EUPHRESCO Final Report (NC)

for Non-Competitive research projects

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Please send the final report to all your project partners, to the NC topic coordinators and to the EUPHRESCO Secretariat (euphresco@fera.gsi.gov.uk).

Project Title and Acronym
Evaluation of factors determining distribution, impact, detection and characterization of fruit tree phytoplasmoses (APOPHYT)

Project Duration:

Start date:	28/09/12
End date:	27/09/14

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2. Executive Summary

Evaluation of factors determining distribution, impact, detection and characterization of fruit tree phytoplasmoses (APOPHYT)

Main objectives

Apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) are caused by '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri' and '*Ca*. P. prunorum', respectively. These are the main phytoplasma diseases on pome and stone fruit that cause severe diseases on commercial crops.
The main goal of APOPHYT project is to improve the understanding of the multitrophic interactions between plants – vectors – phytoplasmas in order to develop disease management strategies and finally to reduce the impact of phytoplasma diseases in Europe.
The different work packages aim to deeper analyse parts of the multitrophic interactions:

1. **Phytoplasma**

Is it possible to improve diagnostics and to develop more sensitive detection methods (WP5) ?
Are phytoplasma present as latent infections in nuclear plantings and nurseries (WP2) ?
How are phytoplasma distributed in Europe (WP2) ?
What are the population dynamics and the diversity of AP strains within apple trees (WP3) ?
Is it possible to evidence virulence differences of AP strains (WP3) ?

2. **Host plant and plant – phytoplasma interactions**

What is influencing the disease development at different locations in Europe (WP4) ?
How different climatic conditions influence symptom expressions (WP4) ?
Can symptom expressions be linked with phytoplasma titre (WP4) ?

3. **Vector and vector – host plant interactions**

Can all vectors be found at all location in Europe (WP6) ?
What is the percentage of vectors infested with phytoplasma (WP6) ?
What is the influence of host plants on vector behaviour (WP6) ?

Principal methods used in the 5 operative WPs

WP2 Screening nuclear plantings and nurseries for latent infections and distribution of AP, PD and ESFY in the EC.
Root or leaf rips samples from apple, pear and *Prunus* species were collected in autumn for DNA extraction. Detection was carried out by standard PCR procedures. Phenotypic data was collected at the time of sampling.

WP3 Molecular classification of virulence of the infecting AP phytoplasma.
DNA (root and shoot) from AP-diseased plants was extracted and *hflB* gene fragments amplified by specific primers. Polymorphic PCR fragments were cloned, sequenced and compared by cluster analysis. Spatial and temporal diversities of the strains within plants were monitored using multiplex real time PCR and were related to virulence.

WP4 Monitoring disease development of uniformly inoculated trees under various climatic conditions and insect-proof containment.
Apple rootstocks M9, Kirchensaller pear seedlings and stone fruit rootstocks INRA II were inoculated with infected scions by grafting at JKI-OW. Inoculated plant material was sent to the partners and kept under insect-proof conditions. Symptom appearance and disease progression were monitored for two years and phytoplasma titre was assessed by real-time PCR. In parallel, climatic factors (temperature, humidity, precipitation, solar radiation) were recorded.

WP5 Improving detection of fruit tree phytoplasma by more efficient and more sensitive diagnostic procedures (LAMP).
Gene database was screened to define target genes for LAMP primer selection. Initial target was a gene shared by all three fruit tree phytoplasma, followed by selection of species-specific primers. Different detection chemicals and DNA extraction procedures were tested for performance. Finally LAMP procedure was validated with field plant material.

WP6 Monitoring of insect vectors and assessment of the vector host plant interactions.
The density of psyllids in nurseries and orchards was monitored by sweeping techniques and species were identified. The presence of phytoplasma in the insects was performed by PCR. Behavioural responses of ESFY psyllids to apricot volatile emissions was evaluated using a 6-arm olfactometer. Discriminant attractive or repellent volatile compounds were identified and characterized.

Results

WP2 Visual inspection for phytoplasma symptoms was negative for all controlled plants in nuclear plantings and nurseries. However, phytoplasma contaminations were demonstrated. These latent infections represent a potential risk factor for the long distance dispersal of fruit phytoplasmas. Infection level was low for apples and apricots, but very high in some countries for pears. In this view, a harmonization of the control measures at EU level is desirable for a more effective control of the diseases spread by trade activities (e.g. phytosanitary passport inspection and/or certification schemes).
WP3 All apple trees examined harbour a mixture of AP strains. In all trees examined strains differ between roots and shoots, at least temporarily and they also vary over the year and between years. AP strains exhibit genetic differences which seem to be correlated with virulence. However, The importance of the plants' physiological condition, remain unknown in the disease development.
WP4 Visual assessment of symptoms is not a reliable indicator for phytoplasma infection. Symptom expression seems to be more related to the virulence of the phytoplasma strains rather than to the climatic conditions. Phytoplasma detection should be conducted in winter time and using root materials and not midribs. An observation period up to 10 growing seasons is recommended for future works in order to correlate symptoms expression with the phytoplasma titre and local climatic conditions.
WP5 A LAMP PCR method (loop-mediated isothermal amplification of DNA) for the detection of AP, PD and ESFY has been developed. The method is reliable, fast and sensitive and has been validated on-site. Information for inspectors, extended ring tests and training courses should be organized in a future project in order to implement the technique.
WP6 Plum, pear or apple *Cacopsylla* sp. are widely present in fruit growing regions of the participating countries. The level of infested psyllids, especially in Norway and Czech Republic, reveals the importance of disease spreading by vectors. On another hand, apricot cultivars and their related volatile compound influenced the psyllid preferences. Therefore, in order to decrease phytoplasma diseases dissemination orchard protection against psyllids should be optimized in an effective and sustainable way.

Conclusions

- **Phytoplasma are present as latent infections in certified propagation stock for scions and nurseries in Europe.**
- **Visual assessment did not permit to detect symptoms in infected plants and is not suitable for an effective control of sanitary status of certified propagation stock for scions and nurseries.**
- **This status should be determined by molecular methods, however, the tissue sampling should be conducted in winter time and using root materials and not midribs.**
- **A LAMP PCR method that is reliable, fast and sensitive and can be used on-site, for the detection of AP, PD and ESFY has been developed.**
- **A mixture of apple proliferation strains has been found in apples tress.**
- **AP strains exhibit genetic differences which seem to be correlated with virulence of the strain.**
- **Symptom expression seems to be more related to the virulence of the phytoplasma strains rather than to the climatic conditions.**
- **Furthermore, phytoplasma titre is varying a lot in the different organs of the plants and over the season.**
- **Phytoplasma insect vectors can be found at all locations in Europe.**
- **The percentage of vectors infested with phytoplasma is very high in some countries and demonstrates the importance of controlling vector insects to avoid disease spread.**
- **Plant genotypes and their related volatile compound influence the vector behaviour that could be used to control psyllids by developing mass trapping.**

3. Report

Contents

1. Main goal of APOPHYT project

Apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) are the main phytoplasma diseases on pome and stone fruit that cause severe diseases on commercial crops. The main goal of APOPHYT project is to improve the understanding of the multitrophic interactions between plants – vectors – phytoplasmas in order to develop disease management strategies and finally to reduce the impact of phytoplasma diseases in Europe.
The different work packages and tasks aim to deeper analyse parts of the multitrophic interactions:

2. WP2 Screening nuclear plantings and nurseries for latent infections and distribution of AP, PD and ESFY in the EC (lead Italy)

2.1. Background

The presence of fruit tree phytoplasmas (AP, PD, and ESFY) in pome and stone fruits is often unnoticed when unfavourable conditions for symptom development prevail and trading of plants with latent infections is likely. In order to assess the level of latent infection and the risk of propagating infected material, an extensive screening of nuclear stocks, certified propagation stocks and nurseries of pome (apple and pear) and stone (peach, apricot, plum) fruits was performed over two years at several locations in Italy, Germany, Austria, Switzerland, Czech Republic and Norway. In Norway also commercial orchards were surveyed.

Table 1 *– WP2-Objectives*

Tasks	Status – fully completed
T1 Assessment of the level of latent infection in propagation stocks and nurseries	**YES**
T2 Assessment of the transmission of AP, PD and ESFY by interconnected root system in nurseries	**NO**, trials not conducted after common agreement between partners at the kick-off meeting

2.2. Methods

The screening was performed by visual observation of plants for the recording of specific symptoms and by PCR procedures (direct PCR, nested PCR, real time PCR). Shoot, leaf rips and/or root samples were collected and submitted to extraction of total DNA to be used as target in molecular assays.

2.3. Results and discussion

All results are summarized in **Table 2**.

In Switzerland, six certified propagation stocks and nurseries were controlled and a total of 1417 plants analysed in 2012 and 2013. Only 3 trees at a certified propagation stock for scions in North West of Switzerland were firstly recorded to be infected by *'Ca.* P. prunorum'. Regarding the propagation of healthy materials, all apricot trees planted at Agroscope research station in Conthey were controlled. In 2013, 0.5% of the 635 plants turned out to be positive to *'Ca.* P. prunorum' and 3.37% of the 1060 plants in 2014. These contaminated plants came from one nursery in the South West of Switzerland (Canton of Valais). In addition, a total of 122 5-years old apricot trees located at Agroscope research station in Conthey were screened and 29.5% revealed to be positive to the ESFY phytoplasma. No visible symptoms were recorded for the majority of the plants coming from the nurseries whereas the 5-years old trees showed essentially chlorosis and rolling of leaves.

In Austria, several nurseries were investigated in collaboration with the regional NPPOs. A total of 315 bud wood samples of pome (231) and stone (84) fruits originated from Austria as well as from other EC member states were taken in springtime and in summer. A low infection rate was recorded for *'Ca.* P. prunorum' (1%, positive samples originated from Austria) and '*Ca.* P. mali' (4%, positive samples originated from other EC member states) whereas 33% (4% of the positive samples originated from Austria, 29% from other EC member states) of samples resulted to be latent infected by '*Ca.* P. pyri'. Further investigation in two Austrian mother stock plantations (apricots and pears) revealed rates of infection of over 10%. In the pear orchard also pear psyllids were captured and analysed and 3% were infected by *'Ca.* P. pyri'.

In Germany, a total of 3000 1-year-old apple, 500 1-year-old pear and 79 stone fruit trees of various age and varieties were screened in December 2012. The screening was done during the process of plant relocation to the budwood production site in Weinsberg. The apple and pear plants were located in Southern Germany close to Whyl (nursery Ganther) and Renschen (nursery Kimmig), whereas the stone fruit samples were collected in the budwood production site Weinsberg. Phytoplasmas were found only in three pear trees originating from nursery Kimmig whereas none of the apple and stone fruit samples were found positive. None of the plants showed typical phytoplasma symptoms. In October 2013 shoot samples from 102 apple plants, 104 pear plants and 103 stone fruit plants were collected in the budwood production site Weinsberg. The apple and pear samples were amongst those plants tested one year earlier. None of the plants tested positive for phytoplasmas and no phytoplasma symptoms were observed. Finally, in October 2014 shoot samples from 100 apple-, pear- and stone fruit (6 to 10 years old apricot and peach) trees were collected in the budwood production site Weinsberg. None of the visually inspected trees showed phytoplasma-specific symptoms and all plants tested phytoplasma negative.

In Italy, a screening of stone fruit propagation material was carried out in 5 nurseries of Latium region (central Italy). In each surveyed nursery a representative number of

plants per plot were sampled. A total of 60 leaf samples were collected from 1-2 year old asymptomatic peach and plum trees during September. None of the tested samples were phytoplasma-positive using molecular tests. No similar data are available for pear and apple plants since these crops are of limited interest in Latium region and the local production of propagation material is marginal.

In Norway, an outbreak of AP is ongoing since September 2010. Despite intense surveillance and removal of infected trees since 2010, the disease is still occurring in all important fruit growing areas in Norway (Buskerud, Hordaland, Sogn og Fjordane, and Telemark). In the ongoing national survey program, more than 1700 samples were tested in 2013 of which 235 were infected. Even though there were few direct findings in nurseries, as much as 77 % of orchards close to nurseries (within 500 m) had AP infected trees. AP phytoplasma was detected in 41 different cultivars. After intense testing and eradication of infected propagation stocks in 2012, only one infected sample was detected in 2013. In this year's season (2014), more than 600 samples have been collected, and they are currently being tested.

In Czech Republic, monitoring in fruit trees mother plants and nurseries was performed in different areas during 2012 - 2014. Plants from: 6 apple mother stocks (for production of certified propagation material), 1 apple nursery, 3 pear mother stocks, 1 pear nursery, 3 apricot mother stocks and 1 apricot nursery were tested. Occurrence of latent infection by AP and ESFY phytoplasmas in fruit tree propagation material was very low. Conversely, latent infections by PD phytoplasma in pear plantings of mother plants were found out at very high rate.

Table 2 *– Number of tested samples in 2012 - 2014 and results of molecular analyses performed by each involved Institution*

Country involved	N° of nurseries/ nuclear stocks monitored	N° of tested samples			Tested matrix	Diagnostic protocols	N° of infected samples		
		apple	pear	stone fruits			*'Ca.* P. mali'	*'Ca.* P. pyri'	*'Ca.* P. prunorum'
Austria		188	43	84	Leaves/ roots	Real time PCR	7 *(3.7 %)*	**14 *(32.5 %)***	1 *(1.2 %)*
Germany	4	3202	704	282	Leaves/ roots	Direct PCR	0	3 *(0.4 %)*	0
Switzer-land	- 6 nurseries - Agroscope	-	-	1417 1695	Leaves/ roots	Nested PCR	-	-	3[(a)] *(0.2 %)* 39 *(2.3 %)*
Italy	5	-	-	60	Leaves	Nested PCR	-	-	0
Norway	Commercial orchard and nurseries	3000 *(2012)* 1700 *(2013)*	-	-	Leaves/ roots	Real time PCR	**750[(b)] *(25.0 %)*** **235[(c)] *(13.8 %)***	-	-
Czech Republic	15	474	99	64	Leaves/ roots	Nested PCR	13 *(2.7 %)*	**47 *(47.5 %)***	**14 *(21.9 %)***
TOTAL		**8564**	**846**	**1907**			**1005 *(11.7 %)***	**64 *(7.6 %)***	**57 *(4.6%)***

(a) In one budwood certified production orchard
(b) Few incidences were detected in propagation stocks
(c) Only one infected sample was detected in nuclear stocks

2.4. Main conclusions

Table 3 *– WP2-Deliverables*

Deliverables	Status – fully provided
D1 Testing apple-, pear- and stone fruit plants in nuclear stocks and nurseries for latent infection by phytoplasma	YES

2.4.1. Summary

Visual inspection for phytoplasma symptoms was negative for all plants. The activities carried out within this WP showed that fruit trees in propagation stocks and nurseries can be contaminated by latent infection of phytoplasmas that are common in Europe. This represents a **potential risk factor for the long distance dispersal** of fruit tree phytoplasmas. Excluding the high infection percentages recorded in commercial orchards from Norway, latent infections by *'Ca.* P. mali' in nurseries and certified propagation stocks were generally low (0 - 3.7 %). Conversely, '*Ca*. P. pyri' was detected in a very high percentage both in Austria and Czech Republic. Regarding stone fruits, *'Ca.* P. prunorum' was widely spread only in nurseries and certified propagation stocks from Czech Republic (21.9 %) whereas in the other countries it was sporadically detected (0 - 2.3 %). Nevertheless, the occurrence of ESFY especially in certified propagation stock for scions, as found in Switzerland, suggests to keep a high attention even in the presence of low infection rates.

2.4.2. Benefits and technology transfer

- Dissemination suitable for scientific community: A **peer-reviewed article** about apple proliferation in Norway will be submitted.
- Dissemination suitable for PPOs (EU level) and for stakeholders (National level): A **leaflet** entitled *Best practices to control fruit phytoplasmas in nurseries and certified propagation stock plants* containing information about risks for nurseries, possible control strategies (insecticide, netting, rootstocks) will be available in 2015 for NPPO's and control agencies.

2.4.3. Impacts and implication for stakeholders and policy makers

The obtained results appear of particular importance for the phytosanitary policy providing useful data for a more accurate assessment of the potential risk related to the circulation of infected propagation material and to take the necessary measures for production of healthy planting material at European level.

2.4.4. Recommendations for the future

Visual inspections are not appropriate and satisfactory to prevent trading of infected material and should be replaced by molecular diagnostics. In the Countries involved in this WP different actions for the management of the infections have been applied such as the **uprooting** of infected plants, establishing of **new mother stocks** (Czech Republic), recommendation to protect the certified propagation stock for scions against psyllids with **hail nets** (Switzerland) or the planned declaration of **contaminated zones** as for ESFY in the Canton of Valais (Switzerland). In this view, a harmonization of the control measures at EU level is desirable for a more effective control of the diseases spread by trade activities (e.g. certification schemes or phytosanitary passport in Directive 2000/29).

3. WP3 Molecular classification of virulence of the infecting AP phytoplasma (lead Germany)

3.1. Background

The virulence of different apple proliferation phytoplasma (AP) accessions varies greatly after experimental inoculation of homogeneous plants material, although *'Ca.* P. mali' is a homogeneous species based on 16Sr RNA sequence data. A close examination of other less conserved genes revealed a considerable genetic heterogeneity. Recently, a variable gene from *'Ca.* P. mali' (ATP00464, *hflB*) was identified which allowed a high resolution of AP strains. A number of related genes from different accessions were identified and analysed thereafter. Disease history of the infected trees could be established in relation with virulence, structure and dynamics of the strains.

Table 4 – *WP3-Objectives*

Tasks	Status – fully completed
T1 Screening of strain collections and nuclear stocks on the presence of virulent AP strains and the occurrence of multiple infections	YES
T2 Monitoring the dynamics of strains in infected plants	YES

3.2. Methods

Eight AP-infected trees from the JKI-Dossenheim strain collection displaying a different symptomatology were selected for examination (Accession numbers: 1/93, 3/6, 3/93, 6/93, 8/93, 10/93, 12/93, 14/93). The DNA of roots and shoots from AP-diseased plants was extracted at three different times (spring, summer and autumn) starting October 2012, with the last sample collection in June 2014. *HflB* gene fragments and a partial ATPase gene were amplified by a set of gene-specific primers. *HflB* PCR fragments were cloned and inserts of about 20 clones per accession were subjected to SSCP analyses. The primers and probes to amplify the ATPase gene were used as a real time PCR assay to assess the proportion of virulent and non-virulent strains.

3.3. Results and discussion

From the eight AP accessions, seven were under observation since 1993 and were monitored on a yearly basis. A rating scheme in which the most severe AP symptom was considered led to a clear phenotypic distinction. The strains 1/93 and 8/93 showed very little or no symptoms over the past 20 years, while the accessions 3/93, 6/93, 12/93 and 14/93 showed each year typical apple proliferation symptoms. The disease severity of accession 10/93 was rated in between. The accession 3/6 was introduced later and rated as strongly virulent. The symptom expression in the years 2013 and 2014 resembled those of the previous years with the exception of accession 1/93. The tree developed in 2013 one witches' broom and four witches' brooms in 2014.

The gene fragments examined are referred to a 464 (hflB fragment) and 460 (ATPase fragment). Both genes differ in sequence between virulent and non-virulent strains. However, at the moment, it is impossible to say if the genes are directly or indirectly involved in symptom expression or if the linkage is coincidental. To achieve a representative coverage of the phytoplasma population from the stem and root system the DNA of three samples from each part of the tree was extracted. The resulting fragments were either pooled for the cloning experiment or the DNA

samples were used individually for real time-assays. In most of the samples a PCR fragment was obtained or the real time-assay resulted in a reading (**Table 5 and 6**).

Table 5 – *SSCP results of different accessions at time of sampling. The number of profiles and the relation of the number of patterns are given.*

		Oct. 12		Apr. 13		Aug. 13		Nov. 13		Apr. 14		Jun. 14	
1/93	**Root**	nd		2*	3-17~	4	1-1-3-15	1	19	4	2-3-5-10	1	20
	Stem	nd		-		3	1-2-17	2	3-17	2	3-17	1	18
3/6	**Root**	1	19	3	1-1-18	1	20	1	20	1	20	3	1-7-12
	Stem	1	20	2	1-18	2	1-18	2	2-18	1	20	3	4-8-8
3/93	**Root**	-		-		nd		2	10-10	4	1-4-6-9	2	15-15
	Stem	-		-		nd		2	4-16	5	1-1-1-17	3	1-8-11
6/93	**Root**	3	2-5-12	2	2-18	4	2-2-4-6	4	1-2-4-13	2	9-11	3	2-7-11
	Stem	-		3	1-8-9	3	1-2-14	2	3-17	3	1-6-8	2	9-11
8/93	**Root**	2	4-14	2	2-18	1	20	1	20	2	1-18	2	3-17
	Stem	-		2	1-19	-		1	20	5	1-3-4-5-6	2	10-10
10/93	**Root**	-		2	1-19	2	5-15	4	2-3-4-6	2	1-19	2	2-18
	Stem	-		1	20	2	8-12	2	7-10	2	5-13	3	1-8--11
12/93	**Root**	2	1-18	3	1-2-15	2	1-19	3	1-2-17	1	18	2	1-1-18
	Stem	1	19	2	4-16	1	20	1	20	2	1-19	3	2-2-16
14/93	**Root**	3	1-3-16	3	1-5-14	3	1-5-14	2	1-19	3	1-4-15	2	5-14
	Stem	2	2-18	2	8-12	2	2-17	2	1-19	2	5-13	3	1-1-15

**= number of distinct patterns; ~ = relation of distinct patterns; nd, no sampling; -, no amplification.*

Table 6 *– Summary of real time-PCR results with virulence-specific primers.*

		Oct. 12		Apr 13		Aug 13		Nov 13		Apr 14		Jun 14	
		Avirul.	**Virul.**	**Avirul.**	**Virul.**	**Avirul.**	**Virul.**	**Avirul.**	**Virul.**	**Avirul.**	**Virul.**	**Avirul.**	**Virul.**
1/93	**Root**	nd	nd	1E+07*	2E+08	3E+06	1E+08	7E+04	1E+04	3E+06	8E+07	4E+07	1E+07
	Stem	nd	nd	-	-	-	2E+10	-	3E+05	5E+05	3E+10	1E+05	1E+05
3/6	**Root**	-	2E+09	-	9E+10	-	6E+10	-	9E+10	-	9E+08	-	2E+10
	Stem	-	9E+10	-	3E+05	-	6E+10	-	9E+12	-	2E+07	-	2E+10
3/93	**Root**	-	-	-	-	nd	nd	-	-	-	8E+03	-	-
	Stem	-	-	-	-	nd	nd	-	-	-	2E+04	-	-
6/93	**Root**	-	3E+07	-	7E+09	7E+07	2E+09	4E+06	9E+07	-	9E+09	-	1E+09
	Stem	-	-	-	3E+09	-	2E+08	-	5E+08	-	5E+09	-	4E+11
8/93	**Root**	-	5E+07	2E+06	1E+09	-	9E+08	-	2E+07	-	1E+10	-	7E+07
	Stem	-	-	-	3E+06	-	-	-	-	-	6E+04	-	-
10/93	**Root**	1E+06	3E+07	5E+07	2E+09	1E+08	2E+10	6E+07	2E+10	8E+07	6E+10	9E+09	5E+10
	Stem	-	-	5E+06	1E+07	2E+08	3E+08	4E+06	2E+07	9E+06	3E+08	2E+07	1E+07
12/93	**Root**	-	2E+08	-	2E+10	-	5E+09	-	4E+13	2E+08	2E+11	-	5E+10
	Stem	-	4E+09	-	1E+10	-	3E+10	1E+03	8E+10	-	5E+12	3E+05	2E+10
14/93	**Root**	3E+06	4E+08	2E+08	3E+10	1E+08	9E+09	8E+06	2E+07	1E+08	9E+10	2E+09	3E+08
	Stem	4E+07	2E+08	2E+08	5E+09	1E+09	-	1E+07	-	1E+08	2E+11	5e+07+	1E+08

**, Phytoplasma titre was determined relative to the amplification of a cloned standard of the ATPase gene from a virulent and non-virulent strain. The copy number given represent the number of organisms in 1mg of phloem tissue; nd, no sampling; -, no phytoplasmas of this virulence type present.*

All PCR fragments amplified by the *hflB* primers were identical in size. The resulting SSCP patterns were of high complexity and samples taken from one tree at different times of the year mostly showed different patterns indicating shifts in the population structure. The accession 1/93 for example, which never showed symptoms, gave negative PCR results in the stem samples at the beginning of the project but not at the end. In the root system phytoplasmas were always present.. The situation in the stem changed the year the first witches' broom was visible. The year later phytoplasmas in the stem phloem could already be amplified in April. The SSCP patterns from root and stem samples were always complex, except for the last sampling date where both showed a uniform pattern. The result does not imply that the other strains completely disappeared, but shows that this strain is outnumbering the other strains. The situation in the other AP accessions is similar. Complex SSCP profiles were present and the proportion of profiles differed between the roots and the shoots. In real time-assays the simultaneous presence of virulent and non-virulent strains was demonstrated and shifts in their proportion were monitored.

3.4. Main conclusions

Table 7 *– WP3-Deliverables*

Deliverables	Status – fully provided
D1 Sequence information of hflB genes	**YES, already published**
D2 Selection of new primers for the simultaneous detection by real-time PCR	**YES**
D3 Determination of single and multiple infection by cluster analysis of sequences	**YES**
D4 Real-time PCR for demonstration of population dynamics	**YES**
D5 Overview on population structure and comparison to phenotypic data	**YES**

3.4.1. Summary

The exact mechanism of how phytoplasmas re-colonize the stem in spring is unknown. From results of this and previous work it is conceivable that a mixture of strains is present in the roots but not all are equally well fitted to colonize the stem. This might either be related to the physiological condition of the plant phloem and/or influenced by the genetic endowment of strains. The varying patterns between and within a year also reveals that there is a constant flow in the population. Trees infected long time ago might show strain equilibrium, however, the re-colonization of the new years' phloem might favour the mild or the virulent strains leading to an asymptomatic condition or disease.

A real time assay was developed to assess the presence and number of virulent and non-virulent strains using two sets of primers. Among all strains tested, the accession 3/6 was the only strain that showed exclusively virulent strains. **All other samples comprised a mixture of virulent and non-virulent types.** During the observation period a few peculiarities were noticed. The roots of accession 1/93 contained virulent and non-virulent strains. Later as the tree started to develop symptoms the stem was phytoplasma-positive but only virulent strains were detected. This observation suggests a correlation of witches' broom development and stem colonization.

3.4.2. Benefits and technology transfer

- Dissemination suitable for scientific community: **3 peer-reviewed articles** about hflB gene diversity and about population dynamics and diversity of apple proliferation strains have been published in 2013 and 2014.
- Dissemination suitable for stakeholders (National level): National report in Germany.
- Dissemination suitable for project partners: Exchange of protocols on sequence information and primers used.

3.4.3. Impacts and implication for stakeholders and policy makers

The potential to differentiate strains of the AP pathogen was developed recently. The availability of the full genome sequence of one AP strain opened the possibility to design primers that amplify highly variable genes. It is now admitted that infected trees are colonized by multiple strains and that the strains show genetic differences which seem to be correlated with virulence.

3.4.4. Recommendations for the future

Although, strain differentiation is now possible the factors for symptom development are still unclear. Stem colonization seems to be necessary for symptom development. The presence of a virulent strain in the roots is not sufficient. **The importance of the physiological condition of the plant,** as well as phenomena like systemic acquired resistance, cross protection or suppressive action of strains, needs to be further elucidated.

4. WP4 Monitoring disease development of uniformly inoculated trees under various climatic conditions and insect-proof containment (lead Germany)

4.1. Background

Fruit tree phytoplasma diseases are known for a long time but the factors influencing disease development are poorly understood. Infected plants follow a succession of disease stages in the course of colonization by the pathogen. How fast and to what extent symptoms appear seems not only to depend on the rootstock/cultivar combination but also on other biotic and abiotic factors, as well as on horticultural practices. The cyclic appearance of phytoplasma symptoms poses a large problem for the visual inspection of propagation material according to the EC **directive 2000/29/EC**. Within this work package the influence of environmental factors on the development of disease symptoms was studied.

Table 8 – *WP4-Objectives*

Tasks	Status – fully completed
T1 Uniformly infected plant material will be evaluated for symptom expression at different locations	YES

4.2. Methods

5 plants of uniformly infected material of apple-, pear- and apricot-rootstock/cultivar combinations were provided to all partners in October 2012 by the lead partner JKI (except for the Czech partner who received only two individuals per combination in

September 2013) and compared to controlled plants. For apple the combination M 9/Golden Delicious, for pear Kirchensaller Mostbirne/Williams and for stone fruits the rootstock INRA II/Luizet was used. The inoculum was tested prior to grafting. The experimental inoculation was performed in March 2012. All material was kept under insect proof conditions. The trial plants were monitored for symptom appearance and disease progression for up to two years. Real time-PCR was performed by some partners to monitor phytoplasma titre using primers from Christensen et al., 2004.

4.3. Results and discussion

In Austria, symptom appearance and disease progression were monitored on 3 out of 5 inoculated apple trees displaying witches´ broom in the first year, and on all trees showing witches´ brooms, enlarged stipules and bursted bark reddish-brown coloured in the second year. Conversely, the PD- and ESFY-infected trees developed no symptoms in the first year. In the second year, all PD trees displayed more or less typical symptoms of leaf reddening, premature development of axillary buds and stunted growth. Two out of three ESFY-infected plants showed abnormal shoots growth, small leaves and chlorosis, but no typical leaf rolling.
Real time-PCR tests were performed to examine the trial plants for the presence of phytoplasmas, roots, stem parts, midribs or buds were screened (Table 1). All five AP-infected trees were positive for *´Ca.* P. mali´ in the first year. In the second year the situation was identical. In all plant parts analysed phytoplasmas were detected. For the PD- and ESFY-infected plants the situation was different. In only one of five pear trees phytoplasmas were detected in the root samples. In the second year four of the five trial plants were positive. However, not all plants gave positive results for the same plant part tested. In two trees the PD phytoplasma was detected in the roots, whereas in two other plants the phytoplasmas were only detected in the upper plant parts. In the first year two of three ESFY-infected trees were positive in the root but not in stem samples. In the second year all three plants tested positive, but like PD-infected material, the positively tested plant material was inconsistent.
In conclusion, the symptom expression seems to be more related to the virulence of the phytoplasma strains rather than to the climatic conditions.
In Germany, symptom appearance and disease progression were monitored on a total of four AP-infected, 14 PD-infected and 20 ESFY-infected plants. In 2012 only the AP-infected plants showed typical disease symptoms with witches' broom formation and enlarged stipules. In April 2013 all AP-infected plants showed enlarged stipules and developed visible witches' brooms in summer, which became more pronounced in autumn. Additionally, leaf reddening was observable. The PD- and ESFY infected plants did not show specific symptoms in spring. However, the ESFY-infected material was more stunted compared to the healthy controls. In autumn PD-infected material showed typical leaf reddening and ESFY-infected material typical leaf roll symptoms. In 2014 the AP- infected plants showed again enlarged stipules when leaves emerged but only one plant developed a witches broom and showed leaf reddening. The plants showed an adequate increase in size. None of the 14 PD-infected plants developed pear decline-specific symptoms. The plants showed an adequate gain in size. Ten ESFY-infected plants showed an early bud burst in February and at the end of the observation period 18 of 20 plants were stunted and showed leaf roll symptoms.
DNA for phytoplasma detection was extracted from root, stem and midrib. Roots were the most reliable tissue for phytoplasma detection followed by stem and midrib tissue. The reliability of detection increased during the observation time. In 2014 all

AP samples were positive. From 14 PD-infected pear trees 11 could be verified as phytoplasma infected in August 2014. In four plants phytoplasmas were only detected in root tissue and in only six plants midrib tissue tested positive. 18 out of 20 ESFY-infected plants were positively tested in 2014. In August the pathogen was detected in roots and shoot but in only two cases midribs were positive. The winter 2013/2014 was mild and negative temperatures were recorded only once. The average summer temperatures in 2014 were slightly lower than in 2013 (**Fig. 1**).
In real time assays Ct values for the different tissue samples ranged between 17 and 29 for AP, PD and ESFY-infected plants. The Ct values of ESFY-infected stone fruit roots were slightly higher indicating a lower phytoplasma titre. In Fig. 1 the mean average Ct value of the examined plants and tissue is given at the sampling dates.
In conclusion, the dynamics of colonization and re-colonization under the local conditions in Dossenheim was as observed earlier. The root was always a place where phytoplasmas could be detected. The re-colonization of the stem took place when the new phloem tissue was produced. The early detection in April/Mai is most likely due to the small size of the trial plants. Significant differences exist in the colonization of leaf midribs. For the detection of the AP phytoplasma, midribs were a reliable source but for PD- and ESFY-phytoplasmas the material gave inconsistent results. Therefore, midribs cannot be recommended as a reliable source for these pathogens. At the end of the observation period the phytoplasma titre was high in all infected individuals.

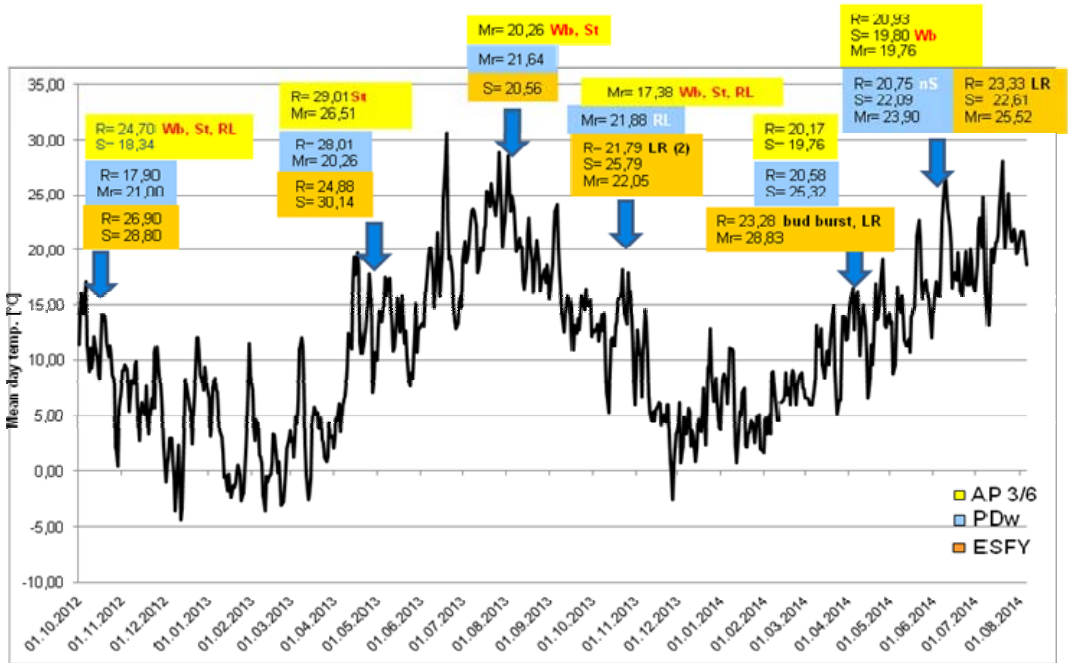

Fig. 1 – *Graphical display of mean day temperature from October 2012 to August 2014. Sampling dates are indicated by arrows. Real time PCR results are given as average Ct value for AP, PD and ESFY-infected plants tissue (R, root tissue; S, stem tissue; M, midrib tissue). Observed disease symptoms are indicated (Wb, witches' broom; St, enlarged stipules; RL, reddening of leaves; LR, leaf roll). Ct (threshold cycle) is the intersection between an amplification curve and a threshold line and is used as a relative measure of the concentration of target in the PCR reaction.*

In Italy, symptom appearance and disease progression were monitored during the two growing seasons (end of February – end of October) and local climatic data were recorded. After 1 year, no symptoms were observed on the inoculated plants and no differences in plant size and vegetation were noticed compared to the healthy

controls. Only symptoms referable to Apple chlorotic leaf spot virus (ACLSV) were observed on rootstock INRA II and confirmed by ELISA test. Slight phytoplasma symptoms started to appear only in the second year of observation during summer. Real time-PCR tests were performed to evaluate the seasonal variation of phytoplasma titre during the growing period. Leaf samples were collected from individual plants of each inoculated tree during 2013 in early summer (June 28), late summer (September 9) and autumn (October 31). Preliminary results show a fluctuation of the phytoplasma titre in leaf tissues for all three phytoplasmas with a lower concentration during the late summer compared to early summer and autumn (**Fig. 2**).
In conclusion, compared to the results obtained from the other partners, symptom expression under the climatic conditions of Latium region (Central Italy; average temp. in August 24°C to 28°C) appeared delayed for about one year. This delay might be related to the significant differences of the local climate compared to the climate of the northern European countries involved in this WP.

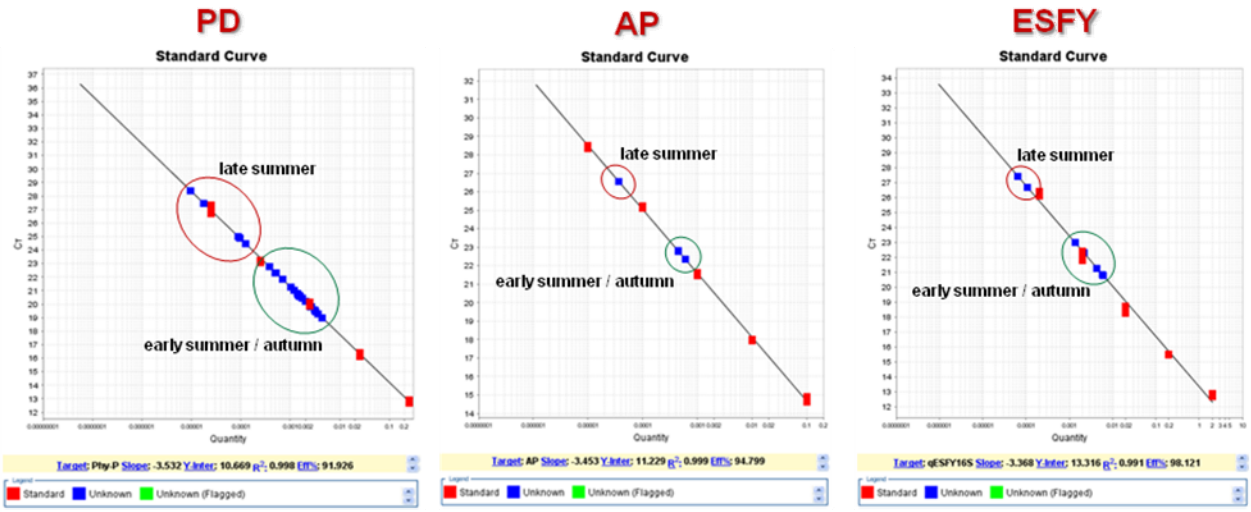

Fig. 2 – *Preliminary results of quantification show a fluctuation of the phytoplasma titre in leaf tissues for all three phytoplasmas with a lower concentration during the late summer compared to early summer and autumn.*

In Czech Republic, symptom appearance and disease progression were monitored in September 2013 and in 2014. The ESFY-infected stone fruit plants showed typical ESFY symptoms in both years. One cultivar died in 2014 but the rootstock survived. The AP-infected plants showed milder symptoms in 2014 compared to 2013. The PD-infected plants showed reddening of leaves in 2013 but were not monitored in 2014.
Real time-PCR tests revealed that the ESFY phytoplasma titre was higher in March than in May. Also the AP phytoplasma titre was significantly higher in March than in April. For PD-infected plants a high number of phytoplasmas were detected in March but no phytoplasmas could be detected in May.
In conclusion, the Czech Republic encountered a very hot and dry summer in 2014 with mean day temperatures up to 34°C which could have been the reason for the mild symptoms in the AP-infected plants and the absence of PD phytoplasmas at the date of testing. Detection by PCR was more successful in winter months (December to March), that is in agreement with previous studies and confirms that propagation

material should be tested during dormancy, at least under the climatic conditions of the Czech Republic.

In Belgium, symptom appearance and disease progression were monitored only with AP-infected trial plants. Symptom development was monitored from January to September 2014. All of them showed thickened parts or nodules on their roots. In March leaves started to develop and showed enlarged stipules and shortened petioles. Secondary shoots developed from axillary buds. In May a reddening of either the midrib or leaf blade started. The formation of leaf rosettes was visible and the witches' brooms became more pronounced. Until the end of the observation period the described symptoms aggravated.

Real time-PCR tests were performed only on 3 out of 6 AP-infected plants. Root and shoot samples were tested the first time in March 2013 and from September 2013 to August 2014 on a monthly basis. In October and November 2013 samples were collected at up to three different dates. All plants examined showed similar Ct values in comparable samples. The lowest Ct value for the leaf midrib was 15.8 and the highest 26.6 measured in November and May, respectively. The Ct values for the root samples ranged from 15.8 to 20.6.

In conclusion, under Belgium conditions all AP-infected plants showed typical disease symptoms in 2014. The phytoplasma titre in the roots and leaves was high throughout the testing period.

4.4. Main conclusions

Table 9 *– WP4-Deliverables*

Deliverables	Status – fully provided
D1 The main partner at JKI-OW will select the appropriate inoculum	**YES, already done**
D2 Inoculated plants are sent to participating partners	**YES, already done**
D3 The symptom development is monitored for two growing seasons	**YES**
D4 The phytoplasma titre will be determined by real-time PCR in autumn	**YES, already done**
D5 Exchange of data	**YES, already done**

4.4.1. Summary

Visual assessment of symptoms is not a reliable indicator for phytoplasma infection. Symptom expression seems to be more related to the virulence of the phytoplasma strains rather than to the climatic conditions (see differences in Italy). Hot temperature in summer seems to inhibit symptoms or leads to a postponed symptom development. Molecular tests should concentrate on plant material with the highest probability of phytoplasma presence (**roots** and not midribs) and also on the time of the year when the organism **titre is highest in winter** (with low Ct values). Flexible protocols need to be established for temperate zones.

4.4.2. Benefits and technology transfer

- Dissemination suitable for project partners: Used of **common protocols** for symptom appearance and disease progression and for Real time-PCR.

4.4.3. Impacts and implication for stakeholders and policy makers

The trial revealed that specific disease symptoms, even for experimentally infected plants, are no reliable indicator for infection. Therefore the practiced visual inspection under the **directive 2000/29/EC** is not an appropriate mean to unambiguously assess plant material. However, also molecular techniques fail when inappropriate

plant material is collected (roots better than midribs). The results improve our knowledge on the latent period and dynamics of phytoplasmas in fruit plants under local climatic conditions and provide useful information particularly for the inspection authorities monitoring fruit trees for phytoplasma symptoms especially on nuclear plantings and nurseries.

4.4.4. Recommendations for the future

On the basis of the obtained results, an **extension of the observation period up to 10 growing seasons** is recommended for future works in order to correlate symptoms expression with the phytoplasma titre and local climatic conditions.

5. WP5 Improving detection of fruit tree phytoplasma by more efficient and more sensitive diagnostic procedures (LAMP) (lead Belgium)

5.1. Background

In correspondence with the overall goal of this work package, a fast and sensitive on-site isothermal assay for detecting these three 16S RFLP group X phytoplasmas has been developed and validated for on-site use.

Table 10 – *WP3-Objectives*

Tasks	Status – fully completed
T1 A LAMP procedure will be developed to detect fruit tree phytoplasma with high sensitivity and specificity for routine application	YES

5.2. Method development, results and discussion

The method is relying on 4 primers, 2 outer primers F3 (forward) and B3 (reverse) and two hybrid inner primers (FIP and BIP) which are added to a reaction mix and submitted to an optimized incubation step (30 minutes at 65°C). The isothermal reaction which takes place is highly specific for the group X phytoplasmas and the massive amount of DNA which is only produced when the target organism (AP, PD and ESFY) are present can then be visualized for interpretation. For the validation of the method developed in this WP, the choice has been made to use capillary electrophoresis and a real-time PCR instrument in the laboratory and compare this to the water-proof portable GenieIII instrument from Opigene Ltd. (UK) to assess the on-site practical use of the LAMP procedure. In the laboratory, the optimized LAMP procedure underwent an extensive validation procedure for AP and PD on the parameters trueness, specificity, analytical sensitivity, repeatability, reproducibility and robustness. With respect to the last parameter, an important aspect for on-site detection is a reduced extraction method. To assess this aspect, the standard CTAB based method was compared to the commercial Plant material Lysis kit (Optigene). Both extraction methods gave good results, indicating that the method can be applied in the field. Additionally, comparison with general and group X phytoplasma detection methods relying on probe-based real-time PCR methods (which are considered as the most sensitive routine detection methods to date), also reveals a comparable sensitivity level. An additional validation procedure for the detection of ESFY is still on-going, but looks promising.
The result is a 1h protocol that can be used in the field.

5.3. Main conclusions

Table 11 *– WP5-Deliverables*

Deliverables	Status – fully provided
D1 Partners will select group- and species-specific LAMP primers	YES
D2 Different detection chemistry will be tested	YES
D3 Various DNA extraction protocols will be tested for routine application	YES
D4 Field tests will be performed to verify applicability of the LAMP/DNA extraction procedure	YES

5.3.1. Summary

A LAMP PCR method (loop-mediated isothermal amplification of DNA) for the detection of AP, PD and ESFY has been developed. **The method is reliable, fast and sensitive and has been validated on-site for AP and PD.** The method developed for ESFY detection still need to be validated, therefore plant material and ESFY strains have been exchange between project partners.

5.3.2. Benefits and technology transfer

- Dissemination suitable for scientific community: **A peer-reviewed article** about LAMP method will be submitted.
- Dissemination suitable for PPOs (EU level) and for stakeholders (National level): **Information for inspectors** during meetings will be conducted in Belgium and Germany.
- Dissemination suitable for project partners: **Exchange of protocols** (incl. efficient DNA extraction, primers, type of cycler…) and of material.

5.3.3. Impacts and implication for stakeholders and policy makers

A reliable LAMP PCR method is available for on-site screening and monitoring of the three phytoplasma pathogens.

5.3.4. Recommendations for the future

After information for inspectors in every participating country about the reliability of the **LAMP method for routine diagnosis,** extended ring tests and training course should be organized. The implementation of these techniques should be an important part of a future project.

6. WP6 Monitoring of insect vectors and assessment of the vector host plant interactions (lead Switzerland)

6.1. Background

The fruit tree phytoplasmas *Ca.* P. mali, *Ca.* P. pyri and *Ca.* P. prunorum are transmitted from plant to plant by insects. The knowledge of potential vectors and their biology is crucial for managing the disease and preventing their spread. Although considerable progress has been made recently by identification of AP phytoplasma vectors and elucidation of their life cycle, important questions remain, like the interaction between the vector and host plants. Furthermore, the transmission capacity of different *Cacopsylla* species as alternative vector for the AP or ESFY phytoplasma is not fully understood.

Table 12 – *WP6-Objectives*

Tasks	Status – fully completed
T1 Monitoring the occurrence of potential vectors	YES
T2 Assessment of the vector host plant interactions	YES

6.2. Methods

The occurrence of psyllid species was monitored by sweeping techniques. Adult psyllids captured in the orchards or in the natural host land were analysed by PCR (fO1/rO1) for their infection status. Plum psyllids were determined by direct PCR (ITS2 amplicon size, Peccoud et al., 2013). Glasshouse trial was conducted in order to determine the acquisition capability of apple psylla *C. melanoneura* for the pear decline ('*Ca.* P. pyri'). 6 potted pear plant (2 healthy and 4 infected trees), protected with Plexiglas-container, and were used. The influence of apricot genotypes on the vector behaviour of *C. pruni* was performed using a six-arm olfactometer allowing simultaneous observation of insect behaviour and odour trapping (Turlings et al., 2004).

6.3. Results

In Switzerland, the study was essentially conducted for the plum psyllid because of the importance of apricot cultivation in the region (South West) where phytoplasma diseases were highly observed. In addition, little information is available about the occurrence and the magnitude of this vector in all the country. The monitoring of plum psyllid (*Cacopsylla pruni*) revealed that this vector is present in all regions (18 localities in 11 different cantons, 59 % were captured in the South West; 33 % in the North East; and 8% in the North West). The captured population consisted in 55% of *C. pruni* and 45% of C. pinihiemata. Nevertheless, in 2014, C. pinihiemata did not carry the phytoplasma. The percentage of infested adults psyllids was low (3.43% of *C. pruni* infested with *Ca.* P. prunorum.

Flower branches of 13 apricot genotypes were used to assess their attractiveness for psyllids. The psyllid frequency were classified into 5 clusters according to the percentage of their choice (0%, 1 to 10 %, 11 to 20 %, 21 to 30% and up to 30 %). GC-MS profile analysis of volatile compounds and stepwise linear regression ($R2 = 0.82$, p-value ≤ 0.05) allowed to differentiate 8 volatile compounds that were important for the psyllid choices. 3 of them were positively attractive for the psyllid and 5 other volatile compounds were repulsive. In the future, the identification of these volatiles will permit developing attractive or repulsive traps to control psyllid migration in the orchards. Differences of attractiveness were established between apricot cultivars; however the differences were not significant. This could perhaps be explained by the phenological stage of the branches for the olfactometer test. Volatile compounds at full bloom are probably similar for the different apricot cultivars. In the future, olfactometer tests should be conducted using vegetative branches that could reveal stimuli that can affect psyllid behaviours.

In Austria, the occurrence of psyllids as potential vectors of European stone fruit yellows (ESFY), pear decline (PD) and apple proliferation (AP) was investigated in different orchards during the last years. *Cacopsylla pruni* was captured on apricot trees whereas high occurrence of *C. melanoneura* and some *C. picta* were occurred on apple trees. *Cacopsylla pyricola*, *C. pyri* and *C. pyrisuga* were present on pear trees from 4 investigation sites in Lower Austria.

The percentage of infested adults psyllids was low (3.64%-6.77%), except for *C. picta* that was infested at 16% with '*Ca.* P. mali'.
Surprisingly, results obtained from detailed analysis of 402 pear psyllids from 4 investigation sites in Lower Austria revealed that the females of all 3 species were more frequently infected by '*Ca.* P. pyri' than the males. It means that plum and pear psyllid females could be more important for vectoring the phytoplasmas than the males.
Molecular analysis of psyllids demonstrated that apple psylla is able to acquire '*Ca.* P. pyri' under artificial conditions at a level of 2 %. This result was the same in both individuals female and male.
In Norway, apple proliferation (AP) is currently occurring in all important fruit growing areas in Norway. Since the disease seem to have spread considerably the last few years, potential vectors have been monitored during the last two years using sticky traps and beat-tray samples at thirty different localities with confirmed AP. More than 2000 specimens have been investigated and so far, only the potential psyllid vector *Cacopsylla melanoneura* has been observed in Norway. *C. melanoneura* was detected in all but one orchard. A few *C. affinis*, pear psyllids and other psyllids were also observed, but no *C. picta*.
The percentage of infested adult psyllids was very high with 20% of *C. melanoneura* infested with '*Ca.* P. mali'.
In Czech Republic, control of AP, PD and ESFY vectors in apple, pear and apricot plantings of mother plants and nurseries, respectively, has been realized by repeated monitoring in 3 plantings during 2013 - 2014 and by one-time captures in 5 other plantings. Higher occurrence rate of *C. pyri* has been recorded in two monitored pear plantings in both years of research. Contrarily, AP and ESFY vectors were rarely found in the main controlled orchards. The percentage of infested adult psyllids was very high with 16% of *C. pyri* infested with '*Ca* P. pyri'.

Table 13 – *Percentage example of infested psyllid species in the different project member countries (2014).*

Country	Host plant	Psyllid species	% infested adults psyllids	Phytoplasma species
Norway	Apple orchards	*C. melanoneura*	20 % (n=163)	*Ca.* Phytoplasma mali
Switzerland	Apricot orchard and natural host land (*Prunus spinosa*)	*C. pruni*	3,43 % (n=321)	*Ca.* Phytoplasma prunorum
Austria	Pear orchards	*C. pyricola* *C. pyri* *C. pyrisuga*	3,85 % (n=104) 6,77 %(n=133) 3,64 %(n=165)	*Ca.* Phytoplasma pyri
	Apricot orchards	*C. pruni*	Few (data ND)	*Ca.* Phytoplasma prunorum
	Apple orchards	*C. picta*	16% (n=6)	*Ca.* Phytoplasma mali
Czech Republic	Pear nuclear stock	*C. pyri*	16% (n=24)	*Ca.* Phytoplasma pyri

ND = Not determined

6.4. Discussion

The psyllid occurrence in each country demonstrates the presence of *Cacopsylla* sp. in Europe, even in Norway where a great range of climatic parameters between winter and summer are evident. It reveals the capacity of psyllids to survive in any particular weather patterns. In the future, modelling of psyllid epidemiology is needed to better understand the influence of climatic conditions on the behaviour of phytoplasma vectors. The level of infested psyllids in Norway and Czech Republic reveals the importance of disease spreading by vectors. Elimination of infected trees

could be effective but the management of *Cacopsylla* species should be considered with attention. Besides, it is important to note the results from Austria where plum and pear psyllid females could be more important for phytoplasma vectoring than males. Furthermore, the discovery of acquirement of pear decline by apple psylla should be deeply explored in order to consider the importance of this alternative vector in the dispersal of pear decline in orchards and nurseries. These findings should be considered for the development of new control strategies against vectors. For every fruit phytoplasma and in every country, the necessity to optimize the orchard protection against psyllids, and thus to decrease phytoplasma diseases dissemination can be recommended. Finally, the importance of apricot cultivars on the vector behaviour was demonstrated. Herein, the study was focused on the volatile compound profiles. The results showed the psyllid preferences for some volatile compounds and suggest a possible psyllid control in the orchards by developing mass trapping.

6.5. Main conclusions

Table 14 *– WP6-Deliverables*

Deliverables	Status – fully provided
D1 Time of occurrence and number of psyllids will be identified	**YES**
D2 PCR assays will identify the percentage of infested adults	**YES**
D3 Preliminary study on potential alternative vectors	**YES**
D4 Determination of host plant volatile profiles and of the influence on the vector behaviour	**YES**
D5 Overview on vector host plant interactions	**NO, to be completed with other potential stimuli that influence psyllid behaviour (reproduction, oviposition, mature and immature adult feeding**

6.5.1. Summary

Plum, pear or apple *Cacopsylla* sp. are widely present in fruit growing regions of the participating countries and are vectors of the phytoplasma diseases. The level of infested psyllids, especially in Norway and Czech Republic, reveals the importance of disease spreading by vectors. On another hand, apricot cultivars and their related volatile compound influenced the psyllid preferences. Psyllid infestation could be avoided by covering mother stock plantations and nurseries with nets or by protecting orchards using appropriate pesticides.

6.5.2. Benefits and technology transfer

- Dissemination suitable for scientific community: **3 peer-reviewed articles** about the occurrence of psyllids in Austria, about the influence of apricot genotype on psyllid attraction and about the influence of climatic factors on plum psyllid epidemiology will be submitted.
- Dissemination suitable for PPOs (EU level) and for stakeholders (National level): **meeting** will be organized for PPOs and stakeholders to inform about the density and the vectoring capacity of psyllids (Norway, Switzerland, and Czech Republic).
- Dissemination suitable for project partners: **Exchange of protocol** about molecular determination of *Cacopsylla pruni* and *Cacopsylla pinihiemata*. A **meeting** will be organized in 2015 in order to exchange about the importance of volatile compounds in future control strategies of psyllids (Dossenheim with J. Gross). In order to better understand the very high percentage of infested *C.*

melanoneura in Norway, plant protection strategies in Europe will be exchanged and compared to Norwegian strategies.

6.5.3. Impacts and implication for stakeholders and policy makers

Climatic conditions influence the behaviour of phytoplasma vectors and modelling of their epidemiology is needed. Furthermore, the discovery of acquisition of pear decline by apple psylla should be deeply explored in order to consider the importance of this alternative vector in the dispersal of pear decline in orchards and nurseries.

6.5.4. Recommendations for the future

For every fruit phytoplasma and in every country, **the necessity to optimize the orchard protection against psyllids,** and thus to decrease phytoplasma disease dissemination, can be recommended. In the future, meteorological parameters have to be considered in order to **develop epidemiological models** and thus to determine precisely the adequate moment for controlling psyllids in an effective and sustainable way. For this purpose, interesting compounds based on attractive and repulsive volatiles could allow the **development of mass trapping**.

7. Project Management, dissemination and impacts

7.1. Project meetings

Kick-off Meeting, JKI Dossenheim, Germany, 28 September 2012
Annual meeting, AGES Vienna, Austria, 5 December 2013
Final meeting, Agrocope Conthey, Switzerland, 11-12 September 2014

7.2. Technology Transfer and Dissemination

A project **Web site** was created using the domain name www.apophyt.org . A Web page was designed including key data of the project. Beside general project data and an introduction about phytoplasma diseases, the main objectives of the project are described and illustrated by images. All participating institutes are listed including their logos, links to their institutes and contact persons. Recent publications of the project are up-loaded and available to the public. This Web page strongly promotes joint research activities in the field of fruit phytoplasma diseases gained through the research network which was established in the framework of the ERA-NET Euphresco programme. This Web page makes research topics of fruit phytoplasmoses accessible to the stakeholders, interested producer, other researcher and students (lead Austria).
A **3-pages article** was produced in the journal “International Innovation” that is a publication of a series of reports covering fundamental plant, crop and food science and is distributed worldwide to over 30'000 stakeholders from government, policy, science, research and private sector community. Hard copies of the journal, as well as a leaflet of the article were distributed to the partners (lead Switzerland).

7.2.1 National reports

- Blystad, DR, Birkenes, SM, Brurberg, MB (Norway). Kartlegging av heksekost i eple i 2012. Bioforsk Report 8(91), Ås (NO), November 2013 (Norwegian).
- Schneider, B. (Germany). Erfassung von Faktoren und Determinanten, die im Zusammenhang mit der Verbreitung, der Bedeutung und dem Nachweis von Kern-

und Steinobstphytoplasmen in der Europäischen Gemeinschaft stehen. February 2013 (German).
- Blystad, DR; Birkenes, SM; Brurberg, MB (Norway). Kartlegging av heksekost i eple i 2013. Bioforsk Report Vol. 9(52), Ås (NO), March 2014 (Norwegian).
- Camps et al. (Switzerland). Towards a long term management of European stone fruit yellows (ESFY), with particular insight into the interactions between the vector *Cacopsylla pruni* and the different host plant *Prunus* spp. (SWISS-APOPHYT). Submission in 2015 (French/English)
- Suchá et al. (Czech Republic). The results of the research activities of the project APOPHYT. December 2014 (Czech).

7.2.2. Scientific publications

- Camps et al. Influence of apricot genotype on psyllid attraction using a six-arm olfactometer (submission in 2015)
- Camps et al. Influence of climatic factors on plum psyllid epidemiology (submission in 2015)
- De Jonghe et al. Development of a LAMP PCR procedure for the AP group phytoplasmas (submission in 2015)
- Firrao et al. 2013. Genome wide sequence analysis grants unbiased definition of species boundaries in 'Candidatus Phytoplasma mali'. Systematic and Applied Microbiology 36:539-548.
- Reisenzein and Lethmayer. Studies on fruit tree phytoplasmas and the vectoring psyllids in Austria. IOBC-Bulletin (submission in 2014).
- Schneider et al. 2014. Suppression of Aggressive Strains of 'Candidatus Phytoplasma mali' by Mild Strains in Catharanthus roseus and Nicotiana occidentalis and Indication of Similar Action in Apple Trees. Phytopathology, Vol. 104(5), 453-461.
- Seemueller et al. 2013. The AAA+ ATPases and HflB/FtsH Proteases of 'Candidatus Phytoplasma mali': Phylogenetic Diversity, Membrane Topology, and Relationship to Strain Virulence. Molecular Plant-Microbe Interactions, Vol. 26(3), 367-376.
- Vizzaccaro et al. 2013.Investigation on molecular variability of 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma prunorum' and 'Candidatus Phytoplasma pyri' by multilocus sequence analysis.Petria 23(1), 131-134

7.2.3. Scientific oral presentations

- Blystad et al. Heksekost - en trussel for norsk epleproduksjon. Bioforsk FOKUS 8:2 (286-286), Ås (NO), 6-7 February 2013 (Norwegian)
- Christen et al. Projet APOPHYT – Enroulement chlorotique de l'abricotier. Annual report internal Agroscope, Changins (CH), 3 December 2013 (French).
- De Jonghe, Steyer et al. Status and epidemiology of pear decline (Candidatus Phytoplasma pyri, PD) and apple proliferation (Candidatus phytoplasma mali, AP) in Belgium. International Symposium on Crop Protection, Gent University, Gent (B), 20 May 2014 (English).
- Ferretti et al. Investigation on molecular variability of 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma prunorum' and 'Candidatus Phytoplasma pyri' by multilocus sequence analysis.VI Italian Meeting on Phytoplasmas and Phytoplasmas Diseases. Alma Mater Studiorum - University of Bologna, Bologna (Italy), 17-19 June 2013 (Italian)

- Peusens, De Jonghe, Steyer et al. Phytoplasma diseases and their vectors in the UK, The Netherlands and Belgium. Meeting COST action FA0807, Lisbon (Portugal), October 2013 (English).
- Reisenzein and Lethmayer. Studies on the interaction between Candidatus Phytoplasma pyri and the vectoring psyllids. IOBC Working Group "Integrated Plant Protection in Fruit Crops", Sub Groups "Pome fruit arthropods" and "Stone fruits". IOBC & AGES, Vienna (AT), 7 October 2014 (English)
- Schneider and Seemüller. Prämunisierung (cross protection) als neue Strategie zur Bekämpfung von Phytoplasmosen im Obstbau am Beispiel der Apfeltriebsucht. 59th Deutsche Pflanzenschutztagung, Julius Kühn-Institut, Freiburg (D), 2014 (German)
- Seemueller et al. Effect on disease development of suppressive strains of 'Candidatus Phytoplasma mali' and their molecular identification". Meeting COST action FA0807, Lisbon (Portugal), October 2013 (English).
- Seemueller et al. Promising approaches to control apple proliferation disease: Plant resistance and cross protection using mild Phytoplasma strains. Proceedings of the 5th international symposium of plant protection and plant health in Europe, German Society for Plant protection and plant Health, Berlin (D), 2013 (English)

7.2.4. Technical publications

- All partners. APOPHYT project: Fruitful endeavours – Vector investigations. Ed. Research Media Ltd., Bristol (UK). International Innovation, November 2013 (English). Target scientific community.
- De Jonghe et al. Appelproliferatiefytoplasma, perenaftakelingsfytoplasma en "little cherry" in België: een probleem in de sierteelt ? Sierteelt en groenvoorziening (submission in 2015)
- Suchá et al. Evaluation of factors determining distribution, impact and detection of fruit tree phytoplasmoses. Phytopatologists, Profi Press Ltd, Prag (CZ), submission in 2015 (Czech). Target growers, phytosanitary inspectors.

7.2.5. Technical oral presentations

- Bünter et al. Information über APOPHYT-Projekt. Tag der Obstbaumproduzenten JardinSuisse, Düdingen (CH), 26 November 2014 (German). Target nurseries.
- Bünter et al. Information über Obst-Phytoplasmosen. Tag der Obstbaumproduzenten JardinSuisse, Bischofszell (CH), 13 November 2013 (German). Target nurseries.
- Camps et al. Vers une gestion à long terme de l'enroulement chlorotique de l'abricotier en Suisse. Journée phytosanitaire Cultures spéciales, Agroscope Changins (CH), 7 January 2015 (French). Target stakeholder, PPO, consultant, funder.
- Christen et al. Anbau von Aprikosen in der Schweiz. 52. Jahrestagung Arbeitskreis Steinobst Deutschland, Wintersingen (CH), 2 July 2013 (German). Target German consultant.
- Christen et al. Anbau von Aprikosen in der Schweiz. Fachbereich Zertifizierung, Pflanzen- und Sortenschutz, Conthey (CH), 14 August 2013 (German). Target PPO, FOAG (funder).
- Christen et al. Projet APOPHYT – Enroulement chlorotique de l'abricotier. Conférence professionnelle sur l'abricot, Martigny (CH), 5 October 2013 (French). Target producer and consultant, OCA-VS (co-funder).

- De Jonghe et al. Plant virology and phytoplasmology vegetable and fruit research - a 2012-2013 overview. Annual meeting - production sector vegetables and fruit, ILVO, Merelbeke (B), 25 November 2014 (Dutch). Target consultants, growers, stakeholders.
- De Jonghe et al. Status van appelproliferatiefytoplasma, perenaftakelingsfytoplasma en "little cherry" in België. Annual Meeting of the Ornamental sector, PCS Ornamental Research Station, Destelbergen (B), 16 June 2014 (Dutch). Target consultants, growers, stakeholders.
- De Jonghe, Steyer et al. Eindrapport REPEDAP project (Onderzoek naar verspreiding en epidemiologie van de quarantaine organismen Pear decline (*Ca.* Phytoplasma pyri, PD) en Apple proliferation (*Ca.* Phytoplasma mali, AP). Closing meeting REPEDAP project, Federal public service Health, Food chain safety and Environment, Brussel (B), February 2015 (Dutch). Target government, NPPO, FAFSC (Federal Agency for the Safety of the Food Chain) and other stakeholders.
- Reisenzein et al. APOPHYT – Project. Forschungsgespräche Obstbau, Lehr-und Forschungszentrum Obst- und Weinbau (LFZ), Klosterneuburg (AT), 29 November 2013 (German). Target stakeholder, producer, researcher.
- Suchá et al. Phytoplasmas of fruit trees and their harmfulness. Seminar the Traditional Fruit Growing Days, Research and Breeding Institute of Pomology Holovousy Ltd., Hradec Králové (CZ), 22 January 2015 (Czech). Target growers, phytosanitary inspectors.

7.2.6. Workshop

- Blystad, D-R; Hatteland, BA; Brurberg, MB. Survey for Apple proliferation in Norway; 2. Apple proliferation and psyllid vectors. One day meeting on apple proliferation phytoplasma. Bioforsk, Lofthus (NO), 13 May 2014 (English). Target authorities and extension service.

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9. References

- Christensen NM, Nicolaisen M, Hansen M, Schulz A, 2004. Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. Molecular Plant-Microbe Interactions 17:1175–84.
- Peccoud J, Labonne G, Sauvion N. 2013. Molecular test to assign individuals within the *Cacopsylla pruni* complex. PlosOne. 8(8): e72454.
- Turlings TCJ, Davidson AC, Tamò C. 2004. A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. Physiological Entomology. 29: 45-55.

10. Appendices

- Assessment of the transmission of AP, PD and ESFY by interconnected root system in nurseries (WP2, task 2) **was not conducted after common agreement** between partners at the kick-off meeting.
- It was not possible to publish *Recommendation to the inspection authorities about the influence of local climatic conditions on the symptoms symptom development* (from WP4). **Only 2 years of monitoring were not enough** to make such recommendations.
- It was not possible to publish a general *Overview on vector host plant interactions* (WP6, deliverable 5), **because of missing knowledge** about other potential stimuli that influence psyllid behaviour (reproduction, oviposition, mature and immature adult feeding).