Euphresco

Final Report

Project Title (Acronym)
***Pseudomonas syringae* pv. *actinidiae* (PSA): diagnosis, detection, identification and study of epidemiological aspects (PSADID)**

Project duration:

Start date:	2013-07-01
End date:	2015-07-30

1. Research consortium partners

Coordinator – Partner 1			
Organisation	Consiglio per la Ricerca in agricoltura e l'analisi dell'economia agraria (CRA) - Plant Pathology Research Center		
Name of Contact	Stefania Loreti, Massimo Pilotti	**Gender**:	F, M
Postal Address	Via C.G. Bertero 22 00156 Rome, Italy		
E-mail	stefania.loreti@crea.gov.it.it, massimo.pilotti@crea.gov.it		
Phone	+390682070341 or +390682070357		

Partner 2			
Organisation	Laboratoire National de la Protection des Végétaux - ANSES-SLV		
Name of Contact	Françoise Poliakoff	**Gender**:	F
Postal Address	7 rue Jean Dixméras, 49044 ANGERS Cedex 01, France		
E-mail	francoise.poliakoff@anses.fr		
Phone	+ 33 (0)241 723240		

Partner 3			
Organisation	Istituto Valenciano de Investigaciones Agrarias - IVIA Centro de Proteccion Vegetal y Biotechnologia		
Name of Contact	Maria Milagros Lopez	**Gender**:	F
Postal Address	Carretera Moncada - Náquera, Km. 4,5 Apartado Oficial ES - 46113 Moncada, Valencia, Spain		
E-mail	mlopez@ivia.es		
Phone	+ 34 96 342 407		

Partner 4			
Organisation	Instituto Nacional de Investigação Agrária e Veterinária (INIAV) UEIS-SAFSV Laboratório de Fitobacteriologia		
Name of Contact	Maria Leonor Cruz	Gender:	F
Postal Address	Av. da República, Quinta do Marquês, 1749-016 Oeiras, Portugal		
E-mail	leonor.cruz@iniav.pt		
Phone	+351 21 4463760		

Partner 5			
Organisation	Investigation and Diagnostic Centres and Response, Plant Health and Environment Laboratory, Ministry for Primary Industries, (MPI-PHEL)		
Name of Contact	Robert K. Taylor	**Gender**:	M
Postal Address	PO Box 2095, Auckland 1140, New Zealand		
E-mail	robert.taylor@mpi.govt.nz		
Phone	+64 9 909 3548		

Partner 6			
Organisation	The New Zealand Institute for Plant & Food Research		
Name of Contact	Joel L. Vanneste	**Gender**:	M
Postal Address	Research Bisley Road, Hamilton, 3214, New Zealand		
E-mail	joel.vanneste@plantandfood.co.nz		
Phone	+64 7 959 4547		

Partner 7			
Organisation	Università degli Studi di Modena e Reggio Emilia - UNIMORE Department of Agricultural and Food Sciences		
Name of Contact	Emilio Stefani	**Gender**:	M
Postal Address	Via J. F. Kennedy 17 42100 REGGIO EMILIA		
E-mail	stefani.emilio@unimore.it		
Phone	+ 39 (0)522 522013		

Other participants to the interlaboratory comparison (ITL)

Partner 8			
Organisation	Benaki Phytopathological Institute (BPI) Department of Phytopathology Laboratory of Bacteriology		
Name of Contact	Maria C. Holeva	**Gender**:	F
Postal Address	Stefanou Delta 8 14561 Kifissia, Athens Greece		
E-mail	m.holeva@bpi.gr		
Phone	+30 210 8180259 or +30 210 8180239		

Partner 9			
Organisation	Laboratorio Fitopatologico Regione Lombardia Servizio Fitosanitario/Fondazione Minoprio		
Name of Contact	Francesca Gaffuri	**Gender**:	F
Postal Address	V.le Raimondi 5422070 Vertemate con Minoprio, Como, Italy		
E-mail	f.gaffuri@fondazioneminoprio.it		
Phone	tel +39 031 320520		

Partner 10			
Organisation	Università 'La Tuscia' di Viterbo - DAFNE Department of Science and Technology for Agriculture, Forestry, Nature and Energy		
Name of Contact	Giorgio M. Balestra	**Gender**:	M
Postal Address	Via S. Camillo de Lellis - 01100 VITERBO		
E-mail	balestra@unitus.it		
Phone	+ 39 (0) 761 357474		

Partner 11			
Organisation	Austrian Agency for Health and Food Safety (AGES) Institute for Sustainable Plant Production- Department of Molecular Biological Diagnosis of plant diseases		
Name of Contact	Richard Gottsberger	**Gender**:	M
Postal Address	Spargelfeldstr. 191, 1220 Wien		
E-mail	richard.gottsberger@ages.at		
Phone	+ 43-0-50 555-33343		

Partner 12			
Organisation	Deputation de Pontevedra - Fitopatoloxía - Depo Xefa unidade de virus, bacterias e nematodos		
Name of Contact	Adela Abelleira Argibay	**Gender**:	F
Postal Address	Subida a la Robleda, s/n 36153 Pontevedra, Spain		
E-mail	adela.abelleira@depo.es		
Phone	+34 986 804 100		

Partner 13			
Organisation	Plant Protection Central Research Institute (PPCRI)		
Name of Contact (incl. Title)	Aynur Karahan	**Gender**:	F
Postal Address	Gayret Mah. Fatih Sultan Mehmet Bulvari No: 66, Yenimahalle, Ankara, Turkey		
E-mail	akarahan@zmmae.gov.tr		
Phone	+ 90 312 344 5994/152		

2. Short project report

2.1. Short executive summary

Pseudomonas syringae pv. *actinidiae* (Psa) is the causal agent of bacterial canker of kiwifruit. This pathogen affects *Actinidia* species (*Actinidia deliciosa* and *A. chinensis*) worldwide. The main symptoms are oozing of whitish or reddish exudates from cankers present along the trunk and branches, spots surrounded by yellow halos on the leaves, twig dieback, fruit collapse, leaf and plant wilting. The disease is a serious threat for kiwifruit production, due to high tree mortality and reduced production and, consequently, having an increasing socio-economic impact. The recent severe outbreaks of bacterial canker of kiwifruit in the European and Mediterranean Plant Protection Organization (EPPO) regions and in New Zealand has been related to the appearance of a local, very aggressive aplotype of Psa.called Psa biovar 3.

Possible pathways of pathogen introduction and disease spread into new territories are *Actinidia* spp. plants for planting, which represent the main pathway for long distance dissemination of Psa. However, positive pollen samples were recovered in New Zealand and in Italy. Therefore, the possibility that infected pollen could be a pathway for Psa introduction and disease spread was investigated and confirmed (EPPO, 2012; Tontou *et al*., 2014). It was suggested that pollen, as a pathway, should be certified free from Psa (Zespri, 2012; EPPO, 2016). Other dissemination pathways are wind and wind driven rain, spring frost, equipment and tools.

Recently an EPPO standard has been published as formal guidance on procedures for the detection of Psa (EPPO, 2014). Screening and identification methods are mainly based on conventional PCR (single and duplex PCR's) (Rees-George *et al*., 2010, Gallelli *et al*., 2011a) and of repetitive-PCR (rep-PCR). Considering the worldwide high impact of this pathogen on kiwifruit, during the last few years several authors have developed new molecular methods (Biondi *et al*., 2013; Balestra *et al*., 2013; Gallelli *et al*., 2014). However, these latter methods need to be validated for their inclusion in the procedure for detection of Psa as screening and/or identification tests.

2.2. Project aims

The project aims to develop innovative diagnostic tools to improve Psa detection and identification in symptomatic and symptomless kiwifruit plant material, including pollen and to improve the knowledge on the epidemiology of *Pseudomonas syringae* pv. *actinidiae* in different areas of Europe.

In particular a test performance study will be organised to produce validation data for relevant detection methods to be used for the detection and identification of Psa on symptomatic and symptomless kiwifruit materials (leaves, pollen and wood tissues).

2.3. Description of the main activities

2.3.1. Test performance study for validation of diagnostic and detection methods

National Reference Laboratories (NRL) need to verify the performance of detection and identification methods developed in-house or choose from those available. The most reliable methods should be taken into consideration during the development of official diagnostic

procedures. In order to meet these needs, a test performance study (TPS) was performed among ten European laboratories (from France, Spain, Greece, Austria, Portugal, Italy), two laboratories from New Zealand and one from Turkey. The TPS allowed to compare the available Psa detection methods: isolation on selective NSA and KB media, single, duplex, nested, multiplex, real-time PCR tests (Rees-George *et al*., 2010, Gallelli *et al*., 2011a, Biondi *et al*., 2013; Balestra *et al*., 2013; Gallelli *et al*., 2014). The performance of these tests was assessed on blind samples consisting of 13 woody extract and 11 pollen samples of kiwifruit spiked with Psa bacterial suspensions at different concentrations (from 10^7 up to 10 CFU/mL of plant extract). The TPS was organised in accordance to the EPPO Diagnostic Standards PM7/76(4) (EPPO, 2017), PM7/98(2) (EPPO, 2014a), and PM7/122(2) (EPPO 2014b).
Data from the TPS was statistically analysed to assess the performance of each test. In order to provide complete validation data, both for detection and identification, this TPS was supplemented by a further study of identification from pure culture of phylogenetically closely related *Pseudomonas* spp., Psa, and bacterial strains associated with kiwifruit.
All details about methods are reported in the article Loreti *et al*., 2018.

2.3.2. Epidemiological studies on Pseudomonas syringae pv. actinidiae in different areas of Europe

Italy - In northern Italy, an experimental orchard was planted in 2011, in order to perform experiments to confirm the epidemiological role of contaminated pollen in the introduction and survival of Psa in the field and its possible association to a disease outbreak. The orchard was located 90 km away from the nearest kiwifruit area, to be sure that no natural Psa inoculum could contaminate the experimental area, through wind and rain driven cells, possibly present in infected kiwifruit orchards.
During the growing season 2014 the experimental orchard (4-year old trees) was artificially pollinated, according to the common application procedures, and disease development monitored. As a control, a neighbouring kiwifruit orchard planted at the same time as a negative control (same age, same cultivar), was pollinated with pollen certified free from Psa. During the five months after pollination, the experimental orchards were fortnightly inspected, in order to monitor disease development.

Portugal - In 2010, Psa was first detected in the North region of Portugal in *Actinidia deliciosa* orchards, as well as in propagation material used to plant new orchards. Since then, yearly national surveys have been performed by the Portuguese Phytosanitary Authority and samples analysed by the INIAV phytobacteriology laboratory. Disease incidence and progression was also assessed in the North and Central regions, where kiwifruit orchards are economically important.

France - Psa was detected for the first time in 2010. Since 2011, a national survey was performed to determine the orchards and nurseries affected by Psa. No extension of the epidemic was observed in 2012 (Bourgouin & Fritsch, 2013). However, the climatic conditions during spring 2013 favoured the spread of Psa and by the end of that year it was estimated that 10 to 15% of the orchards were affected (Bourgouin & Fritsch, 2013). A study on the characterization of Psa isolated from France was performed as reported in Cunty *et al*. (2015).

Spain - The presence of Psa was first discovered in Pontevedra, Galicia region, the main kiwi fruit producing region of Spain. The isolates were identified as similar to the virulent Psa biovar 3 reported in other countries (Abelleira *et al*. 2013). In the course of surveys performed in the following years, strains of *P. syringae* pv. *actinidifoliorum* were found in the close region of A Coruña (Abelleira *et al*. 2015). Other *P. syringae* strains close but not identical to this new pathovar are under study for their accurate taxonomic classification.
As the asymptomatic plant material has been considered responsible of the introduction of the disease in new areas, regional surveys were performed since then and plant material analysed following the EPPO protocol, with small modifications.

2.4. Main results

2.4.1. Test performance study for validation of diagnostic and detection methods

Results on the detection and isolation of Psa from pollen samples were unexpected (little success in isolating the pathogen or to detect its DNA from experimentally infected samples), probably because Psa cells died during sample transportation. The hypothesis taken into consideration was that the artificial inoculation of pollen makes Psa more vulnerable to external conditions, with respect to the natural colonization, and Psa died and was degraded. Wood samples allowed the participating laboratories to apply all the required methods reliably, thus obtaining an overview of the performance criteria either for Psa detection and identification for all tested protocols.
The results showed that simplex PCRs gave good results, whereas duplex-PCR and real time PCR were the most reliable tools for detection and identification of Psa. Nested and multiplex-PCR gave false-positive results.
A detailed description of the obtained results is reported in Loreti *et al*., 2018.

2.4.2. Epidemiological studies on Pseudomonas syringae pv. actinidiae in different areas of Europe

Italy - Disease symptoms, (leaf spots) developed during 8-10 weeks after pollination with contaminated pollen; no canker developed. No symptoms were detected in the control orchard. In spring 2015, typical bleeding cankers started to develop in the orchard pollinated with Psa-contaminated pollen the previous year. Until September 2015, the disease progressed dramatically: a few trees died and several others showed cankers on wines, cordons and trunks. No symptom related to Psa was detected in the control orchard during 2015. Therefore, according to our experimental results, it was possible to confirm that pollen is an efficient pathway for Psa dissemination into new areas and might be the cause of severe disease outbreaks through artificial pollination. We also observed, that the highest disease intensity may appear not during the months following pollination, but the following year, when a sufficiently high population of Psa may have built in the pollinated orchard.

Portugal - Between 2010 and 2013, more than 100 bacterial isolates were collected along the country from infected plants of different cultivars and ages. The use of two conventional PCR protocols allowed identifying all known Psa biovars. Further, characterization based on genes coding for coronatin (Cfl) and/or phaseolotoxin (argK) allowed excluding the presence of biovar 2 among the Portuguese strains. Additionally, BOX-PCR fingerprinting profiles and the phylogenetic tree based on rpoD were characteristic of biovar 3 for most of the strains tested.

The lack of avrD1 amplification indicated the presence of a small population of biovar 4 strains recently allocated to *Pseudomonas syringae* pv. *actinidifoliorum*. Conclusions were drawn on the presence of two different pathovars of Psa affecting Portuguese kiwi orchards. *P. syringae* pv. *actinidifoliorum* might have been introduced in the early 2000, while the main population, highly aggressive, is present in the infected plants within the major production areas, leading to relevant yield losses (Cruz *et al*., 2014).

France - The deep characterization of the Psa strains collected during the monitoring activity performed in France, confirmed that Psa bv. 4 strains differed from Psa strains belonging to bv. 1, 2, 3 and was renamed *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov., (Cunty *et al*. (2015). *P. syringae* pv. *actinidifoliorum* differed mostly from *Pseudomonas syringae pv. actinidiae* at pathogenic level, as this pathovar cannot induce canker on wood. *Pseudomonas syringae* pv. *actinidifoliorum* seems to be present in France before the detection of recent outbreaks of Psa.

Spain - In general, a good correlation has been observed among the results obtained by the different techniques reported in EPPO protocol. Results from Galicia, where around 851 ha of kiwi are currently cultivated, indicate that in 2012-2014, 31 asymptomatic samples from nurseries (out of 164) and 47 from orchards (out of 165) were Psa positive. In Asturias, where around 168 ha of kiwi are currently cultivated, in 2012-2014, 5 samples from nurseries (out of 104) were Psa positive. In Comunidad Valenciana, where the estimated area of kiwi has increased from 23 ha in 2011 to 262 ha in 2013, in 2012-2014, 276 samples from nurseries and 36 from orchards were negative for the target. In Cantabria, where around 40 ha of kiwi are currently cultivated, in 2013-2014, 39 samples from orchards were negative for the target. In País Vasco, where around 75 ha of kiwi are currently cultivated, in 2013-2014, 5 samples from orchards analysed were also negative. These results confirm the frequent dissemination of the bacterium with asymptomatic plant material.

2.5. Conclusions and recommendations to policy makers

The TPS activity allowed to obtain validation data for the most suitable methods for the detection and identification of Psa. In particular, whereas simplex PCRs (Rees-George *et al.,* 2010) gave good results, duplex-PCR (Gallelli *et al*., 2011) and real time PCR (Gallelli *et al*., 2014) demonstrated to be the most reliable tools for both the detection and identification of Psa. Nested (Biondi *et al*., 2013) and multiplex-PCR (Balestra *et al*., 2012) gave false-positive results. The use of the most reliable detection test is suggested for routine analyses, but when Psa free status needs to be accurately assessed, it is recommended that at least two detection tests are used as reported in Loreti *et al*. (2018). These evidences are useful for the National Plant Protection Organisation (NPPO) laboratories (references laboratories, plant protection services laboratories) and for a possible revision of the official diagnostic protocols (e.g. European and Mediterranean Plant Protection Organization (EPPO) protocol PM7/120 for the detection of Psa).

The epidemiological studies showed several evidences. First, Psa bv. 4 was a new pathovar, named P. syringae pv. actinidiofoliorum. This aspect is crucial because of the higher phytosanitary impact of Psa (i.e. destruction of infected material) with respect to *P. syringae* pv. *actinidiofoliorum*, notoriously less aggressive on kiwifruit, not included in the A2 list of

EPPO or considered a quarantine pathogen. Another important aspect revealed by this study is the risk of Psa dissemination by asymptomatic material and by pollen. This suggests the importance of using plant propagation material and pollen controlled, for the absence of Psa, by reliable laboratory analyses. The use of Psa-free certified material avoids the large-scale dissemination of this pathogen.

2.6. Benefits from trans-national cooperation

The results obtained by the TPS provide a wide comparison of the available diagnostic methods. The involvement of 13 laboratories experienced in the Psa analysis permitted to share information about the most used methods for Psa detection and identification. Through this collaboration a complete and harmonized diagnostic protocol was developed, taking into account all the methods used by the different labs in different regions of the world.

Cited references

- Abelleira A, Ares A, Aguin O, Picoaga A., López MM, Mansilla JP, 2013. Current situation and characterization of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Galicia (north west Spain). Plant Pathology (2013) Doi: 10.1111/ppa.12125.
- Abelleira A, Ares A, Aguin O, Peñalver J, Morente MC, López MM, Sainz MJ, Mansilla JP, 2015. Detection and characterization of Pseudomonas syringae pv. actinidifoliorum in kiwifruit in Spain. J Appl Microbiol.,119(6):1659-71.
- Balestra GM, Taratufolo MC, Vinatzer BA, Mazzaglia A, 2012. A multiplex PCR assay for detection of *Pseudomonas syringae* pv. *actinidiae* and differentiation of populations with different geographic origin. Plant Disease 97: 472-478.
- Biondi E, Galeone A, Kuzmanovic N, Ardizzi S, Lucchese C, Bertaccini A, 2013. *Pseudomonas syringae* pv. *actinidiae* detection in kiwifruit plant tissue and bleeding sap. Annals of Applied Biology 162, 60–70.
- Cruz L, Cruz J, Fernandes C, Chicau G, Tenreiro R. 2014. http://simposio.spf.scap.pt/images/Livro%20Resumos_SCAP-spf_wev_20nov2014.pdf
- Cunty A, Poliakoff F, Rivoal C, Cesbron S, Saux FL, Lemaire C, Jacques M, Manceau C, Vanneste J. 2015. Characterization of Pseudomonas syringae pv. actinidiae (Psa) isolated from France and assignment of Psa biovar 4 to a de novo pathovar: Pseudomonas syringae pv. actinidifoliorum pv. nov. Plant Pathology 64:582-596
- EPPO, 2012. Pest risk analysis for Pseudomonas syringae pv. actinidiae. EPPO, Paris, France. http://www.eppo.int/QUARANTINE/Pest_Risk_Analysis/PRAdocs_bacteria/12-18034%20 Express_PRA_PSA.pdf (accessed on January 25th, 2016).
- European Plant Protection Organization. (2014a). PM7/98 (2) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bulletin/Bulletin OEPP, 44, 117.
- European Plant Protection Organization. (2014b). PM7/120(1)Pseudomonas syringae pv. actinidiae. EPPO Bulletin/ Bulletin OEPP, 44(3), 360–375. European Plant Protection Organization. (2014c).
- PM7/122(1)Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories. EPPO Bulletin/ Bulletin OEPP, 44(3), 390–399.
- European Plant Protection Organization. (2017). PM7/76(4)-Use of EPPO diagnostic protocols. EPPO Bulletin/Bulletin OEPP, 47,7

- Gallelli A, L'Aurora A, Loreti S, 2011. Gene sequence analysis for the molecular detection of *Pseudomonas syringae* pv. *actinidiae*: developing diagnostic protocols. Journal of Plant Pathology 93, 425–35.
- Gallelli A, Talocci S., Pilotti M and Loreti S., 2014. Real-time PCR for detecting *Pseudomonas syringae* pv. *actinidiae* isolates causing the recent outbreaks of kiwifruit bacterial canker. Plant Pathology. [Doi: 1111/ppa.12082]
- International Standardization Organization 2003. ISO 16140:2003 Microbiology of food and animal feeding stuffs - Protocol for the validation of alternative methods.In. Geneva, Switzerland.
- Loreti S., A. Cunty, N. Pucci, A.Chabirand, E. Stefani, A.Abelleira, G. M. Balestra, D. A. Cornish, F.Gaffuri, D. Giovanardi, R. A. Gottsberger, M. Holeva, A.Karahan, C. D. Karafla, A. Mazzaglia, R. Taylor, L. Cruz, M. M. Lopez, J. L. Vanneste and F. Poliakoff, 2018. Performance of diagnostic tests for the detection and identification of *Pseudomonas syringae* pv.*actinidiae* (Psa) from woody samples. *Eur J Plant Pathology* https://doi.org/10.1007/s10658-018-1509-5
- PM 7/98 (2), 2014. Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. *EPPO Bulletin* 40(1): 5-22.
- PM 7/122(1), 2014.Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories. EPPO Bullettin 44 (3): 390–399
- Rees-George J, Vanneste J, Cornish DA, Pushparajah IPS, Yu J, Templeton MD, Everett KR, 2010. Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S-23S r DNA intertrascribed spacer region and comparison with PCR primers based on other gene regions. *Plant Pathology* 59, 453–464.
- Tontou R, Giovanardi D, Stefani E, 2014. Pollens a possible pathway for the dissemination of *Pseudomonas syringae* pv. *actinidiae* and bacterial canker of kiwifruit. *Phytopathologia Mediterranea* 53, 333-339.
- Zespri, 2012. Mitigating the risk of *Pseudomonas syringae* pv. *actinidiae* introduction by pollen. In: Zespri Innovation Project V11285 report of February 2012, pp. 28. Available online at: http://www.kvh.org.nz/vdb/document/1144

3. Publications

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

None

3.2. Article for publication in the EPPO Reporting Service

None

3.3. Article(s) for publication in other journals

The results of the test performance study organized in the framework of the present project was published in a peer-reviewed scientific journals as folllowing reported:
Loreti S., A. Cunty, N. Pucci, A.Chabirand, E. Stefani, A.Abelleira, G. M. Balestra, D. A. Cornish, F.Gaffuri, D. Giovanardi, R. A. Gottsberger, M. Holeva, A.Karahan, C. D. Karafla, A. Mazzaglia, R. Taylor, L. Cruz, M. M. Lopez, J. L. Vanneste and F. Poliakoff, 2018. Performance of diagnostic tests for the detection and identification of Pseudomonas syringae pv.actinidiae (Psa) from woody samples. Eur J Plant Pathology https://doi.org/10.1007/s10658-018-1509-5

Meeting talks:
Loreti S., F. Poliakoff, E. Stefani, A. Abeillera, G. M. Balestra, L. Cruz, F.Gaffuri, D. Giovanardi, P.E. Glynos, R. Gottsberger, M.C. Holeva, A.Karahan, C. D. Karafla, M. M. Lopez, A.Mazzaglia, N. Pucci, M. Pilotti, R. K. Taylor, M. C. Taratufolo, Joel L. Vanneste. A test performance study (TPS) on the detection and identification of Pseudomonas syringae pv. actinidiae from wood and pollen blind samples. II International PSA Symposium, 10-13 giugno 2015, Bologna, Italia.

4. Open Euphresco data

None