Euphresco

Final Report

Project title (Acronym)
Set up of reliable detection protocols for the specific identification of '*Candidatus* Phytoplasma phoenicium' (DIPCAPP)

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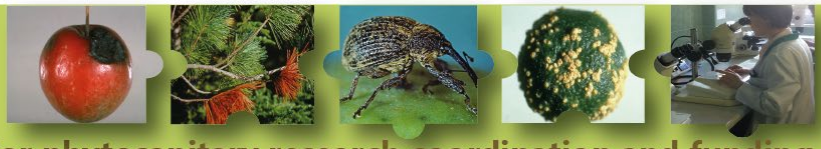
Contents



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2. Short project report

2.1. Short executive summary

'*Candidatus* Phytoplasma phoenicium' ('*Ca*. P. phoenicium') strains are members of the phytoplasma's subgroup 16SrIX-B and its variants (Quaglino *et al*., 2015). These strains are the etiological agents of a lethal disease of almond trees (*Prunus dulcis*). Peach (*P. persica*) and nectarine (*P. persica* var. *nucipersica*) may also be seriously affected by '*Ca*. P. phoenicium' (Jawhari *et al*., 2015). The common name of the disease is almond witches'-broom (AlmWB). Although '*Ca*. P. phoenicium' infection occurs mainly in almond, peach, and nectarine, it has also been occasionally identified in *P. armeniaca* (apricot), *Prunus* x *amygdalo-persica* (main rootstock for almond and peach in Europe), and in wild plants such as *P. orientalis*, *P. scoparia*, *Anhemis* spp., *Smilax aspera* (Fiore *et al*., 2018). '*Ca*. P. phoenicium' is reported from Lebanon and Iran where it is widespread where *Prunus* hosts are grown. Recently, '*Ca*. P. phoenicium' has also been detected on almond plants in southeast Italy (Nigro *et al*., 2019). Early detection is an essential measure to survey the presence and to avoid the introduction and spread of the pathogens into free areas. Detection methods for '*Ca*. P. phoenicium' have been developed and include conventional and real-time polymerase chain reaction. The DIPCAPP project contributed to identify the most suitable protocol for the detection of '*Ca*. P. phoenicium'. Among six already published and evaluated tests, four showed excellent specificity, sensibility, accuracy, and reproducibility and were equally effective in detecting '*Ca*. P. phoenicium'. From the results of the comparison carried out in the project, it appeared that the conventional PCR from Jawhari, *et al.*, (2015) and the real-time PCR from Jawhari, *et al.*, (2015) are the best methods for the detection of the pathogen, also considering the limited time and labour requested for their execution.

2.2. Project aims

The project aimed to provide methods, protocols, and procedures for the detection of '*Ca*. P. phoenicium' (subgroup 16SrIX-B), validated in accordance with the European standards for the harmonization of the detection within the EC.

2.3. Description of the main activities

The activities of the project focused on setting up and carrying out a thorough test performance study (TPS) to validate and compare various diagnostic tests available and published in the literature for the detection of '*Candidatus* Phytoplasma phoenicium'.

A total of six different tests, all based on the amplification of genomic fragments that are either shared by all phytoplasmas or specific to '*Ca.* P. phoenicium', were chosen for the TPS. The included tests are:

- EP1: direct end-point PCR test targeting the 16S-23S rRNA genes, specific for AlmWB phytoplasma (IX-B) (Jawhari, *et al.*, 2015);
- EP2: nested end-point PCR test targeting the *inmp* gene, specific for AlmWB phytoplasma (IX-B) (Quaglino *et al*., 2015);
- EP3: nested end-point PCR test targeting the 16S rRNA gene using generic phytoplasma primers, followed by restricted fragment length polymorphisms (RFLP) analysis of patterns to identify the AlmWB phytoplasma (IX-B) (Molino Lova *et al*., 2011);
- EP4: end-point PCR test using the barcoding primers, followed by sequencing of the amplicons to identify of AlmWB phytoplasma (EPPO PM 7/129);
- RT1: real-time PCR test for the generic detection of phytoplasma, not specific for AlmWB (Christensen *et. al*., 2004, EPPO PM 7/133);
- RT2: real-time PCR test for the detection of AlmWB phytoplasma (IX-B) (Jawhari, *et al.*, 2015).

The 7 project partnerscarried out all or some of the diagnostic tests listed above. Each test was carried out in at least 3 of the participating laboratories.

Each laboratory received an identical set of blind samples, 12 in total, as well as a negative and positive control. The positive control and the samples were spiked samples with nucleic acids extracted from infected peach or almond trees. The negative samples and the controls included also other plant species (grapevine, apple, periwinkle), and non-target phytoplasmas that are either associated with diseases present in Europe (phytoplasma subgroups 16SrX-A – Apple Proliferation disease, and phytoplasma subgroups XII-A – Grapevine Bois Noir disease) or closely related to the target (phytoplasma subgroups subgroup 16SrIX-C). The full list of samples is presented in table 1.

Table 1. Information on the TPS samples and the expected result when (i) using tests for the specific detection of AlmWB (IX-B), (ii) using tests for the generic detection of phytoplasma, or (iii) using tests for the detection of a conserved plant gene (18S rDNA).

Sample	Plant Species	Phytoplasma (16S rRNA subgroup)	Exp. Result		
			AlmWB	Phytoplasma	Plant
Sample 1	Peach	Almond Witches Broom phytoplasma (IX-B)	Pos	Pos	Pos
Sample 2	Apple	Apple Proliferation phytoplasma (X-A)	Neg	Pos	Pos
Sample 3	Grapevine	Bois Noir phytoplasma (XII-A)	Neg	Pos	Pos
Sample 4	Peach	European Stone Fruit Yellows phytoplasma (X-B)	Neg	Pos	Pos
Sample 5	Periwinkle	Almond Witches Broom phytoplasma (IX-C)	Neg	Pos	Pos
Sample 6	Peach	Almond Witches Broom phytoplasma (IX-B)	Pos	Pos	Pos
Sample 7	Almond	None	Neg	Neg	Pos
Sample 8	Almond	Almond Witches Broom phytoplasma (IX-B)	Pos	Pos	Pos
Sample 9	Peach	Almond Witches Broom phytoplasma (IX-B)	Pos	Pos	Pos
Sample 10	Almond	Almond Witches Broom phytoplasma (IX-B)	Pos	Pos	Pos
Sample 11	Almond	Almond Witches Broom phytoplasma (IX-B)	Pos	Pos	Pos
Sample 12	Peach	None	Neg	Neg	Pos
Neg. control	Peach	None	Neg	Neg	Pos
Pos. control	Almond	Almond Witches Broom phytoplasma (IX-B)	Pos	Pos	Pos

Each sample was tested at its initial concentration and diluted 1:10.

The total number of true positives, true negatives, false positives and false negatives were determined for each laboratory and each test.

Based on these results, the diagnostic performance indexes (accuracy, sensitivity, specificity) were calculated according to the recommendations of the EPPO Standard PM7/98.

The accuracy is the proportion of accords between the results obtained with a tested method and reference results on identical samples:

$$AC = 100 \times (TP+TN) / (TP+TN+FP+FN)$$

The diagnostic sensitivity is the capability of the tested method to detect the contaminated samples (based on the positive samples):

$$SE = 100 \times TP / (TP + FP)$$

The diagnostic specificity is the capability of the tested method to not detect the non-contaminated samples (based on the negative samples):

$$SP = 100 \times TN / (TN+FN)$$

Reproducibility was calculated as correspondence, evaluating for each sample the pairwise probability that the results given by different labs were the same and reporting the average value for all samples included in each individual assay.

2.4. Main results

The results of the TPS are reported in Table 2, including for each test the number of partners that used the test, the total number of observations obtained, as well as the number of true positive, true negatives, false positives, false negatives, and indeterminate results.

Table 2. TPS results. True positive (TP), true negative (TN), false positive (FP), false negative (FP), and indeterminate (Ind) results.

Method	N° of labs	Total observations	TP	TN	FP	FN	Ind
EP1	6	70	35	35	0	0	0
EP2	6	47 [a]	23	24	0	0	0
EP3	4	23	12	11	0	0	0
EP4	3	12 [a]	7	4	1	0	0
RT1 [b]	4	46	37	7	0	2	0
RT2	6	47 [a]	23	24	0	0	0

[a]: due to technical difficulties, the results from one or more of the laboratories had to be removed from the analysis of the results for this method.

[b]: this test is not specific for AlmWB, but detects all phytoplasma, which is why the ratio between true positive and true negative results is different compared to other tests, with a higher number of TP results.

With the exception of test EP4, all tests allowed the correct detection of '*Ca.* P. phoenicium' in all the blind samples. With the exception of test RT1, no test failed to detect the presence of phytoplasma in infected samples.

In test RT2 (the only test which included an internal plant gene amplification as control), the amplification of the internal control gene was obtained from all the provided samples, in line with the expected results. This result confirms that the negative results obtained with the tests are not due to low quality of the DNA, but to absence of the target pathogen in the sample.

For the real-time PCR tests, the confidence value (CV) was determined as a measure of the uncertainty of the results, and a statistical analysis (one-way ANOVA) was carried out to determine whether there were significant differences between the threshold cycles (CT) obtained by different labs or using different thermocyclers.

For the results of these analyses, it is important to mention that, while two different concentrations of the samples have been employed in the study (undiluted and 1:10 dilution), the actual concentration of the target pathogen in each sample has not been determined before performing the TPS. Therefore, the use of possibly very different samples can affect the homogeneity of the CT values obtained with real-time PCR.

Despite this possible source of disparity, the results obtained by the partners were very similar, and no significant differences emerged between samples amplified using the same method by different partners.

Regarding the measure of uncertainty, method RT1 had an average CT of 18.19 $\pm$ 1.72 (CV = 9.47%) for undiluted samples and 19.84 $\pm$ 0.97 (CV = 4.97%) for diluted samples. Method RT2

had an average CT value for AlmWB of 17.87 ± 1.63 (CV = 9.14%) with undiluted samples, and of 20.01 + 0.92 (CV = 4.60%) for diluted samples. The endogenous plant gene gave average CT values of 13.65 ± 2.02 (CV = 14.82%) for undiluted samples and 16.26 ± 1.78 (CV = 10.97%) for diluted samples.

These data suggest that both methods RT1 and RT2 have similar ability of detecting phytoplasma and give very similar error rates, which are close to 10% for undiluted samples and approximately 5% for diluted samples. In our experimental results, a CV of almost 10% is not a concern, since all CT values of positive samples remain well below the threshold for certain positive result (CT < 35) even should they be 10% higher. Still, it is worthy to note that with this level of CV, any result with CT 32 or above could be deemed suspicious or unclear.

The overall results regarding the diagnostic performance indexes are reported in Table 3.

Table 3. Tests' performance characteristics. The values are expressed in percentage.

Test	Accuracy	Analytical sensitivity	Analytical specificity	Reproducibility
EP1	100	100	100	100
EP2	100	100	100	100
EP3	100	100	100	100
EP4	91.7	100	87.5	N/A
RT1	95.6	77.8	100	91.7
RT2	100	100	100	100

Tests EP1, EP2, EP3, and RT2 all gave 100% values in all four examined parameters and are therefore equally effective in detecting the presence of '*Ca*. P. phoenicium' in the samples.

2.5. Conclusions and recommendations to policy makers

Among the six tests, four (EP1, EP2, EP3,and RT2)showed 100% values for specificity, sensibility, accuracy, and reproducibility and are therefore equally effective in detecting the presence of '*Ca*. P. phoenicium'. The results of the comparison carried out in the project is that the conventional PCR (Jawhari, *et al.*, 2015) (EP1) and the real-time PCR (Jawhari, *et al.*, 2015) (RT2) are the best tests for the detection of the pathogen for the following reasons: (i) tests EP2 and EP3 are nested-PCRs which include two consecutive PCR reactions, each of which lasts more than two hours, increasing the total time for detection; (ii) method EP3 involves an additional stepi.e. the analysis of the obtained amplicons by RFLP (overnight digestion followed by electrophoretic analysis) which results require specific skills for correct interpretation (presence of specialized technicians with in-depth knowledge of literature). Within the two best methods, the conventional PCR (EP1) is the most easily accessible to laboratories worldwide as it does not require expensive instruments.

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2.6. Benefits from trans-national cooperation

The overarching benefit of trans-national cooperation was to build stronger relationships between international laboratories. These relationships enabled transfer of experience and knowledge in the detection of '*Ca*. P. phoenicium' in the countries involved. The project led to the successful validation of detection protocols, two of which [direct PCR (EP1) and real-time PCR (RT2)] were selected as best protocols for '*Ca*. P. phoenicium' detection. The collaboration allowed the opportunity to validate diagnostic tests within the framework of a collaborative test performance study and demonstrates the usefulness of broad comparative laboratory testing for method validation, which is recommended by the European Plant Protection Organization (EPPO). The data from the TPS supported the development of an EPPO diagnostic protocol on '*Ca*. Phytoplasma phoenicium'.

3. Publications

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

Manuscript in preparation.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

None.

4. Open Euphresco data

Validation data will be published in the EPPO Database on diagnostic expertise.