			
Cylindrocarpon destructans			RU 35		
Dendrophoma obscurans			RU 24, 29, 30		
Diplocarpon earliana (*Marssonina fragariae*)		ES 4,5			AT 20, 21
Discohainesia oenotherae			RU 29		
Fusarium lateritium			RU 35		
Fusarium oxysporum f. sp. fragariae			RU 24		
Fusarium sporotrichiella			RU 34		
Fusarium spp			RU 29		
Gnomonia comari		ES 6,7,8			
Marssonina potentillae			RU 29		
Marssonina potentillae f.sp. var fragariae			RU 24		
Mycosphaerella fragariae	IE 5	ES 9, 10	RU 29		AT 20, 21
Oidium erysiphoiides			RU 29		
Phoma obscurans			RU 35		
Phyllosticta fragaricola			RU 29		
Phytophtora spp.					AT 20

Phytophthora cactorum	IE 6,7	ES 11-16	EPPO 1994 RU 24, 31, 35	EPPO 2004, LT 2	AT 17, 19
Phytophthora fragariae var *fragariae*	IE 1, 4, 6, 7		RU 2,3,4,5,6	EPPO 1992	AT 24
Phytophthora nicotiana var parasitica	IE 3		RU 35		
Podosphaera aphanis (Sphaerotheca macularis, S. humuli, S. aphanis, Oidium fragariae)	IE 2, 10*, 11	ES 19,20	RU 24		AT 20, 17
Pyrenochaeta lycopersici		ES 17,18			
Pythium spp.			RU 32		AT 20
Ramularia tulasnei			RU 24		
Rhizoctonia solani			RU 29, 35		AT 20
Verticillium albo-atrum			RU 23, 24,29	EPPO 1986, LT 6	
Verticillium dahliae			No. RU 22, 24		AT 20, 21, 19, 17
Verticillium latericium			No. RU 24		
Bacteria					
Xanthomonas fragariae	IE 1	ES 1, 2			AT 20, 25
Phytoplasma					
Strawberry phylloid fruit phytoplasma			RU 25		

* referred only to mildew

present
absent

Conclusion

The amount of literature of strawberry diseases at national level is relatively poor (except in Russia). However, a first outline of the occurrence of strawberry diseases in the partner countries of this project can be drawn from the results of the questionnaire and the tests conducted in the field.

The status of virus diseases has only been surveyed in Russia, Austria and Ireland. Their importance has not been surveyed in this project. Leaf diseases occur in Estonia only on fields of small producers. Over a period of 5 years (2008-2013) leaf diseases were deemed important for Ireland, Spain and Lithuania (mainly *P. aphanis*). For 60 % of respondents in Lithuania grey mold is the most important disease, it is also a main disease in Austria and Ireland. 25% of producers in Spain would name grey mold as second most important disease. *Macrophomona phaseolina* was only mentioned by one of five Spanish producers. *Fusarium spp.* was only observed in Spain during the relvant period.

Verticillium-diseases occurred mainly in Austria and Lithuania, during 2008-2013. They were also observed in Spain and Lithuania. Crown rot (*P. cactorum*) emerged (frequently) in Spain, Lithuania, Austria and Ireland. The available publications suggest that *P. fragariae* is of major commercial concern in Ireland. *X. fragariae* only occurred in Austria in field but to date it has also been reported in Ireland and Spain. More field inspections would be necessary to gain a representative picture of the occurrence and relevance of strawberry diseases.

WP3: Review and analysis of available diagnostic methods

Lead: *Evelin Loit, EE-EMU*

Main Partners: *Ulrike Persen, AGES-AT; I. Maria Destefanis, IE-DAFF*

Contributing partners: *all*

Objectives and tasks of the project

The primary objective of this work package (WP3) was to provide a comprehensive overview of the existing scientific literature available on PCR-based diagnostic techniques for the detection and quantification of the most important strawberry pathogens including *Fusarium* spp., *P. fragariae*, *Colletotrichum acutatum*, *V. dahliae*, *B. cinerea*, *M. phaseolina* and *X. fragariae*. A secondary objective was to determine the pre-analytical and analytical requirements of PCR assays. Finally, we provided an updated list of published PCR protocols as a systematic review of methods for the detection and quantification of strawberry pathogens. The aim was to generate a common diagnostic PCR based-method for routine testing by looking the factors that affect the efficiency of the different test formats and comparing their performance in pathogen detection in plant material and soil.

Methods used and results obtained

Study design was a systematic review of PCR-based techniques used for detection and quantification of strawberry pathogens. Using appropriate subject headings, AGRICOLA, AGRIS, BASE, Biological Abstracts, CAB Abstracts, Google Scholar, Scopus, Web of Knowledge, Science Direct and Springer Link databases were searched from their inception up to April 2014. The articles were selected if the investigation included PCR methods applied on strawberry pathogens. All references of the selected articles were further investigated if the title of the article mentioned the use molecular diagnostic methods on strawberry pathogens. Moreover, some experts on the subject were identified from relevant publications in order to receive advice on relevant literature about diagnostic methods in strawberry pathogens. Grey literature (conference abstracts and unpublished studies) and duplicate publications of the same data were disregarded. Thereafter, relevant information of articles was extracted, summarized and schematically outlined. We synthesized results according to PCR protocol, primer sets and target DNA employed in each study and pathogen treatment.

Specificity and sensitivity of methods were also identified by systematically summarizing the available literature. As result, each method was assessed on the basis of three criteria that were defined a priori to answer the research questions: PCR-based methods used for detection and quantification of important pathogens on strawberry; available methods were compared by through detection sensitivity and specificity; pre-analytical and analytical requirements were related to accuracy of each method. Statistical metaanalysis was not justified because of the heterogeneity of the included studies in detecting strawberry pathogens.

In sum, the original systematic search strategy identified 259 unique citations of which 200 articles were excluded based on the content of title and/or abstract. Fifty-nine articles were read and evaluated for inclusion criteria. This resulted in the inclusion of twenty articles. Ten articles were read based on references, of which three were included, bringing the sum of included relevant articles to 23. Our systematic review identified 10 different protocols for *X. fragariae*, eight for *P. fragariae*, four for *B. cinerea*, six for *C. acutatum*, three for *V. dahliae*, and only one protocol for *F. oxysporum*. No PCR-based detection method for *M. phaseolina* in strawberry could be identified.

Discussion of results and their reliability

The majority of the studies included in this review, investigated conventional PCR (cPCR) methods (detection based on agarose gels) for detection/identification of strawberry pathogens. In this regard, several methods were developed to improve sensitivity of cPCR. Nested PCR with both internal and external primers was reported to increase detection sensitivity and reduce the effect of PCR inhibitors. In fact, the use of a nested approach is useful when the pathogen is present in very low levels or the infestations need to be detected in complex environmental samples. However, the risk of false positives due to cross-contamination of reaction mixtures in routine analysis increases by the introduction of a second round of amplification. Multiplex PCR (mPCR) was also applied for detection of strawberry pathogens. Although, mPCR is useful for the simultaneous and specific detection of different DNA targets, it requires a tedious and time-consuming optimization processes. Furthermore, it seems to be less reliable for quantitative analyses. Decrease in sensitivity and limited number of interested targets are the most significant drawbacks of multiplex PCR.

In the other side, some studies focused on quantification of pathogen using real-time PCR technique, in which sensitivity was increased. The higher sensitivity of rtPCR compared to cPCR; firstly, data are available in real time, do not require time-consuming post-PCR processing and can be analysed quantitatively. Secondly, rtPCR commonly amplify very short DNA fragments (70-100 bp) which favours a higher level of PCR efficiency and sensitivity compared to cPCR. Only twelve rtPCR protocols were referred for detection and quantification of strawberry pathogens. But, their numbers increased from only one in 2004 to six between 2007- 2012. Since, primers designed for conventional PCR can be utilized in real-time PCR assays, existing cPCR protocols can be adapted for real time detection. Real-time PCR using TaqMan probe and SYBR green dye are the most widely used for diagnostic purposes, but in our systematic review all protocols utilized probe-based method (TaqMan), which provide greater sensitivity and specificity than other PCR techniques. However, availability of instrumentation, the degree of diversity among target and non-target sequences, and the need for multiplexing are primary factors in the choice of real-time platforms.

Several other research needs were under investigation. First of all, most included studies used commercial kits to extract DNA from strawberry tissue and soil, because of their simplicity and rapidity together with the absence of harmful chemical compounds. However, DNA isolation kits can be expensive and inefficient when handling plants with high polyphenolic content. Second, sample collection and long-term storage procedures were reported, but procedures for sample transportation were missed in most studies. Indeed, the absence of common pre-analytical procedures might affect final results. Third, rtPCR was mostly used, but not always with the same materials and methods, resulting in a reduced comparability.

Output

Concise literature overview and meta-analysis of the available methods that could be used for strawberry pathogen diagnostics.

All deliverables have been met. The results have been published:

- Mirmajlessi SM, Destefanis M, Gottsberger RA, Mänd M, Loit E. PCR-based specific techniques used for detecting the most important pathogens on strawberry: a systematic review. Syst Rev. 2015 Jan 15; 4:9.

WP4: Development of a common diagnostic method to detect quarantine and emerging strawberry pathogens

Lead: *I. Larena; Co-lead: A. De Cal, ES-INIA*

Main Partners: *Eha Kruus, Mahyar Mirmajlessi, EE-EMU*

Contributing partners: *IE-DAFF, AGES-AT, FGBU VNIIKR*

Early diagnosis and accurate detection of pathogens is an essential step in plant disease management. Moreover, fast and accurate tests are necessary to characterize the distribution of the pathogens, prevent their introduction into new areas and minimize their spread within affected areas. There is a need for an improved testing and diagnostic methods for emerging and quarantine strawberry pathogens (listed in 2000/29 EC) in EU.

The objective of this study was to describe the use of PCR and/or real-time PCR to detect and/or quantify the main strawberry pathogens in EU: *F. oxysporum* f. sp. *fragariae, F. solani, M. phaseolina, P. fragariae, P. cactorum, V. dahlia, V. albo-atrum, X. fragariae (B. cinerea)*, and strawberry viruses.

Isolates of *F. oxysporum* (FOF), *M. phaseolina* (MP), and *F. solani* (FS)

The fungal isolates used in this study: 6 isolates of FS, 18 isolates of FOF and 14 isolates of MP. FS and FOF isolates were isolated from strawberry plants with disease symptoms from Spanish nurseries (Avila and Segovia) and were identified in INIA laboratory, except TOR1, TO11 and F-POST81 kindly provided by Nieves Capote group from IFAPA of Seville. All isolates were stored at -80 ºC in 20% glycerol (long-term storage) and at 4 ºC in tubes containing sterile sand in the dark (short-term storage). The isolates were grown on Czapek Dox Agar (CDA) (Difco; Detroit, MI, USA) in darkness at 25 ºC for mycelial and conidial production

MP isolates have been kindly provided by Manuel Aviles of the University of Seville and by Nieves Capote from IFAPA, Seville. All isolates were stored at -80 ºC in 20% glycerol (long-term storage) and at 4 ºC on dried plates APD. For conidial and mycelial production, MP isolates were grown on PDA in Petri dishes in the dark at 20-25 ºC for seven days.

DNA extraction

Total DNA from the mycelia and conidia of each fungal isolate was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. A 10-mg sample of mycelia and conidia that were grown on PDA at 22°C for 7 to 10 days was collected with a spatula, and then transferred into a microfuge tube that contained 400 µl of lysis buffer. The DNA from each isolate was eluted into 100 µl of sterile water, and its concentration was measured using a Nanodrop 2000 (Thermo Scientific). DNA concentration varied depending on isolate between 3-300 ng μl^{-1}. DNA samples were stored at –20°C until required. The extracted DNA from all isolates was used as the template for PCR.

In order to check DNA quality and amplifiability, DNA template was amplified by PCR with universal primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) that amplify the region ITS1-5.8S-ITS2 rDNA (White et al 1990). The PCRs were performed in a 25-µl reaction mixture that contained 10 mM Tris-HCl (pH 8.3),50 mM KCl, 100 mM of each dNTP, 2 mM MgCl2, 1 mM of each primer, 1 U of *Taq* DNA Polymerase (Biotools B&M Labs, S.A., Madrid, Spain), and 10 ng of template genomic DNA. The PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 1 min 30 s, 55°C for 1 min, and 72°C for 2 min in an iCycler thermocycler (BioRad Laboratories Ltd.). The reaction was terminated by a final elongation step at 72°C for 10 min. Control reactions, in which no DNA template was present, were performed to test for possible contamination of the reagents with fungal DNA. The PCR products were electrophoresed through 1% agarose gels, stained with GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium), and then visualized under ultraviolet light. A 1-kb Plus DNA ladder (Invitrogen Life Technologies, Carlsbad, CA, USA) was used as a size marker. All PCRs were repeated at least twice. The amplification products are approximately 600 bp (Fig. 15).

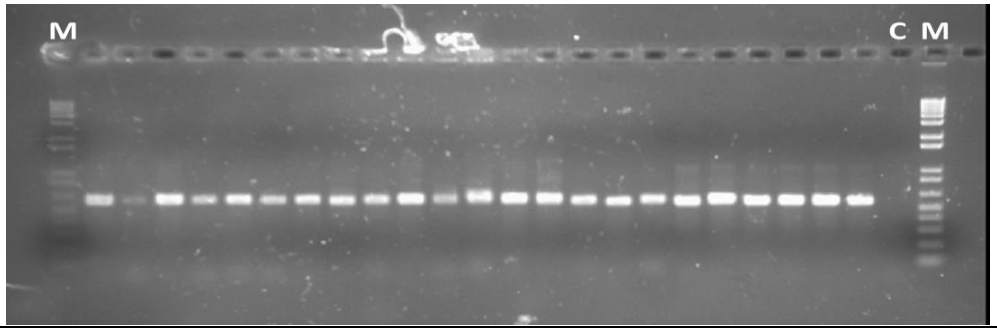

Fig. 14. DNA quality from DNA isolates from various pathogens amplified with ITS4/5 primer pair. M: Molecular Marker 1KB Plus DNA ladder (Invitrogen), C: control without DNA. The amplification products are approximately 600 bp.

Detection of *F. solani* by Arif *et al* (2012) with some modifications:

Primer pair and PCR conditions are described in Table 16 y 17. All PCRs were performed in a reaction volume of 25 µL. The DNA was diluted a 100-fold prior to conventional PCR.

Table 16: Primers

Pathogen	Primers	Sequence (5'- 3')	Amplified size
Fusarium solani	TEF-Fs4f	ATCGGCCACGTCGACTCT	658 bp
	TEF-Fs4r	GGCGTCTGTTGATTGTTAGC	
Macrophomina phaseolina	MpKF1	CCGCCAGAGGACTATCAAAC	350 bp
	MpKR1	CGTCCGAAGCGAGGTGTATT	
F. oxysporum* f.sp. *fragariae	FOFRI-1F		171 bp
	FOFRI-1R		

Table 17: The PCR conditions

REACTIVES	Final Concentration	24 µl mix y 1 µl DNA (1:100)
Buffer 10x	1x	
dNTPs (10 Mm)	0,2 mM	
TEF-Fs4f (5 µM)	0,3 µM	
TEF-Fs4r (5 µM)	0,3 µM	
Taq- pol TAKARA (5U/µl)	2 U	
mili Q	Up to the final volume	

	Tª	Time	
Pre-denaturation	94 ° C	2 min	
Denaturation	94 ºC	30 s	x 25 cycles
Annealing	56 ºC	30 s	
Elongation	72 ºC	2 min	
Final elongation	72ºC	3 min	

In all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination

The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium). A 1-kb Plus DNA Ladder (Invitrogen Life Technologies) was used as a molecular size marker. The PCR with the specific primer pair yielded a 658-bp DNA band. All PCR were repeated at least twice (Fig. 15).

To determine the sensitivity of the PCR with *F. solani*-specific primer TEF-Fs4f/TEF-Fs4r, a solution that contained 1.2×10^{-11} ng μl^{-1} genomic *F.solani* DNA was diluted serially until the final DNA concentration was 1.2×10^{-8} ng μl^{-1}. A 1-µl aliquot of each dilution was used in the PCR (Fig. 16).

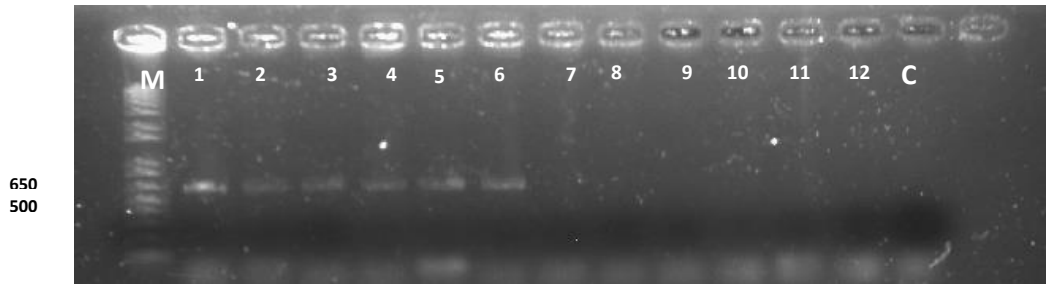

Fig. 15. PCR amplification in which Lanes 1-6, corresponding to DNA from 6 isolates of *F. solani;* Lanes 7-9, DNA from isolates of *F.oxysporum f.sp. fragariae;* Lanes 10-12, DNA from isolates *M. phaseolina.* Lane C, negative control without DNA template. Lane M, molecular weight standard (1-kb Plus DNA Ladder).

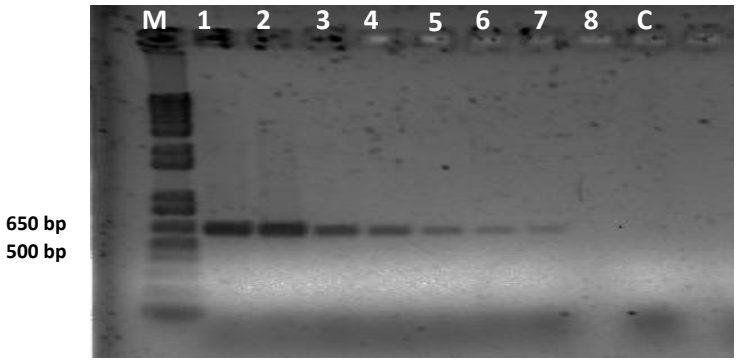

Fig. 16. PCR amplification in which increasing concentrations of DNA from an isolate of *F. solani* were used as template with the TEF-Fs4f/TEF-Fs4r primer pair and annealing at 56°C. Lanes 1 to 6, DNA amplification products with the following concentrations of DNA template Lane 1, 1.2×10^{-11} g; lane 2, 6.0×10^{-10} g; lane 3, 1.2×10^{-10} g; lane 4, 6.0×10^{-9} g; lane 5, 3.0×10^{-9} g; lane 6, 1.2×10^{-9} g; lane 7, 6.0×10^{-8} g; lane 8, 1.2×10^{-8} . Lane C, negative control without DNA template. Lane M, molecular weight standard (1-kb Plus DNA Ladder)

Detection *of M. phaseolina* by Babu et al (2007) with some modifications:

All PCRs were performed in a reaction volume of 20 µL. The DNA wasn´t diluted prior to PCR. Specific primer were MpKF1 (5' CCGCCAGAGGACTATCAAAC 3')/ MpKR1 (5' CGTCCGAAGCGAGGTGTAT 3'). PCR condition were described in Table 18.

Table 18: The PCR conditions

REACTIVES	Final Concentration	
Buffer 10x with Cl_2MG	1x	19 µl mix y 1 µl DNA (no dilution)
dNTPs (10 Mm)	0,2 mM	
MpKF1 (5 µM)	0,3 µM	
MpKR1(5 µM)	0,3 µM	
Taq- pol TAKARA (5U/µl)	2,5 U	
mili Q	Up to the final volume	

	Tª	Time	
Pre-denaturation	95 º C	2 min	
Denaturation	95 ºC	30 s	x 25 cycles
Annealing	50 ºC	1 min	
Elongation	72 ºC	2 min	
Final elongation	72ºC	3 min	

In all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination

The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium). A 1-kb Plus DNA Ladder (Invitrogen Life Technologies) was used as a molecular size marker. The PCR with the specific primer pair yielded a 350-bp DNA band. All PCR were repeated at least twice (Fig. 17).

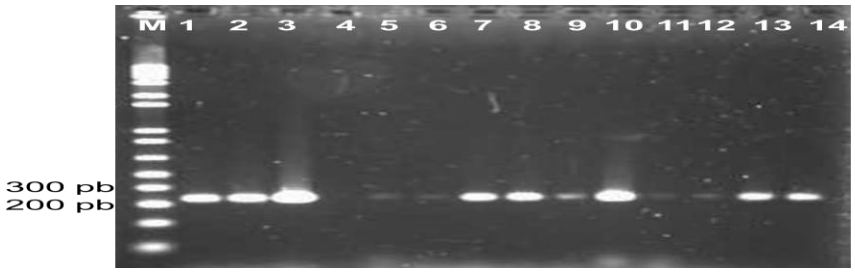

Fig. 17. PCR amplification of DNA from 14 isolates of *M. phaseolina*. Lane M, molecular weight standard (1-kb Plus DNA Ladder).

To determine the sensitivity of the PCR with *M. phaseolina* -specific primer MpKF1/MpKR1, a solution that contained 1.6×10^{-10} ng μl^{-1} genomic *M. phaseolina* DNA was diluted serially until the final DNA concentration was 1.6×10^{-7} ng μl^{-1}. A 1-µl aliquot of each dilution was used in the PCR (Fig. 18).

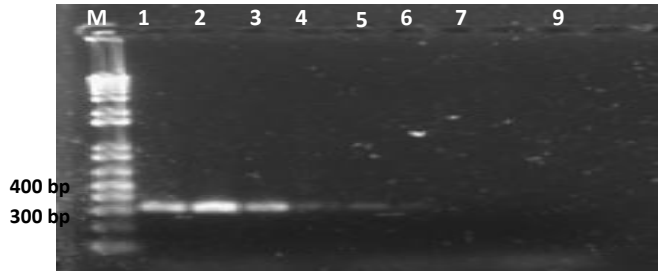

Fig. 18. PCR amplification in which increasing concentrations of DNA from an isolate of *M. phaseolina* were used as template with the MpKF1/MpKR1 primer pair and annealing at 50°C. Lanes 1 to 6, DNA amplification products with the following concentrations of DNA template Lane 1, 1.6×10^{-10} g; lane 2, 7.9×10^{-9} g; lane 3, 1.6×10^{-9} g; lane 4, 7.9×10^{-8} g; lane 5, 1.6×10^{-8} g; lane 6, 7.9×10^{-7} g. Lane 7, 1.6×10^{-7} g; Lane 9, negative control without DNA template. Lane C, negative control without DNA template. Lane M, molecular weight standard (1-kb Plus DNA Ladder).

Detection of *F. oxysporum f.sp. fragariae* by Larena et al (in preparation):

Five specific primers for *F. oxysporum f.sp. fragariae* were designed on the basis of differences in the nucleotide sequences of the ITS1-5.8S-ITS2 region of *F. oxysporum f.sp. fragariae* isolates and other Fusarium species whose sequences are deposited in GenBank. Genomic DNA from five isolates of *F. oxysporum f.sp. fragariae* was screened in order to determine the optimal conditions for each forward/reverse primer combination. Finally we selected the primer pair FOFRI-1F/FOFRI-1R as the best one.

All PCRs were performed in a reaction volume of 25 µL. The DNA was diluted a 1000-fold prior to conventional PCR (Table 19).

Table 19: The PCR conditions

REACTIVES	**Final Concentration**	
Buffer 10x minus Cl_2Mg BIOTOOLS	1x	20 µl mix y 5 µl DNA
dNTPs (10 Mm)	0,1 mM	
Cl_2Mg (50 mM)	2 mM	
FOFRI-1F (10 µM)	0,25 µM	
FOFRI-1R (10 µM)	0,25 µM	
Taq- pol BIOTOOLS (5U/µl)	2,5 U	
mili Q	Up to the final volume	

	Tª	Time	
Pre-denaturation	95 º C	3 min	
Denaturation	95 ºC	1 min 30 s	x 30 cycles
Annealing	60 ºC	1 min	
Elongation	72 ºC	2 min	
Final elongation	72ºC	10 min	

In all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination

The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium). A 1-kb Plus DNA Ladder (Invitrogen Life Technologies) was used as a molecular size marker. The PCR with the specific primer pair yielded a 171-bp DNA band. All PCR were repeated at least twice (Fig. 19).

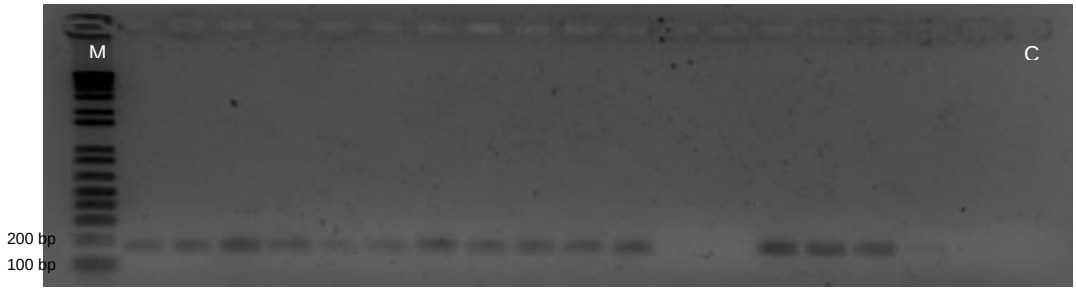

Fig. 19. PCR amplification of DNA from 18 isolates of *F. oxysporum f.sp. fragariae*. Lane M, molecular weight standard (1-kb Plus DNA Ladder). Lane C, negative control without DNA template

To determine the sensitivity of the PCR with *F.oxysporum f.sp. fragariae* -specific primer FOFRI-1F/FOFRI-1R, a solution that contained 2.4 × 10^{-9} ng μl^{-1} genomic *F.oxysporum f.sp. fragariae* DNA was diluted serially until the final DNA concentration was 1.2 × 10^{-7} ng μl^{-1}. A 5-µl aliquot of each dilution was used in the PCR (Fig. 20).

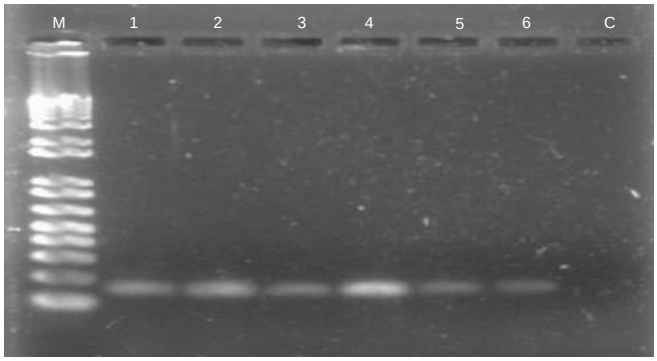

Fig. 20. PCR amplification in which increasing concentrations of DNA from an isolate of *F.oxysporum f.sp. fragariae* used as template with the FOFRI-1F/FOFRI-1R primer pair and annealing at 50°C. Lanes 1 to 6, DNA amplification products with the following concentrations of DNA template Lane 1, 2.4× 10^{-9} g; lane 2, 1.2 × 10^{-9} g; lane 3, 2.4 × 10^{-8} g; lane 4, 1.2 × 10^{-8} g; lane 5, 2.4 × 10^{-7} g; lane 6, 1.2 × 10^{-7} g. Lane C, negative control without DNA template. Lane M, molecular weight standard (1-kb Plus DNA Ladder).

Specificity of primer pairs

The specificity of each primer pair was evaluated using the isolated DNA of the 6 strains of FS, 14 strains of MP and 14 strains of FOF and other fungi in our laboratory collection

Table 20. Fragments amplified from different fungi with specific primer pair to *M. phaseolina* (MpKF1/MpKR1), *F. oxysporum f.sp. fragariae* (FOFRI-1F/FOFRI-1R), and *F. solani* (TEF-Fs4f/TEF-Fs4r)

Fungi		MpKF1/MpKR1	FOFRI-1F/FOFRI-1R	TEF-Fs4f/TEF-Fs4r
Aspergillus nidulans		-	-	-
Aspergillus spp.		-	-	-
Penicillium oxalicum		-	-	-
P. chrysogenum		-	-	-
Phoma betae		-	-	-
Cladosporium cucumerinum		-	-	-
F. gramineum		-	-	-
F. o. f.sp. lyscopersici		-	-	-
F. melonis		-	-	-
F. niveum		-	-	-
Verticillium albo-atrum		-	-	-
V. dahliae		-	-	-
P. rubens		-	-	-
One Isolate from strawberry		-	-	-
F. solani 1	*F. solani*	-	-	+
F. solani 2		-	-	+
F.solani 3		-	-	+
F. solani 4		-	-	+
F. solani 5		-	-	+
F. solani 6		-	-	+
FOF1	*F. oxysporum* f.sp *fragariae*	-	+	-
FOF2		-	+	-
FOF4		-	+	-
FOF5		-	+	-
FOF6		-	+	-
FOF7		-	+	-
FOF8		-	+	-
FOF9		-	+	-
FOF 10		-	+	-
FOF 11		-	+	-
FOF.12		-	+	-
FOF 13		-	+	-
FOF 14		-	+	-
M.1	*M. phaseolina*	+	-	-
M.2		+	-	-
M.3		+	-	-
M.4		+	-	-
M.5		+	-	-
M.6		+	-	-

M.7	+	-	-
M8	+	-	-
M9	+	-	-
M10	+	-	-
M11	+	-	-
M12	+	-	-
M13	+	-	-
M14	+	-	-

+ amplification; - No amplification

Identification of *Phytophthora fragariae* by real-time PCR

The protocol was developed by Kopina et al. (2012). Nucleic acid source is mycelium, plant tissue. The assay is designed for ras-related protein (Ypt1) gene sequences producing an amplicon of 431 bp. The following oligonucleotides are used: forward primer PHL (5'- CAA-GAC-YAT-CAA-GCT-SCA -3'), reverse primer PHR (5'-GTT-GTT-GAA-CGA-HGA-CTC-YGT-G -3') and (Taqman) probes Ph.FR (FAM-CAT-TTC-GCC-GGC-TAA-GCG-TG- RTQ1). Molecular grade water (MGW) is used to make up reaction mixes; it should be purified (deionised or distilled), be sterile (autoclaved or 0.45lm filtered) and nuclease-free. 10X MagMix PCR buffer (LLL "Dialant Ltd", Moscow) containing Taq poly-merase, reaction buffer containing $MgCl_2$ and nucleotides are used for PCR. Amplification is performed using the iCycler iQ 5 (Bio-Rad, USA). The analytical specificity of the assay was assessed using 13 *Phytophthora spp*. strains causing Phytophthora root rots. All *P. fragariae* isolates reacted positive. No cross-reactions with other species were observed.

Nucleic Acid Extraction and Purification

DNA can be extracted from pure cultures using the DNA extraction kit of the "DNA-Extran" series № NG-511-100 (CJSC "Syntol", Moscow). The method is based on processing the sample with proteinase K followed by removal of proteins without organic solvents used for extraction. Isopropyl alcohol with glycogen as a precipitator is used for DNA deposition.

DNA can be extracted from plant tissue using Doyle and Doyle (1990) methodology. Plant tissue (50 mg) was placed in 1.5-mL microtubes containing 400 µl of 2% CTAB extraction buffer with modifications [20mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, plus 1% 2-mercaptoethanol added just before use]; microtubes were then vortexed for 10 s and incubated at 60ºC for 30 min; 60 µl of chloroform-isoamylalcohol (24:1) was then added to the solution which was vortexed for 10 s and centrifuged at 10,000 rpm for 3 min; the supernatant was transferred to a fresh tube

and this stage was repeated once; cold isopropanol (-20ºC) was added to the supernatant (0.7 of the total volume of supernatant collected); samples were gently mixed by inversion and centrifuged at 10,000 rpm for 3 min; the DNA pellet adhered to the tube was then visualized; the liquid phase was then released and DNA washed twice with 500 µl 70% ethanol; the pellet was set to dry for approximately 12 h with the tubes inverted upon filter paper at room temperature; the pellet was ressuspended in 100 µl TE buffer solution plus 5 µl RNAse (10 mg mL^{-1}); the solution was then incubated at 37ºC for 1 h, and after stored at –20ºC.

DNA can be extracted using commercially available DNA extraction kits, e.g. DNeasy Plant Kit (Qiagen) or QuickPick Plant DNA kit (Bionobile, Parainen, FI) according to the manufacturer's instructions.

DNA purification using spin columns filled with polyvinylpyrolidone (PVPP) is necessary for DNA isolated using the DNeasy Plant kit. The columns are prepared by filling Axygen Multi-Spin columns (Dis-polab, Asten, The Netherlands) with 0.5 cm PVPP, placing it in an empty reaction tube and washing twice with 250 µl MGW by centrifuging the column for 5 min at 4,000 g. The DNA suspension is applied to a PVPP column and centrifuged for 5 min at 4,000 g. The flow through fraction is used as input for the PCR. For DNA isolated using the QuickPick kit no DNA purification is necessary. Either use extracted DNA immediately, store over-night at 4^0C or at -20^0C for longer periods.

Polymerase Chain Reaction

Master mix (concentration per 25 µl single reaction). 1X TaqMan MagMix PCR buffer (LLL "Dialant Ltd", Moscow), 10 pM of each primer, 5 pM TaqMan probe, Molecular grade water is added to 20 µl, 5.0 µl extracted DNA obtained as described above.

- PCR cycling parameters: 1 cycle at 95^0C – 5 min; 40 cycles at 95^0C – 15 sec, 56^0C – 40 sec.
- A cycle threshold (Ct) value <40 with probe Ph.FR indicates the presence of *P. fragariae* DNA.

Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

• Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample collected from uninfected plane tree wood. Clean extraction buffer can also be used to monitor contamination;

• Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a pure culture of *P. fragariae* a matrix sample that contains *P. fragariae;*

• Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water and of the supermix solution that was used to prepare the reaction mix;

• Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from a pure culture of *P. fragariae*, DNA extracted from an infected host tissue, or a synthetic control (e.g. the cloned ITS region). The PAC should preferably be near to the limit of detection.

Methods proven suitable at AGES lab (for detailed protocols see WP5)

- Detection of P. fragariae (2 protocols): According to Ioos et al. (2006), RAS-like, TRP1 (single copy genes containing introns)

  - *P. fragariae* (*TRP*1): expected amplicon 403 bp

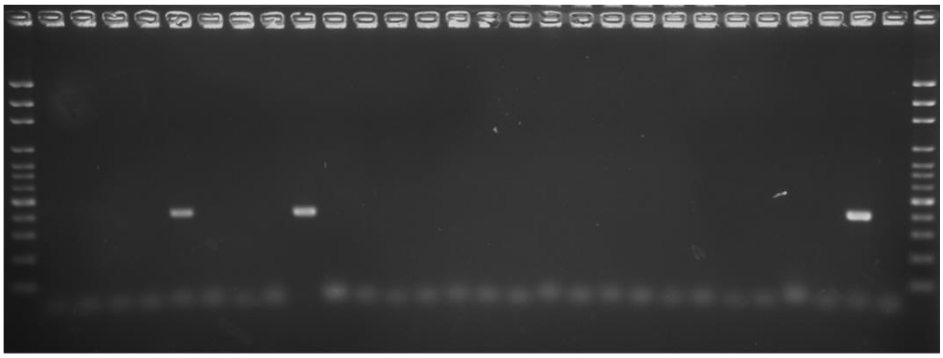

Fig. 21

- Detection of P. cactorum: According to Causin et al. (2005), primers developed from specific RAPD fragment.
  - *P. cactorum*: expected amplicon 450 bp

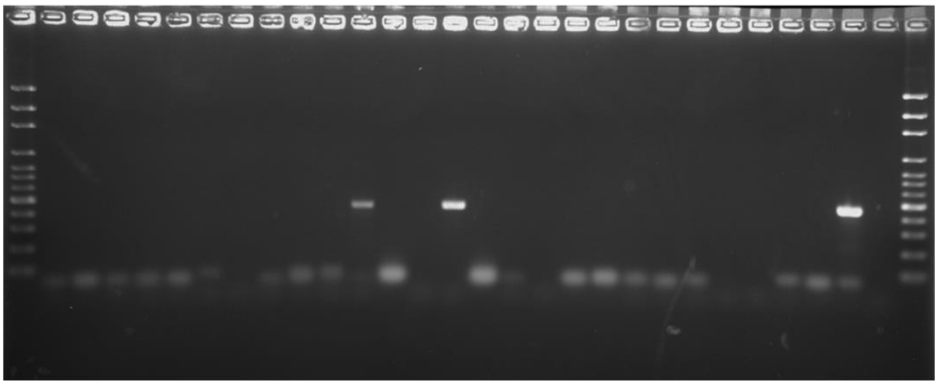

Fig. 22

- Detection of V. albo-atrum and V. dahliae: According to Carder et al. (1994), included in the EPPO Diagnostic Standard PM 7/78 (1): Verticillium albo-atrum and V. dahliae
  - *V. dahliae*: expected amplicon 580 bp

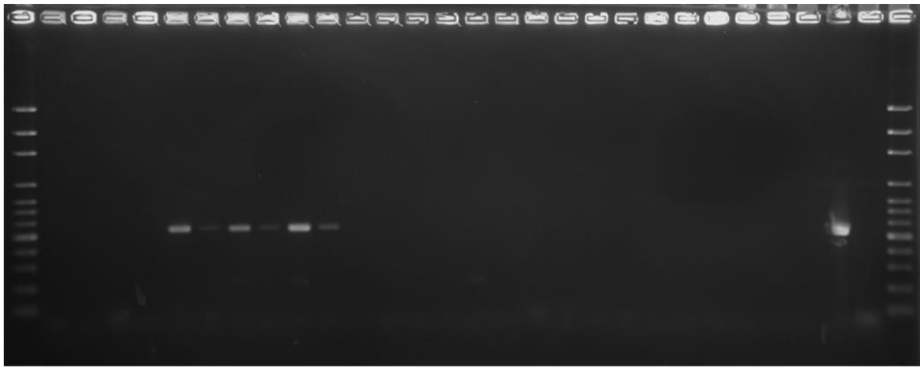

Fig. 23

- *V. albo-atrum*: expected amplicon 300 bp

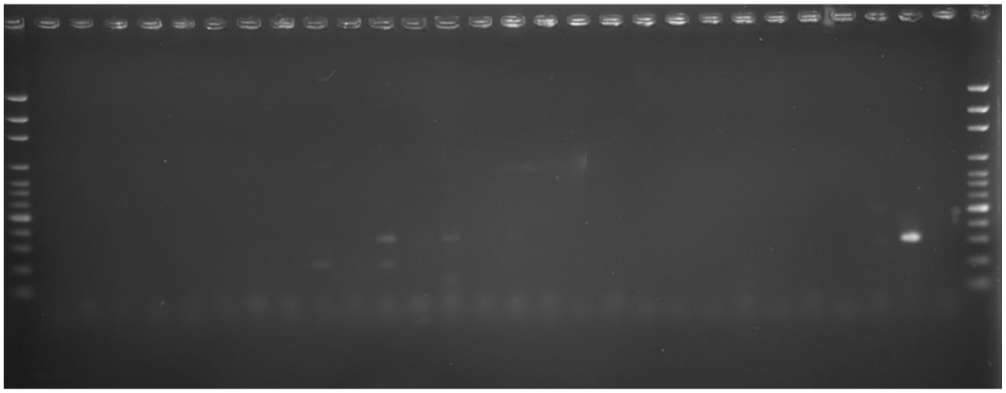

Fig. 24

Quantification of *V. dahliae*: According to Bilodeau et al. (2012), Taqman, IGS (intergenic spacer):

Quantification of *V. dahliae* from strawberry fields (1.0)

Molecular method used: According to Bilodeau et al. (2012), Taqman, targeting the IGS (intergenic spacer). Extraction directly from field samples with PowerSoil® DNA Isolation Kit (MO BIO)

Drawback: > 10 microsclerotia / g soil is considered a high number for certain strawberry cultivars. 250 mg of soil is extracted 4 x: total DNA of 1 g soil eluted in 400µl. Template for PCR is 1-4 µl. Bellow the detection limit!

- Way out?

Concentrating elution volume to 40 µl (Vacuum concentrator + PowerClean® DNA Clean-Up Kit, MO BIO). Using fraction from wet sieving technique for DNA extraction and quantification with molecular methods. (no improvement!)

Initial soil quantity too low!

Quantification of V. dahliae from strawberry fields (2.0)

Soil quantity was increased to 10 g. Extraction with Phenol-Chloroform-Isoamylacohol in 50 ml vials. DNA pellet was eluted with 150 µl water

- Drawback: PCR inhibition!

Purify DNA using PowerClean® DNA Clean-Up Kit (MO BIO), elution with 30 µl

Concentrating elution volume to 10 µl (Vacuum concentrator). Detection limit lower than eight microsclerotia/g soil, but higher than 5 microsclerotia/g soil compared with wet sieving method results. Ct values on the detection limit of 35-38 in our hands. Enrichment in wet sieving method cannot be compensated by real-time PCR sensitivity, even when an increased soil quantity is used! Sensitivity is still too low! Further improvement runs counter to efford needed

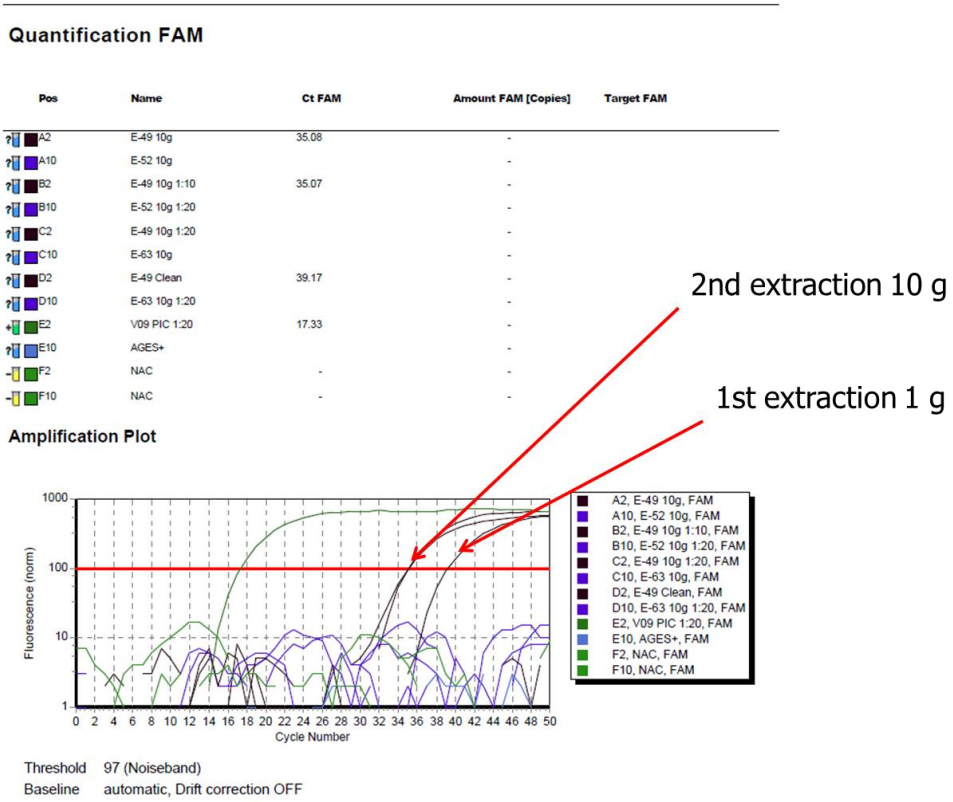

Fig. 25. Example real time run V. dahliae from soil

Conclusion on *V. dahliae* quantification from soil

Wet sieving technique is to date the only suitable method for quantification of *V. dahliae* microsclerotia from soil. Real-time PCR is much faster, but sensitivity of a practicable procedure is too low for quantification from soil in order to give recomendations on the choice of strawberry varieties concerning susceptibility to *V. dahliae*.

WP5: Method validation in ringtests

Lead: *Richard Gottsberger, AT-AGES*
Contributing partners: *all*

Objective

The objective of the workpackage was comparing and validating novel diagnostic methods developed/reviewed in WP4 in different laboratories. Methods for detection of important strawberry pathogens were selected by the different partners for protocols testing and optimization. A selection of protocols for the detection of 7 fungal pathogens was chosen, including *Phytophthora fragariae, P. cactorum, Verticillium dahliae, V. albo-atrum, M. phaseolina, Fusarium oxysporum f. sp. fragariae, F. solani and Botrytis cinerea*. From three partner labs, samples were sent to all participants together with primers and protocols. All PCR reagents were provided by each lab. The end point PCR cyclers were used under the conditions indicated in the protocols. Every deviation from the protocols was indicated in the results sheet.

Following protocols were provided by the partners after testing and optimization in WP 2, 3 and 4:

Protocols provided by the project partners:

Protocols of diagnostic methods to *F. solani, M. phaeolina, F. oxysporum f.sp. fragariae*; *Provided by Spain*:

DNA Extraction from pure culture

Total DNA from 10 mg of mycelia and conidia of each fungal isolate was extracted using the DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration from each isolate was then measured using a Nanodrop 2000 (Thermo Scientific). DNA concentration varied depending on isolate between 3-300 ng / µl. DNA quality was checked by PCR with universal primers ITS4 and ITS5 (White et al 1990) that amplify the region ITS1- 5.8S -ITS2 rDNA. The amplification products are approximately 600 bp. DNA samples were stored at –20°C until required.

Table 21: Primers

Pathogen	Primers	Sequence (5'- 3')	Amplified size
Fusarium solani	TEF-Fs4f	ATCGGCCACGTCGACTCT	658 bp
	TEF-Fs4r	GGCGTCTGTTGATTGTTAGC	
***Macrophomina phaseoli*na**	MpKF1	CCGCCAGAGGACTATCAAAC	350 bp
	MpKR1	CGTCCGAAGCGAGGTGTATT	
F. oxysporum* f.sp. *fragariae	FOFRI-1F	GATGAAGAACGCAGCAAAATG	171 bp
	FOFRI-1r	AACGCGAATTAACGCGAG	

F. solani with some modifications (Arif *et al*. 2012)

All PCRs were performed in a reaction volume of 25 µL. The DNA was diluted a 100-fold prior to proceed with the conventional PCR.

Table 21: The PCR conditions

REACTIVES	Final Concentration	
Buffer 10x	1x (2.5 µl)	
dNTPs (10 Mm)	0,2 mM (0.5 µl)	24 µl mix y 1 µl DNA (1:100)
TEF-Fs4f (5 µM)	0,3 µM (1.5 µl)	
TEF-Fs4r (5 µM)	0,3 µM (1.5 µl)	
Taq- pol TAKARA (5U/µl)	2 U (0.25 µl)	
mili Q	Up to the final volume (13.75 µl)	

	Temprature	Time	
Pre-denaturation	94 ° C	2 min	
Denaturation	94 ºC	30 s	
Annealing	56 ºC	30 s	x 25 cycles
Elongation	72 ºC	2 min	
	72ºC	3 min	

Final elongation	

In all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination. The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium). A 1-kb Plus DNA Ladder (Invitrogen Life Technologies) was used as a molecular size marker. The PCR with the specific primer pair yielded a 658-bp DNA band. All PCR were repeated at least twice.

M. phaseolina with some modifications (Babu *et al*. 2007)

All PCRs were performed in a reaction volume of 20 µL. The DNA was not diluted prior to perform the PCR.

Table 22: The PCR conditions

Reactives	**Final Concentration**	
Buffer 10x with Cl_2MG	1x	
dNTPs (10 Mm)	0,2 mM	19 µl mix y 1 µl DNA (no dilution)
MpKF1 (5 µM)	0,3 µM	
MpKR1(5 µM)	0,3 µM	
Taq- pol TAKARA (5U/µl)	2,5 U	
mili Q	Up to the final volume	

	Temprature	**Time**	
Pre-denaturation	95 º C	2 min	
Denaturation	95 ºC	30 s	
Annealing	50 ºC	1 min	x 25 cycles
Elongation	72 ºC	2 min	
Final elongation	72ºC	3 min	

In all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination.

The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium). A 1-kb Plus DNA Ladder (Invitrogen Life Technologies) was used as a molecular size marker. The PCR with the specific primer pair yielded a 350-bp DNA band. All PCR were repeated at least twice.

F. oxysporum f.sp. *fragariae* by Larena *et al*.

All PCRs were performed in a reaction volume of 25 µL. The DNA was diluted a 1000-fold prior to conventional PCR.

Table 23: The PCR conditions

Reactives	**Final Concentration**	
Buffer 10x minus Cl_2MG BIOTOOLS	1x	
dNTPs (10 Mm)	0,1 mM	
Cl_2MG (50 mM)	2 mM	
FOFRI-1F (10 µM)	0,25 µM	20 µl mix y 5 µl DNA
FOFRI-1R (10 µM)	0,25 µM	
Taq- pol BIOTOOLS (5U/µl)	2,5 U	
mili Q	Up to the final volume	

	Temprature	**Time**	
Pre-denaturation	95 ° C	3 min	
Denaturation	95 ºC	1 min 30 s	
Annealing	60 ºC	1 min	x 30 cycles
Elongation	72 ºC	2 min	
Final elongation	72ºC	10 min	

In all experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination. The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium). A 1-kb Plus DNA Ladder (Invitrogen Life Technologies) was used as a molecular size marker. The PCR with the specific primer pair yielded a 171-bp DNA band. All PCR were repeated at least twice.

Protocols for detection of Phytophthora spp. and Verticillium spp. in strawberry; *provided by AGES, Austria:*

Phytophthora cactorum

Detection of *Phytophthora cactorum* in symptomatic/ asymptomatic samples of *Strawberry* by *conventional PCR.*

Reagent, Solutions, Control organisms:

Standard- and reference substances

- Positiv control:
- Optional-internal control

Table 24: Chemicals / Reagent

Product	Purity	Trademark for instance	Storage	Shelf life
FIREPol® 5 x Master Mix (7.5 mM MgCl2)		Solis BioDyne	-18°C	According to manufacturer

INSTRUCTION

Sample preparation

Step by step description of sample preparation-DNA extraction assay or reference to another SOP.

PCR

Table 25: Primer sequences

Name of forward and reverse primers		Primer sequence	
PC1	5´-	GAAACGGGTGTTGATATCGGAC	3‘
PC2	5´-	GTTTCGGGTGCTGCCAAAAACT	3‘

Table 26: Preparation of (RT-PCR and PCR) master mixes

Reagent (plus concentration of stock solution)	Final concentration	µl per sample
Aqua bidest		8,6
FIREPol Master Mix [5x]	1x	3
PC1 [10µM]	0,47 µM	0,7
PC2 [10µM]	0,47 µM	0,7

Preparation of reaction mixtures (adding samples to be tested);

Mastermix: *13µl*
Template-DNA: *2µl*

Controls:
Positive control (pc): *2µl*
Aqua bidest (nc): *2µl*

Table 27: Temperature profile for amplification

PCR phase	cycle	temperature	time
initial denaturarion	1	95°C	3 min
denaturation		95°C	30 sec
annealing	35	61°C	30 sec
elongation		72°C	30 sec
Final elongation	1	72°C	5 min
	1	15°C	∞

Amplification product: 450 bp

Phytophthora fragariae

Detection of Phytophthora fragariaein symptomatic/ asymptomatic samples of Strawberry by conventional PCR

Reagent, Solutions, Control organisms:

Standard- and reference substances

Positiv control: z.B. CBS 209.46

Table 28: Chemicals / Reagent

Product	Purity	Trademark for instance	Storage	Shelf life
FIREPol® 5 x Master Mix (7.5 mM MgCl2)		Solis BioDyne	-18°C	According to manufacturer

INSTRUCTION

Sample preparation

Step by step description of sample preparation-DNA extraction assay or reference to another SOP.

PCR

Table 29: Primer sequences

forward and reverse primers		**Primer sequence**	
TRP-PPF309a9F	5´-	CTACCTCCCTAAGCTTATCA	3‘
TRP-PPF309a9R	5´-	ACGCAGCATCATAGAAAAT	3‘

Table 30: Preparation of (RT-PCR and PCR) master mixes

Reagent (plus concentration of stock solution)	**Final concentration**	**µl per sample**
Aqua bidest		8,6
FIREPol Master Mix [5x]	1x	3
TRP-PPF309a9F [10µM]	0,47 µM	0,7
TRP-PPF309a9R [10µM]	0,47 µM	0,7

Preparation of reaction mixtures (adding samples to be tested);

Mastermix: *13µl*

Template-DNA: *2µl*

Controls:

Positive control: *2µl*

Aqua bidest: *2µl*

Table 31: Temperature profile for amplification

PCR phase	**cycle**	**temperature**	**time**
initial denaturarion	1	95°C	**3 min**
denaturation		95°C	**30 sec**
annealing	**35**	58°C	**30 sec**
elongation		72°C	**30 sec**

Final elongation	1	72°C	**5 min**
	1	15°C	∞

Amplification product: 403 bp

Verticillium albo-atrum

Detection of *Verticillium albo-atrum* in symptomatic/ asymptomatic samples of *Strawberry* by *conventional PCR*

Table 32: Chemicals / Reagent

Product	**Purity**	**Trademark for instance**	**Storage**	**Shelf life**
FIREPol® 5 x Master Mix (7.5 mM MgCl2)		Solis BioDyne	-18°C	According to manufacturer

Sample preparation

Step by step description of sample preparation-DNA extraction assay or reference to another SOP

Table 33: PCR primers

forward and reverse primers		**Primer sequence**	
Verticilium2albo	5´-	ATGGACCGAACAGCTAGGTA	3‘
Verticilium3albo	5´-	TCTCAGATATATGCTGCTGC	3‘

Table 34: Preparation of (RT-PCR and PCR) master mixes

Reagent (plus concentration of stock solution)	**Final concentration**	**µl per sample**
Aqua bidest		8,6
FIREPol Master Mix [5x]	1x	3
Verticilium2albo [10µM]	0,47 µM	0,7
Verticilium3albo [10µM]	0,47 µM	0,7

- Preparation of reaction mixtures (adding samples to be tested);

Mastermix: *13µl*
Template-DNA: *2µl*
Controls:
Positive control: *2µl*
Aqua bidest: *2µl*

Table 35: Temperature profile for amplification

PCR phase	Cycle	Temperature	Time
Initial denaturarion	1	95°C	3 min
Denaturation		95°C	30 sec
Annealing	35	54°C	30 sec
Elongation		72°C	30 sec
Final elongation	1	72°C	5 min
	1	15°C	∞

Amplification product: 300 bp

Verticillium dahliae

Detection of *Verticillium dahlia* in symptomatic/ asymptomatic samples of *strawberry* by *conventional PCR.*

Standard- and reference substances

Table 36: Chemicals / Reagent

Product	Purity	Trademark for instance	Storage	Shelf life
FIREPol® 5 x Master Mix (7.5 mM MgCl2)		Solis BioDyne	-18°C	According to manufacturer

Sample preparation

Step by step description of sample preparation-DNA extraction assay or reference to another SOP

Table 37: PCR primers

Forward and reverse primers		**Primer sequence**	
Verticilium19-dahliae	5´-	CGGTGACATAATACTGAGAG	3‘
Verticilium22-dahliae	5´-	GACGATGCGGATTGAACGAA	3‘

Table 37: Preparation of (RT-PCR and PCR) master mixes

Reagent (plus concentration of stock solution)	Final concentration	µl per sample
Aqua bidest		8,6
FIREPol Master Mix [5x]	1x	3
Verticilium19-dahliae [10µM]	0,47 µM	0,7
Verticilium22-dahliae [10µM]	0,47 µM	0,7

Preparation of reaction mixtures (adding samples to be tested);

Mastermix: *13µl*

Template-DNA: *2µl*

Controls:

Positive control: *2µl*

Aqua bidest: *2µl*

Table 38: Temperature profile for amplification

PCR phase	Cycle	Temperature	Time
Initial denaturarion	1	95°C	3 min
Denaturation		95°C	30 sec
Annealing	35	54°C	30 sec
Elongation		72°C	30 sec
Final elongation	1	72°C	5 min
	1	15°C	∞

Amplification product: 580 bp

Protocols for detection of *Botrytis cinerea* in strawberry; provided by LRCAF, Lithuania:

Botrytis cinerea

Fungal isolates

Botrytis spp. was collected from strawberry plants in different regions of Lithuania. All isolates were purified by single spore: isolates were grown on Potato dextrose agar (PDA) at 22±2°C. The incubation time varied from 7 to 20 days, until the fungi colonized the surface of the medium. After purification all isolates were stored on PDA slopes at 4°C.

DNA extraction

Botrytis spp. isolates for DNA extraction were grown on Potato dextrose agar (PDA), at 22±2°C under alternate light (12h/12h). The incubation time varied from 7 to

14 days, until the fungi colonized the surface of the medium. Fungal genomic DNA was extracted from 200 mg of mycelium material collected from Petri dish with spatula. Mycelia were grounded in liquid nitrogen using a mortar and pestle. DNA was extracted according to Genomic DNA Purification Kit (Fermentas, Lithuania) (Genomic DNA Purification Kit). Samples were incubated in Grant Bio PHMT Thermoshaker (Grant). DNA were dissolved in 100 µl of distilled water and stored at -20°C. DNA concentration measured with Eppendorf photometer (Eppendorf, Germany) and NanoDrop 1000 spectometer (ThermoScientific).

Genomic DNA Purification Kit protocol:

1. Prepare precipitation solution by mixing 720 µl of sterile deionized water with 80 µl of supplied 10X concentrated Precipitation Solution.
2. Mix 200 µl of sample with 200 µl of TE buffer. Add 400 µl of lysis solution and incubate at 65°C for 5 min. Then the sample is incubated at 65°C for 10 min with occasional inverting of the tube.
3. Immediately add 600-620 µl of chloroform, gently emulsify by inversion (3-5 times) and centrifuge the sample at 10,000 rpm for 2 min.
4. Transfer the upper aqueous phase containing DNA to a new tube and add 800 µl of freshly prepared precipitation solution (1 step.), mix gently by several inversions at room temperature for 1-2 min and centrifuge at 10,000 rpm (~9400 x g) for 2 min.
5. Remove supernatant completely (do not dry) and dissolve DNA pellet in 100 µl of NaCl solution by gentle vortexing. Make sure that the pellet is completely dissolved.
6. Add 300 µl of cold ethanol, let the DNA precipitate (10 min at -20°C, up to 20 hours) and spin down (10,000 rpm (~9400 x g), 3-4 min). Remove the ethanol. Wash the pellet once with 70% cold ethanol and dissolve DNA in 100 µl of sterile deionized water by gentle vortexing.

PCR amplification

PCR amplification was performed in a 25µl reaction volume containing 1 µl of DNA, 12.5 µl PCR master mix 2x (Fermentas, Lithuania), 9.5 µl DNase/Rnase-free Water, 1µl of each primer (Fermentas, Lithuania). In one reactions were used primers sequences 5'- AGCTCGAGAGAGATCTCTGA-3' (C729+) and 5'- CTGCAATGTTCTGCGTGGAA- 3' (C729-) (Rigotti et al., 2002; Khazaeli et al., 2010),

and in other reactions new primers (Bc108+, 5'-ACCCGCACCTAATTCGTCAAC-3'; Bc563–, 5'-GGGTCTTCGATACGGGAGAA-3') (Rigotti et al., 2006). PCR reactions were performed in a Mastercycler (Eppendorf, Germany). The program applied for amplification was as: 1 cycle of 2 min at 94 ^{o}C, 35 cycles of 45 s at 94 ^{o}C, 50 s at 50 ^{o}C, 50 s at 72 ^{o}C; 1 cycle of final extension for 5 min at 72 ^{o}C. The PCR product was separated by electrophoresis on a 1.5 percent agarose gel in 1x TAE buffer and visualized by staining with RedSafe Nucleic Acid Staining Solution (20,000x) (iNtRON Biotechnology). The C729 +/– primers (Rigotti et al., 2002), amplifies a DNA fragment of 0.73 kb and new primer Bc563 –, Bc108+ - 0.48 kb and 0.36 kb. Size marker used GeneRuler DNA Ladder mix (Fermentas).

Table 39: PCR master mix

PCR reaction	**1x**
DNase/Rnase-free Water	9.5 µl
PCR Master Mix (2x)	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
DNR	1 µl
Total volume	**25 µl**

RESULTS

From the 6 laboratories participating in the ringtest, 5 provided results in the distributed results sheet. The expected results from the samples sent out were:

LAB Code:					insert + or -						
	Thermocycler:				Polym. Mix:						
	PCRs:										Realtime
Samples:		Ph.cact.	Phyt fr.	Vert- dahl.	Vert. Albo-atr.	Fus. solani	Fus. oxy f sp. frag	Macrophomina	Botryt C729	Botry Bc108/563	Phyt. fr.
E1		-	-	-	-						
E2		-	-	-	-						
E3		-	+								
E5		-	+								
E10		+	-								
E11		+	-								
E12		+	-								
E15		+	-								
E16		-	+								
E23		-	+								
E29				+	-						
E30				+	-						
E31				+	-						
E32		+	-								
E33		+	-								
E45		-	-								
E46				-	+						
E47				-	+						
Judia						+					
B42(89)						+					
Tor1						+					
Tor11						+					
•6.14						+					
For 4							+				
25.19.1.2							+				
25.6.2.2							+				
71.1							+				
•53.10							+				
MAC4 MC1								+			
M2 MONO 3								+			
MAC 8 MC1								+			
M15								+			
•M 22								+			
1										+	
23										+	
26										+	
27										+	
28										+	
29										+	
35										+	
BCx										-	
Changes from the protocol											

Fig. 26

The results sent out by the partner laboratories were gathered and only analysed if any of the expected results was reported (true positive and true negative)

Calculated results per assay (percentage of correctly detected results):

Assay Nr. 1 (*Phytophthora cactorum*)

Only the results from 2 labs could be considered for evaluation this method. The results from the remaining labs were not reliable (not one sample was detected positive).

	Lab6	Lab2	Lab1	Lab4	Lab3
E1	-	-	-	-	-
E2	-	-	-	-	-
E3	-	+	-	-	-
E5	-	-	-	-	-
E10	+	+	-	-	-
E11	+	+	-	-	-
E12	+	+	-	-	-
E15	+	+	-	-	-
E16	-	-	-	-	-
E23	-	-	-	-	-
E29					
E30					
E31					
E32	+	+	-	-	-
E33	+	+	-	-	-

Fig. 27

The percentage of correctly detected results was 95.84% when considering only 2 labs. Including all results (5 labs) the correct average percentage was 63.08% for this method.

Assay Nr. 2 (*Phytophthora fragariae*)

Results from 4 labs were taken into account for the evaluation of this assay.

	Lab6	Lab2	Lab1	Lab4	Lab3
E1	-	+	-	-	-
E2	-	-	-	-	-
E3	+	-	-	-	-
E5	+	+	-	+	+
E10	-	-	-	-	-
E11	-	-	-	-	-
E12	-	-	-	-	-
E15	-	-	-	-	-
E16	+	-	-	-	+
E23	+	+	-	+	+
E29					
E30					
E31					
E32	-	nt	-	-	-
E33	-	nt	-	-	-

Fig. 28

The percentage of correctly detected results was 87.5% when considering 4 labs. Including all results (5 labs) the correct average percentage was 83.33% for this method.

Assay Nr. 3 (*Verticillium dahliae*)

Results from 4 labs were taken into account for the evaluation of this assay.

	Lab6	Lab2	Lab1	Lab4	Lab3
E1	-	-	-	-	-
E2	-	-	-	-	-
E3					
E5					
E10					
E11					
E12					
E15					
E16					
E23					
E29	+	+	-	-	+
E30	+	+	-	-	+
E31	+	+	-	+	+
E32					
E33					
E45		-			-
E46	-	-	-	-	-
E47	-	-	-	-	-

Fig. 29

The percentage of correctly detected results was 94.29% when considering 4 labs. Including all results (5 labs) the correct average percentage was 85.71% for this method.

Assay Nr. 4 (*Verticillium albo-atrum*)

Results from 3 labs were taken into account for the evaluation of this assay.

	Lab6	Lab2	Lab1	Lab4	Lab3
E1	-	-	-	-	-
E2	-	-	-	+	-
E3					
E5					
E10					
E11					
E12					
E15					
E16					
E23					
E29	-	-	-	+	-
E30	-	-	-	-	-
E31	-	-	-	+	-
E32					
E33					
E45	+	+			-
E46	+	+	-	-	-
E47	+	+	-	-	-

Fig. 30

The percentage of correctly detected results was 78.75% when considering 3 labs. Including all results (5 labs) the correct average percentage was 73.25% for this method.

Assay Nr. 5 (*Fusarium solani*)

Results from 3 labs were taken into account for the evaluation of this assay.

	Lab6	Lab2	Lab1	Lab4	Lab3
Judia	+	+	+	-	-
B42(89)	+	+	+	-	-
Tor1	+	+	+	-	-
Tor11	-	+	+	-	-
•6.14	+	+	+	-	-

Fig. 31

The percentage of correctly detected results was 93.20% when considering 3 labs. Including all results (5 labs) the correct average percentage was 56.00% for this method.

Assay Nr. 6 (*Fusarium oxysporum f.sp. fragariae*)

Results from all labs could be taken into account for the evaluation of this assay.

	Lab6	Lab2	Lab1	Lab4	Lab3
For 4	+	+	+	+	+
25.19.1.2	-	-	+	-	-
25.6.2.2	-	-	+	-	-
71.1	-	+	+	+	+
•53.10	+	+	+	+	+

Fig. 32

Including all labs the correctly detected average results was 64.00% for this method.

Assay Nr. 7 (*Macrophomina phaeolina*)

Only the results from 4 labs could be considered for evaluation this method. The results from the remaining lab were not reliable (not one sample was detected positive) and therefore excluded from calculation.

	Lab6	Lab2	Lab1	Lab4	Lab3
MAC4 MC1	-	+	+	+	-
M2 MONO 3	+	+	+	+	-
MAC 8 MC1	+	+	+	+	-
M15	+	+	+	+	-
•M 22	+	+	+	+	-

Fig. 33

The percentage of correctly detected results was 96% when considering 4 labs. Including all results (5 labs) the correct average percentage was 76% for this method.

Assay Nr. 8 (*Botrytis cinerea*)

Only 3 labs tested the samples for *Botrytis cinerea* in the ring test. In one lab some results were inconclusive, but included in the calculation as a wrong result.

	Lab6	Lab2	Lab1	Lab4	Lab3
1	+	-	nt	?	nt
23	+	-	nt	+	nt
26	+	-	nt	+	nt
27	+	+	nt	?	nt
28	+	+	nt	-	nt
29	+	-	nt	+	nt
35	+	+	nt	+	nt
BCx	-	-	nt	-	nt

Fig. 34

The percentage of correctly detected results was 74.63% when considering the results of the 3 labs performing the test.

Detailed result calculation per assay (according to Hughes *et al.* 2006):

		Assays							
Target species	Criteria	Ph cact.	Ph fr.	V. dahl.	V. al-at.	Fus. sol.	F. ox. fr.	M. ph.	Bot. cin.
Strawberry diseases	Number of PA	12	11	10	6	14	16	19	15
	Number of NA	29	28	20	22	3	5	4	6
	Number of ND	15	5	1	7	1	9	1	5
	Number of PD	1	1	0	3	0	0	0	0
	Sensitivity	44,4	68,8	90,9	46,2	93,3	64,0	95,0	75,0
	Specificity	96,7	96,6	100,0	88,0	100,0	100,0	100,0	100,0
	Accuracy	71,9	86,7	96,8	73,7	94,4	70,0	95,8	80,8
Labs included in calculation		5	4	4	5	3	5	4	3

Fig. 35

		Standard test		
		+	−	Total
New test	+	69 (PA)	3 (PD)	72
	−	6 (ND)	12 (NA)	18
	Total	75	15	90

Table 3 is adapted from Hughes *et al.*, 2006; Numbers in this table are for demonstration purposes.
PA, positive agreement; PD, positive deviation; ND, negative deviation; NA, negative agreement.
Positive (+) and negative (−) results for 90 samples tested using both tests, illustrating diagnostic sensitivity [PA/(PA+ND)], diagnostic specificity (NA/(NA+PD)), and relative accuracy (PA+NA)/(PA+PD+ND+NA). Diagnostic sensitivity = 92%, Diagnostic specificity = 80%; Relative accuracy = 90%|

Fig. 36

CONCLUSION

Protocols for the detection of seven fungal pathogens (*Phytophthora fragariae, Ph. cactorum, Verticillium dahliae, V. albo-atrum, M. phaseolina, Fusarium oxysporum f. sp. fragariae, F. solani and Botrytis cinerea*) were tested among participating laboratories. There was a pronounced variation in the percentage of correctly detected samples (56-96%) among the participating labs and between the assays tested. False negative results could be attributed to a reduced sensitivity due to processes of lyophilization or vacuum concentration of primers and/or extracted DNA from samples, which were decided on to simplify transportation of the material tested. Contamination during the rehydration of samples and/or primers or handling with the PCR mix may have led to false positive results. However, these assumptions would have to be examined in more detail. Under certain conditions, using freshly extracted DNA and primers, all tested assays should be suitable to detect the selected diseases directly from diseased strawberry plants. This could be shown in WP 4 when methods were optimized in the different labs, respectively. However, the ring test pointed out that for implementation of these molecular methods in different labs some optimization processes may be necessary under the different conditions to obtain an assay which gives satisfactory results in the hands of each lab personal and equipment. The data generated in this ring test can be used for validation purposes. Details on the performance criteria (diagnostic sensitivity, diagnostic specificity and relative accuracy) of each assay in the ring test are pointed out in the chapter “Detailed result calculation per assay (according to Hughes et al. 2006)”.

Output

Optimized PCR and qPCR protocols for effective detection of pathogens: *Phytophthora fragariae, Ph. cactorum, Verticillium dahliae, V. albo-atrum, Macrophomina phaseolina, Fusarium oxysporum f. sp. fragariae, F. solani and Botrytis cinerea* have passed first validation.

Degree of achievement: 100% (common protocol for EPPO is pending)

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SPAT dissemination activities

Table 40: Publications

Authors	Year	Title	Journal
Mirmajlessi SM, Destefanis M, Gottsberger RA, Mänd M and Loit E.	2015	PCR-based specific techniques used for detecting the most important pathogens on strawberry: a systematic review.	Systematic Reviews, 4:9.
Rugienius R., Šikšnianienė J. B., Rasiukevičiūtė N.	2015	Assessment and testing of strawberry pathogens.	Agrarian and forestry science: Recent research results and innovative solutions Nr. 5. Book of scientific conference abstracts. LRACF, 104-105.
Persen U., Gottsberger R.A., Fickert W., Altenburger J., Blümel S.	2014	SPAT-Projekt zu Erdbeerkrankheiten in Europa	Besseres Obst, 12, 4
Persen U., Gottsberger R.A.	2015	Wissenschaftliche Methoden ermöglichen das Erkennen und Nachweisen von Krankheiten zur Unterstützung erfolgreicherer Kulturpraxis	Besseres Obst, 3

Table 41: Conference and seminar presentations

Title	Authors	Conference	Location	Year	Oral/Poster
A systematic review on PCR-based specific methods to detect the most important strawberry pathogens.	Mirmajlessi et al	International Plant Protection Congress	Berlin, Germany.	2015	Oral/Poster
General principles of real-time PCR: A technology for quantitative detection of phytopathogens.	Mirmajlessi et al	International Conference on Environmental Science and Development	Amsterdam, Netherlands.	2015	Oral
Development of quantitative PCR techniques for plant pathogens diagnostic research.	Mirmajlessi et al	European Foundation for Plant Pathology Conference	Cracow, Poland.	2014	Poster
Assessment and testing of strawberry pathogens.	Rugienius R., Šikšnianienė J. B., Rasiukevičiūtė N.	Lithuanian Research Centre for Agriculture and Forestry scientific conference “Agrarian and forestry science: Recent research results and innovative solutions.	30 January, Babtai, Kaunas district	2015	Oral
Development of a method to detect the major emerging pathogens of strawberry plants in Spain	Larena I., Herranz Y., Morales, M.T., De Cal A., Melgarejo P.,	XVII Congress of the Spanish Society of Phytopathology	Lérida (Spain)	2014	Poster

PCR-based techniques used for detecting the pathogens on strawberry.	Mirmajlessi	Estonia University of Life Sciences	Tartu, Estonia	2015	Seminar
Distribution and diagnosis of strawberry pathogens	Persen U., Gottsberger R.A.	Austrian Strawberry Symposium	Graz, Austria	2014	oral
Distribution and diagnosis of strawberry pathogens	Persen U., Gottsberger R.A.	Seminar for strawberry producers	Leonding, Austria	2015	oral
Distribution, assessment and diagnosis of strawberry pathogens	Persen et al	55. Austrian Plant Protection Congress	Seggau, Austria	2014	oral

Table 42: Other activites

Authors	Year	Title	Activity/publication
Anu Riisalu	2015	Prevention and detection of strawberry pathogens	BSc thesis
Neringa Rasiukevičiūtė	2016	Peliminary title: Botrytis spp. genetic and phenotypic diversity, diseases forecasting and control in strawberries and onion.	PhD thesis
Inmaculada Larena	2014	Proyecto SPAT: Strawberry Pathogens Assessment and Testing	Monitoring workshop on project Euphresco

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APPENDIX I

Questionnaire sent to producers

Please fill in or tick where applicable

PART 1. General information

About you

Name:_____

Address:_____

Are you a: ☐ Grower
☐ Nursery producer
☐ Farm advisor
☐ Other _____

Have you availed of the following:

☐ Formal training in strawberry production ☐ Extension services ☐ Information sheets

☐ Producer groups ☐ On-line guides to strawberry diseases

PART 2. Your Production System

Please indicate the production system (s) that best represents that which you work with:

Production System	Yes/No	Area (Ha)	Varieties	Previous Crop (if any)
Standard with straw				
Standard without straw				

Ridges, mulch film/ fleece				
Coverage with plastic foil				
Coverage with plastic foil and fleece				
Greenhouse or plastic tunnel				

Please indicate what you consider to be the main outlet for your produce:

☐ Wholesale ☐ Market stand ☐ Fresh farm produce ☐ Pick-your-own ☐ Processing

☐ Other (please specify) _____

PART 3. Diseases affecting strawberry production

Which are the three most important strawberry diseases, in order, from your point of view, in 2013 and during the last 5 years ?

No.	In 2013	From 2008-2013
1		
2		
3		

Please estimate your personal knowledge about strawberry diseases (especially recognition of symptoms)

☐ High- I consider myself an expert on strawberry diseases and have extensive experience in this area

☐ Medium-I have some experience with the main strawberry diseases

☐ Low- I have little experience with strawberry diseases and would not be able to identify them in the field

If you feel comfortable to answer the following questions, please fill in the table below

Have you observed any of the following strawberry diseases in 2013?

Diseases (causative agent)	Plants affected %	Intensity of damage 1 – 4 *)	Estmated yield loss %	Affected cultivars	Please specify plant protection measures		When planted ? (year / month) origin of plants	Year first noted
					Indirect **	**Direct *****		
Root and crown rot *(Phytophthora cactorum)*								
Red stele/ red core root rot *(Phytophthora fragariae)*								
Verticillium-wilt *Verticillium dahliae*								

Anthracnose *(Colletotrichum acutatum)*								
Grey mold (Botrytis cinerea)								
Powdery mildew *(Sphaerotheca macularis)*								
Leaf spots								
Viruses (please name)								
Gnomonia								
Others								

* Percentage plant tissue affected: 1=0-5 %, 2 =5-25 %, 3= 25-50 %, 4= > 50%
**Indirect measures include: mowing, irrigation, roofing, raised beds etc
***Please give name of plant protection product

Comments and notes

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THANK YOU FOR YOUR CO-OPERATION