MINIREVIEW

DIAGNOSIS AND DETECTION OF THE MAIN BACTERIAL PATHOGENS OF STONE FRUIT AND ALMOND

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SUMMARY

Diagnosis and detection are key aspects related to plant health status. A critical review of the available diagnostic methods utilised for *Agrobacterium rhizogenes* and *A. tumefaciens*, *Pseudomonas amygdali*, *P. syringae* pv. *mors-prunorum*, *P. syringae* pv. *persicae*, *P. syringae* pv. *syringae* and *Xanthomonas arboricola* pv. *pruni,* the main pathogens of the stone fruit trees, is presented. As there is a general lack of updated standardized protocols for the detection of most of these bacteria, the most appropriate media for their isolation are reported along with serological reagents, PCR and real-time PCR protocols with comments on their accuracy for the analysis of these pathogens in plant samples. There are many selective media for isolation, especially for *Agrobacterium* spp., but fewer for *Pseudomonas* spp. and *X. arboricola* pv. *pruni.* Serological techniques are not very useful for these pathogens due to the current lack of specific antibodies commercially available. As to molecular methods, it is surprising to find so many PCR protocols for *Agrobacterium* species, very few and unspecific for the *Pseudomonas* species pathogenic to stone fruit trees, and several recent PCR protocols for *X. arboricola* pv. *pruni*. The new advances in genomics and proteomics will provide information for selecting new targets to develop specific and sensitive techniques for the diagnosis and detection of these bacterial pathogens in plant material.

Key words: isolation, selective media, PCR, real-time PCR, immunofluorescence, ELISA.

INTRODUCTION

The main bacterial pathogens of stone fruit and almond trees in the European Union (EU), taking into account their incidence, economic damages to these crops, and the threat that they represent for the European agriculture are: *Agrobacterium rhizogenes* and *A. tumefaciens*, causal agents of tumours in roots, crown and aerial parts of the trees (Scortichini, 1995; López, 2000; Janse, 2006), *Pseudomonas amygdali*, *P. syringae* pv. *mors-prunorum*, *P. syringae* pv. *persicae*, and *P. syringae* pv. *syringae*, responsible for shoot necrosis, fruit and leaf spots and cankers on trunks and branches (Scortichini, 1995; López, 2000; Janse, 2006), and finally *Xanthomonas arboricola* pv. *pruni*, the agent of bacterial spot disease of stone fruits (Civerolo and Hatting, 1993; Scortichini, 1995; Janse, 2006), recently reported also on almond (Palacio-Bielsa *et al*., 2010).

The main methods for their diagnosis have recently been reviewed by Janse (2010) in a compilation of the different techniques utilised or developed for these and other bacterial pathogens in the framework of the EU-COST Action 873. Moreover, a recent publication by Palacio-Bielsa *et al*. (2009a), reviewed the PCR protocols designed for all species, subspecies and pathovars of plant pathogenic bacteria designed up to 2007. Consequently, as this information is already available, in this mini review we intend to critically analyse the most utilised methods for the diagnosis and detection of *Agrobacterium* spp., *Pseudomonas* spp. and *X. arboricola* pv. *pruni*.

According to López *et al*. (2006) the term "diagnosis" is reserved for identifying the nature and cause of a disease in plants showing symptoms, whereas "detection" is referred to tracing the presence of a target organism in symptomless plant tissues, or in other environmental samples. We will not provide the reader with an exhaustive list of methods and references, but will advise on the most robust techniques, based on our experience and the available literature, indicating also the gaps and the near future perspectives. For each bacterial species (or group of species with similar characteristics) a selection of the available media for isolation, reagents for serological techniques and protocols for PCR and real-time PCR are reported. Comments about their accuracy for diagnosis and detection in stone fruit and almond are added in each case.

Identification methods are not exhaustively reviewed because they should require an entire review by themselves, but some key common aspects are discussed after the diagnostic and detection methods.

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GENERAL ASPECTS AND TECHNIQUES UTILIZED

Diagnostic and detection techniques for plant pathogenic bacteria have traditionally used microscopic observation, isolation on culture media, serological testing, bioassays and more recently, molecular assays. Standard protocols, especially those of the European and Mediterranean Plant Protection Organization (EPPO) rely, in addition to other methods, on the isolation of the target organism followed by its identification and verification of its pathogenicity. This whole process may take several weeks for final confirmation and may be difficult for processing large number of samples when a high number of positives are expected. Another drawback is related to the lack of growth on solid media of the injured, or viable but not culturable bacteria (VBNC). As this state is sometimes reversible (López *et al*., 2006), the direct isolation, apart from problems with its detection threshold, could therefore give false negative results. However, plating efficiency can be improved by a preceding enrichment step through cultivation in liquid medium or by a bioassay *in planta*.

Serological techniques utilised for bacterial pathogens are mainly indirect immunofluorescence staining (IF), several types of enzyme-linked immunosorbent assay (ELISA) and the recent so-called lateral flow devices. Their accuracy is correlated with the quality of the antibodies utilised and when possible, the use of monoclonal antibodies is advisable. In general, serological methods are robust, cheap and appropriate for a first screening and for massive analyses.

The use of DNA-based methods has exponentially increased in the last twenty years, but it has not yet completely replaced traditional methods. Conventional PCR has replaced DNA hybridization very fast, but in the more recent years, real-time PCR is leading to faster and more accurate detection protocols, of high sensitivity and relatively easy to perform. Other DNA or RNA-based methods like Nucleic Acid Sequence Based Amplification (NASBA) or Loop Mediated Isothermal Amplification (LAMP) have also been applied to few bacterial pathogens (López *et al*., 2009) but, till now, not for those affecting stone fruit and almond.

In the diagnosis of these pathogens, the sensitivity for isolation is generally from 1-10^3 CFU ml^{-1}, for IF is about 10^3-10^4 CFU ml^{-1}, for ELISA and lateral flow devices about 10^5-10^6 CFU ml^{-1}, (but can be improved with a previous enrichment step), for conventional PCR is 10^2-10^4 CFU ml^{-1} and from 1-10^3 CFU ml^{-1} for real time PCR. In addition to the sensitivity of the techniques, other important factors to evaluate are specificity and accuracy, which should be compared for the different techniques before designing a protocol. Such evaluation has, to our knowledge, not yet been done for bacterial pathogens of stone fruits. In the absence of enough data about the accuracy of each technique in different types of plant material, there is a tendency to use integrated approaches including conventional, serological and molecular assays (Alvarez, 2004; López *et al*., 2003). This is especially required for quarantine pathogens, like *P. syringae* pv. *persicae* or *X. arboricola* pv. *pruni*, where the use of more than one technique based on different biological principles is advised for maximum accuracy.

For the analysis of symptomless plants, environmental samples, or for detecting latent infections, the maximum sensitivity is required because we intend to detect very low populations of the target bacteria. Consequently, a previous enrichment step is generally advisable before isolation, enrichment or conventional PCR. Real-time PCR can often be used directly with high sensitivity protocols (López *et al*., 2006, 2009).

Microarrays are one of the most promising tools for simultaneous detection of a wide-range of organisms in a single assay and their versatility has been recently explored also for rapid diagnosis. A prototype DNA microarray for diagnosis has been developed for the rapid and simple identification of 22 European quarantine phytopathogenic bacteria (Pelludat *et al*., 2009). The microarray has 38 probes targeted to the 16S rDNA and the housekeeping genes *rpoB*, *groEL* and *ftsZ*, enabling differentiation of quarantine bacteria down to the species and, in some cases, subspecies level. This work was conducted within the European research networking frameworks of EU-COST Action 853 (Agricultural Biomarkers for Array Technology) and EU-COST Action 873 (Bacterial diseases of stone fruits and nuts). Once improved and validated, these chips would be optimal tools to identify quarantine plant pathogenic bacteria and to prevent their dissemination (López *et al*., 2009).

DIAGNOSIS AND DETECTION OF *AGROBACTERIUM TUMEFACIENS* AND *AGROBACTERIUM RHIZOGENES*, CAUSING TUMOURS IN STONE FRUIT AND ALMOND

A. tumefaciens and *A. rhizogenes* are current names corresponding to biovar 1 and biovar 2 strains of pathogenic and non-pathogenic *Agrobacterium*. Strains of both species can cause the typical tumours on roots, crown and, sometimes, on stems or trunks, due to the transfer of the T-DNA of the Ti plasmid from the bacteria to the plant cell. Although several taxonomic studies propose other nomenclature for these species, the above names will be used in this review according to Sawada *et al*. (1993) and with the modification suggested by Bouzar (1994). Both bacterial species can produce tumours in most stone fruit and almond cultivars, several rootstocks being very susceptible. Although the presence of tumours can directly affect the plant growth, losses are primarily related with the prohibition of com-

mercialization of plants with tumours. For these reasons, these bacteria are considered as quality pathogens according the EU legislation (Anonymous, 1993). There is currently a lack of standardized protocols for diagnosis and detection of these pathogens. Disease diagnosis can fail if it is only based on the isolation of *Agrobacterium*-like colonies on selective or non-selective media, followed by identification and confirmation of pathogenicity. This may happen because of the low populations of pathogenic cells in the tumours (Cubero *et al*., 1999), or the possible presence of bacterial cells in the VBNC state, for example when excessive copper chemicals are used for its control (Alexander *et al*., 1999). Consequently, the use of PCR or another DNA-based complementary approach is advisable.

Isolation. The literature reports many media designed for specific *Agrobacterium* isolation, but there are frequent problems in succeeding the isolation, due to the difficulties in obtaining pathogenic strains from tumours, from latent infections and especially from soil (Moore *et al*., 2001), the mentioned low bacterial populations in tumours (and the even lower levels in the plants outside them) and the slow development of the colonies in several of the semi-selective media. Although there is a general lack of true selective media, those described by Schroth *et al.* (1965) for *A. tumefaciens* and New and Kerr (1971) for *A. rhizogenes*, are still among the most useful for isolations and perform very well when compared with those more recently developed (Moore *et al*., 2001). For isolations from soil a tellurite-amended medium is advised (Mougel *et al*., 2001). The isolations must always be followed by the identification of the colonies as pathogenic *Agrobacterium* spp. by biochemical tests, 16S rRNA sequencing, PCR, and inoculation to herbaceous or homologous hosts.

Serological techniques. The serological approach has failed for detection and identification of pathogenic agrobacteria because of the large serological variability of *A. tumefaciens* and *A. rhizogenes* strains and the lack of specific antibodies for pathogenic strains of both species. Alarcón *et al.* (1987) and Benjama *et al.* (1995) raised and evaluated different antisera and used them in IF, ELISA, electrophoresis and other methods, but could not find any species-specific reagent among the studied ones. There are no validated commercial antibodies available.

Molecular techniques. Although a number of PCR protocols have been described for identification and/or detection of tumourigenic *Agrobacterium*, the present work focuses only on those related to stone fruits. An extensive review of PCR protocols for detection and identification of *Agrobacterium* is available in Palacio-Bielsa *et al*. (2009a).

One of the major limitations to the routine use of PCR for *Agrobacterium* detection and diagnosis is the presence of inhibitory compounds in the template, such as polyphenols, which should be eliminated, preferably with a DNA extraction step (Cubero *et al*., 1999). Some PCR protocols include an internal control in order to recognize false negatives due to inhibitors from the hosts of *A. tumefaciens* or soil, thus improving the reliability of PCR for searching for pathogenic *Agrobacterium* in plants (Cubero *et al*., 2002).

Choice of primers is another important factor in the optimisation of PCR. For conventional PCR, Nesme *et al*. (1989) designed several sets of primers within different regions of the Ti plasmid involved in *Agrobacterium* pathogenicity. The most utilised were those from the T-DNA region (FGP*tmr*530/FGP*tmr*701), and the intercistronic region between *virB* and *vir*G, in the virulence region of the pTi (FGP$virB_{11+12}$/FGP*vir*B15). The tmr primers were designed to amplify both nopaline and octopine type pTi, whereas virB-G primers were reported for specific amplification of nopaline, but not octopine pTi type. However, as indicated by Cubero *et al.* (1999), amplification was also obtained using these primers from *A. tumefaciens* strains with an octopine Ti-plasmid. Both sets of primers have been successfully used for detection of *Agrobacterium* spp. from *Prunus* material following a simple DNA extraction with isopropanol (Cubero *et al.*, 1999). In a comparison of techniques performed by Cubero *et al*. (1999), these PCR protocols were more efficient for *Agrobacterium* detection than isolation on selective and non-selective media with or without previous enrichment.

PCR performed with virB-G primers was also effective for detecting the systemic movement of *Agrobacterium* spp. in cherry and other hosts (Cubero *et al.*, 2006). Pathogenic agrobacteria were detected even in symptomless stem fragments of the inoculated plants (between the primary and secondary tumours), and in stems, crown and roots of naturally infected walnut plants showing tumours at the graft union. A modified protocol using virB-G primers has been developed to improve the efficiency in detecting the target sequence from peach roots (Puopolo *et al*., 2007).

Amplification with primers designed from sequences within Ti plasmid regions involved in pathogenicity will be only obtained if DNA from pathogenic *Agrobacterium* is present in the sample. Conversely, with primers from the *tmr* region, the amplified sequence is a fragment inside the T-DNA which is integrated in the plant genome during infection and therefore, amplification could be probably obtained also from the transformed plant cells (Cubero *et al.*, 1999).

Primer set VCF/VCR was designed to amplify fragments of the Ti and Ri plasmid-encoded *virC1* and *virC2* regions from all pathogenic *Agrobacterium* strains (Sawada *et al.*, 1995). The specificity of the original

primer set was improved by designing a new set of primers (VCF3/VCR3) for amplifying tumourigenic *Agrobacterium* strains isolated from apple seedlings (Sawada *et al.*, 2004). Their usefulness for detecting the target in stone fruit has not been tested.

Two sets of primers, based on the *virD2* and *ipt* genes, were designed for specific detection of pathogenic *Agrobacterium* strains (Haas *et al.*, 1995). The former set detects different *Agrobacterium* species, whereas the primer pair corresponding to conserved sequences in *ipt* gene detects only *A. tumefaciens* and distinguishes it from *A. rhizogenes*. Both primer pairs can also be used together in the same PCR amplification, thus allowing simultaneous detection of both genes in a single reaction, but there are no available data about their efficiency in the analysis of plant material.

A multiplex PCR has been designed to aid in identification and differentiation of *A. tumefaciens, A. rhizogenes, A. rubi* and *A. vitis* (Pulawska *et al.*, 2006). Five primer pairs were designed on the basis of the nucleotide sequence of the 23S rDNA. One of them is universal for all agrobacteria, whereas the remaining four are species-specific. Differentiation between biovar 2 and the others requires the use of restriction analysis of the PCR product and the protocol has only been used for strain identification.

Determination of whether a soil is free from tumourigenic *Agrobacterium* could be a crucial aspect for fields selected for establishing a nursery. A semi-nested PCR, using three primer pairs based on the *tms2* gene, was designed for detection of tumour-inducing *Agrobacterium* from naturally infected soils (Pulawska and Sobiczewski, 2005). Primers from Haas *et al.* (1995) have also been used in both conventional and real-time PCR for detection of *A. tumefaciens* in soil (Sudarshana *et al.*, 2006), achieving a sensitivity of 200 and 20 CFU g^{-1} soil, respectively.

A comparative study and validation of different PCR protocols would be advisable for improving the reliability of PCR for detection of pathogenic *Agrobacterium* occurring on stone fruit. This is important because the various DNA amplification-based assays have been developed separately and, in some cases, validated only with a limited number of strains and/or with strains of limited geographical origins. A systematic comparison of the existing assays, on a uniform and representative set of plant samples, will provide the required data to enable a deliberate choice for regulatory and management programs and also to check for *Agrobacterium*-free plant material.

Information about the sequences of the genome of *A. tumefaciens* strain C58 (Wood *et al.*, 2001; Goodner *et al.*, 2001) and of the biological control agent *A. rhizogenes* K84 (available, but still unpublished) should provide more targets for designing specific PCR protocols, or for developing microarrays or other tools.

DIAGNOSIS AND DETECTION OF *PSEUDOMONAS* spp. AFFECTING STONE FRUITS AND ALMOND

The main pseudomonads causing diseases worldwide and producing severe yield losses are *Pseudomonas syringae* pv. *syringae* (*Pss*), *Pseudomonas syringae* pv. *morsprunorum* (*Psm*) and *Pseudomonas syringae* pv. *persicae* (*Psp*) on stone fruit, and *Pseudomonas amygdali* (*Pa*) on almond trees. Some other *Pseudomonas* spp. described as causal agents of different diseases observed in stone fruits are *Pseudomonas marginalis* pv. *marginalis*, *Pseudomonas syringae* pv. *avii* and *Pseudomonas viridiflava* (Bradbury, 1986; Takanashi and Shimizu, 1989). Information of these pathogens and others affecting stone fruits has been reviewed by Janse (2010), a member of the EU-COST 873 group of investigators.

Psp is considered a quarantine organism (Annex II, Part A-Sect. II) in the European Union (Anonymous, 2000 and amendments). EPPO includes it in the A2 quarantine organism list and published a specific Data Sheet [http://www.eppo.org/QUARANTINE/bacteria/Pseudomonas_persicae/PSDMPE_ds.pdf]. For this reason, there is a protocol for sampling, which includes some testing methods, and a more recent one for diagnosis recommended by EPPO (Anonymous, 1992, 2005). Although *Pss, Psm* and *Pa* are not considered quarantine organisms, the damages caused by them to fruit crops worldwide are severe enough to consider here the main aspects of their diagnosis and detection. *Pss* and *Pa* are the only described phytopathogenic pseudomonads reported on almond trees.

Isolation. Selecting the right material in the most favourable sampling conditions for recovering a given pathogen is especially important in the case of stone fruit-infecting *Pseudomonas* species. Only in some months of the year, during the most convenient period (usually early spring), these bacteria are active in infected tissues or occur as epiphytes in large populations. Several semi-selective media have been described, such as D-4 (Kado and Heskett, 1970), Hildebrands' media A, B, C, D and E (Hildebrand, 1971), PSM (Burr and Katz, 1982), KBC (Mohan and Schaad, 1987), SPS (Miyoshi and Tachibana, 1994) and mP3 and MS3 (Vicente *et al.*, 2004). The most commonly used medium however, with less toxicity for the target pathogens is still King's medium B (King *et al.*, 1954). This medium could be supplemented with cycloheximide to avoid fungal growth (Whitesides and Spotts, 1991). *Pss* and some strains of *Psm* produce a fluorescent pigment on King's B under UV light, while *Pa*, *Psp* and some strains of *Psm* do not. Some other media, i.e. CSGA (Luisetti *et al.*, 1972) or CSGM (Lelliot and Stead, 1987) have been described for observing this pigment in *Psp,* but still 20% of the strains failed to produce it

on CSGA medium (Luisetti, 1988).

Isolation must be followed by identification of the isolates. In the case of *Pa* identification could be performed by biochemical and physiological tests, fatty acids methyl-ester profiles (FAME), 16S rRNA sequencing, *rpoB* gene sequences and pathogenicity assays. Such verification is required for confirmatory diagnosis. In the case of *Pseudomonas syringae* pathovars, and due to their phenotypic and genomic heterogeneity, an integrated approach should be used to identify the isolates, selecting among the different techniques available, such as biochemical and physiological tests, toxins and siderophores production, fatty acids methyl-ester profiles (FAME), protein profiling analysis (SDS-PAGE), serological tests, rep-PCR profiles, RFLP, *gyrB* and *rpoD* sequences, 16S rDNA sequences, multilocus sequence analysis (MLSA), and others.

Pathogenicity assays in plants are essential in addition to the well known procedures for HR in tobacco leaves or bioassays on detached organs (small pear and cherry fruits, yellow lemon fruits, lilac leaves or shoots). For a recent overview of these tests, see Bultreys and Kaluzna (2010) and Janse (2010). Another recent publication compares some inoculation procedures for proving pathogenicity (Gilbert *et al*., 2010).

Serological tests. For IF and ELISA, commercial antisera are only available for *Pss* (Neogen Europe, UK). However, some *Psp* strains show common antigens mainly with *Pss* and *P. syringae* pv. *papulans* [http://www.eppo.org/QUARANTINE/bacteria/ Pseudomonas_ persicae/PSDMPE_ds.pdf]. Polyclonal antisera enabled discrimination of 23 O-serogroups among the *P. syringae* pathovars (Saunier *et al*., 1996) and a study with monoclonal antibodies (Ovod *et al*., 1999) revealed that strains belonging to a broad group of pathovars showed cross-reactivity with a panel of core-specific monoclonal antibodies. So, it is necessary to check carefully the available commercial antibodies and to know the possible cross-reactions with other pathovars of *P. syringae* or even with other *Pseudomonas* spp. Due to the lack of specific polyclonal or monoclonal antibodies, serological techniques cannot be advised for diagnosis or detection in these cases.

Molecular techniques. Some PCR protocols have been developed for detecting genes responsible for the production of toxins or siderophores by different *Pseudomonas* species, e.g. for *syrB* and/or *syrD* genes, involved in the synthesis and export of these lipodepsipeptides and conserved among *Pss* strains (Bultreys and Gheysen, 1999; Sorensen *et al*., 1998). Although the toxins syringomycin, syringotoxin, syringostatin and syringopeptin are produced by some *Pss* strains, they are not restricted to this pathovar (since they are also produced by pathovars *aptata* and *atrofaciens*) or this species (because syringotoxin is also produced by *P. fuscovaginae*). PCR assays using the primer pair *syD1* and *syD2* were positive with almost all the *Pss* strains tested, except for one, and also for strains of *P. syringae* pv. *atrofaciens* and *P. syringae* pv. *aptata* but not for *P. fuscovaginae* (Bultreys and Gheysen, 1999). Another PCR protocol was developed by Bereswill *et al*. (1994) for detecting a region of the coronatine biosynthetic gene cluster responsible for coronatine production (*cfl* gene). This toxin is produced by strains belonging to the *P. syringae* pathovars *alisalensis*, *atropurpurea*, *glycinea*, *maculicola*, *mors-prunorum*, *porri* and *tomato* (Braun *et al.*, 2008). The *cfl* gene sequences were also detected in *P. syringae* pathovars *aesculi*, *berberidis*, *ulmi*, *spinaceae* and *zizaniae*, and in *P. cannabina* (Bultreys *et al*., 2008). It is also necessary to consider the possible existence of non-toxin producing strains, as shown for those of *P. syringae* pathovars *glycinea*, *maculicola*, *mors-prunorum* and *tomato*, which do no synthesize coronatine (Bereswill *et al*., 1994).

The development of a PCR protocol for detecting the gene *irp1* involved in the production of the siderophore yersiniabactin (Bultreys and Gheysen, 2008; Bultreys *et al*., 2006), failed because the assay proved not to be specific enough, being the yersiniabactin locus present in many species of enterobacteria (Bultreys and Gheysen, 2008).

The conclusion is that, until now, PCR protocols for toxins and siderophores produced by *Pseudomonas* are not yet specific enough to be used for their diagnosis or detection in plant material. However, they can be used combined with other tests for identification purposes.

As mentioned above, a DNA microarray, has been developed for the identification of 22 European quarantine phytopathogenic bacteria. In the case of *Psp* an accurate discrimination to the pathovar level was not achieved, because the same pattern was obtained for *P. syringae* pv. *pisi*. For its direct identification in plant material further improvement could be necessary to avoid cross-hybridisation with closely related pathovars (Pelludat *et al*., 2009) or to select other housekeeping genes with pathovar-specific sequences.

XANTHOMONAS ARBORICOLA pv. *PRUNI*, CAUSING BACTERIAL SPOT DISEASE OF STONE FRUIT AND ALMOND

Xanthomonas arboricola pv. *pruni* (*Xap*) is the causal agent of bacterial spot disease of stone fruit and almond (Young, 1977; Civerolo and Hatting, 1993; Palacio-Bielsa *et al*., 2010). It causes severe losses on susceptible *Prunus* spp. cultivars in several of the major stone fruit producing areas of the world. Because of its economic importance, *Xap* is considered a quarantine organism by the phytosanitary legislation of the European Union, (EU) (Anonymous, 2000 and amendments) and by EPPO (A2 List of pest recommended

for regulation, Anonymous, 2003).

Xap can have an epiphytic phase and/or be latent and, consequently, it can be transmitted by different types of plant material (Goodman and Hattingh, 1986; Dhanvantari, 1971; Zaccardelli *et al.*, 1998). For setting up effective quarantine measures, a specific, sensitive and rapid detection protocol for this target is required. Currently, only visual inspections for symptoms once a year are required to certify plants as free of *Xap* in nurseries, which is clearly insufficient to prevent dissemination of the pathogen. The only recently published diagnostic protocol is the EPPO Standard PM 7/64 (Anonymous, 2006) which includes isolation and IF, but no molecular methods.

Isolation. Two media, YDC (Stolp and Starr, 1964) and YPGA (Ridé, 1969; Lelliot and Stead, 1987) are cited in the EPPO Standard (Anonymous, 2006) for the isolation of *Xap* from symptomatic samples. This requires some experience, especially for isolation from cankers, but is not really difficult when the symptoms are relatively recent, because the morphology of the *Xanthomonas* colonies is quite typical. However, low concentration of *Xap* together with the presence of naturally occurring, rapidly growing saprophytic bacteria on the plates may hinder isolation from samples of symptomless plant material or in testing for epiphytic populations. In some cases, such difficulty could be surpassed by diluting the extracts to be assayed. To prevent fungal growth, addition of cycloheximide (250 mg l^{-1}) is advisable.

For isolation from symptomless buds and leaf scars, SP medium (Hayward, 1960), used by Zaccardelli *et al.* (1995), is recommended by the EPPO protocol (Anonymous, 2006). This medium has been validated using samples from peach and plum only but not from cherry, almond or apricot (Anonymous, 2006). Selective media XPS (Schaad and Stall, 1988) and XPSM (Civerolo *et al.,* 1982) have been described for isolation from symptomless plant material (Shepard and Zehr, 1994). Nevertheless, Zaccardelli *et al.* (1995) obtained better results from peach using SP medium than XPS. Moreover, XPSM medium may not be appropriate for all *Xap* strains (Civerolo *et al.*, 1982).

Isolation must be followed by identification of the colonies as *Xap* by means of biochemical tests, protein profiling analysis (SDS-PAGE), fatty acids methyl-ester profile (FAME), IF, REP-PCR analysis, sequencing of house keeping genes and confirmed by a pathogenicity test (Anonymous, 2006).

Serological tests. According to the EPPO Standard for *Xap* (Anonymous, 2006), symptomless plant material can be screened using IF. Samples of 100 pieces of tissue containing buds and leaf scars were analyzed by IF and isolation on SP agar (Zaccardelli *et al.*, 1995) with good results. However, as before, these techniques were validated on peach and plum but not on cherry, almond or apricot, although satisfactory results are likely to be achieved also on those species.

Currently, IF and ELISA commercial kits for *Xap* are available (ADGEN, UK; SEDIAG, France; AGDIA, USA). However, none of these polyclonal antibodies is specific for *Xap* since, in our experience, reactions are also obtained with other *Xanthomonas* species such as *X. campestris* pv. *campestris*, *X. hortorum* pv. *pelargonii*, *X. axonopodis* pv. *vitians*, *X. vesicatoria* and *X. cynarae*, and then probably with other *Xanthomonas*. Consequently, they could be advised for a rapid screening of the samples or to detect the presence of *Xanthomonas*-like bacteria, but not for accurate identification of *Xap*.

Molecular techniques. No PCR protocol, neither for detection nor for identification of *Xap* pure cultures, is included in the EPPO Standard (Anonymous, 2006). A special task force of the EU-COST Action 873 is entrusted with developing and validating molecular diagnostic methods for *Xap* in the framework of this Action. Methods that are being evaluated include PCR, quantitative PCR, and proteomics.

A conventional PCR protocol for specific detection of a fragment of a gene sequence from a putative protein related to an ABC transporter ATP-binding system in *Xap* was designed by Pagani (2004), using primers Y17CoF/Y17CoR. This protocol has been optimized and evaluated for EU-COST 873 participants from Spain and Italy (Peñalver *et al.*, 2008; Scortichini, 2008). However, although this method proved to be appropriate for both diagnosis and identification of the pathogen, it is not sufficiently sensitive to allow its reliable detection from symptomless plants. For the development of a specific duplex-PCR, the primers suggested by Pagani (2004) were also chosen by Dallai *et al.* (2009). Other primers designed on the *gyrB* sequences did not give satisfactory results, but could be taken into account in designing probes to be used in real-time PCR.

Another PCR-based method was reported for the rapid detection of *Xap* (Park *et al.*, 2008) using sequences targeting DNA regions related to the *hrp* gene cluster of *Xap*. Primer pair Xap5 and Xap3 directed the amplification of a 548 bp and 246 bp DNA fragment, respectively, from the genomic DNA of all known *Xap* strains tested, but not from other pathovars or bacterial species. The method was reported to be also useful for diagnosis of *Xap* from naturally infected *Prunus* samples, although details are not yet available.

The development of another duplex-PCR for identification of *Xap* from isolated cultures and for direct detection in plant samples has also been reported. One set of primers target a gene involved in quinate metabolism, and pathovar specific primers target a specific DNA se-

quence conserved among *Xap* strains. Reliable application was observed in preliminary tests for direct detection from symptomatic apricot fruit, leaf and wood samples (Pothier *et al.*, 2009a)

For the development and validation of real-time PCR assays, SYBR Green and TaqMan methodologies have been employed using the ABC primers Y17CoF2/ Y17CoR (Pagani, 2004). Both methods have been useful for identification of pure cultures of the pathogen. A real-time PCR with SYBR Green was developed and the same fragment was amplified from all *Xap* isolates. Specificity of this protocol was quite good and it was applied for the detection and diagnosis of *Xap* occurring on *Prunus laurocerasus* (Bergsma-Vlami, 2010).

A real-time PCR protocol with a TaqMan probe to be used for symptomatic as well as for symptomless plant material has been developed using primers and a probe based on the sequence of the transporter protein previously reported (Pagani, 2004). It was specific for *Xap* from different *Prunus* species and geographical origins, with a sensitivity of ca. 10^2 CFU ml^{-1} in leaves and dormant buds using a simple DNA extraction procedure (Llop *et al.*, 1999). In some cases, amplification was even possible using heat-treated leaf washes without the need of a DNA extraction step prior to amplification. The procedure has proved to be robust, rapid and can be automated with high sample throughput potential, permitting analysis of a large number of samples in few hours. This real-time PCR is highly reliable, sensitive and suitable as screening test for *Xap* detection and can be used as a rapid method complementary to isolation (Palacio-Bielsa *et al.*, 2009b).

For the diverse PCR protocols mentioned, further comparative tests, using symptomatic and symptomless plant material of different hosts are necessary to evaluate their sensitivity. The only remark is that all the PCR protocols based on Pagani (2004) primers, as well as the duplex-PCR developed by Pothier *et al.* (2009a) showed undesirable specific amplifications with *X. arboricola* pv. *corylina*, a hazelnut pathogen never reported from *Prunus* species.

Currently, in the framework of the EU-COST 873, the complete genome of *Xap* strain CFBP 5530 has been sequenced, and is being mined to gain insight into the virulence and ecological fitness mechanisms of this pathogen (Pothier *et al.*, 2009b). It is expected that in the near future, the information from this and other genomes of different *Xanthomonas* species and pathovars could provide new tools for designing a more specific and rapid detection and identification methodology.

GENERAL CONCLUSIONS

There are different methodologies available for detection and diagnosis of bacteria pathogenic to stone fruit and almond, ranging from conventional isolation to real-time PCR. Many diagnostic tools have been developed for *Agrobacterium* species and for *X. arboricola* pv. *pruni,* but there is a lack of updated methods and protocols for most of the *Pseudomonas* species. There is also a need of validated protocols for all of them, and more standardization is required.

The advances in these topics will not only have a direct effect in plant health but could be also applied to environmental studies, to obtain new information on the hidden life of these bacterial pathogens, that could help in designing more appropriate sampling and control methods. Although the information provided by the sequencing of several genomes of *Agrobacterium* strains and of one strain of *X. arboricola* pv. *pruni* seems not yet to have a direct effect on the development of new diagnosis and detection tools, it is expected to have a strong impact in the near future. The comparative analysis of plant pathogenic bacterial genomes will identify species-specific sequences, and functional genomics will identify genes involved in pathogenesis and we hope that new technologies will be developed for rapid high-throughput, multiple pathogen detection *in situ*.

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REFERENCES

Alarcón B., López M.M., Cambra M., Ortiz J., 1987. Comparative study of *Agrobacterium* biotypes 1, 2 and 3 by electrophoresis and serological methods. *Journal of Applied Bacteriology* **62**: 295-308.

Alexander E., Pham D., Steck T.R., 1999. The viable-but-non culturable condition is induced by copper in *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. *Applied and Environmental Microbiology* **65**: 3754-3756.

Alvarez A.M., 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. *Annual Review of Phytopathology* **42**: 339-366.

Anonymous, 1992. EPPO Standards. Phytosanitary procedures. *Pseudomonas syringae* pv. *persicae*, sampling and test methods. PM 3/44. *Bulletin OEPP/EPPO Bulletin* **22**: 243-246.

Anonymous, 1993. Commission Directive 93/48/EEC of 23 June 1993 setting out the schedule indicating the conditions to be met by fruit plant propagation material and fruit plants intended for fruit production, pursuant to Council Directive 92/34/EEC. *Official Journal of the European Communities* **L 250**: 1-8.

Anonymous, 2000. Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* **L 169**: 1-112.

Anonymous, 2003. Data sheets on quarantine organisms. *Xanthomonas arboricola* pv. *pruni*.Online:[http://www.eppo.org/QUARANTINE/BACTERIA/Xanthomonas_pruni/XANTRP_ds.pdf]

Anonymous, 2005. EPPO Standards. Diagnostics PM 7/43 *Pseudomonas syringae* pv. *persicae*. *Bulletin OEPP/EPPO Bulletin* **35**: 271-273.

Anonymous, 2006. EPPO Standards PM 7/64 (1) Diagnostics *Xanthomonas arboricola* pv. *pruni*. *Bulletin OEPP/EPPO Bulletin* **36**: 129-133.

Benjama A., Alami N., Saadaovi E.M., 1995. Étude serologique des souches marocaines d'*Agrobacterium tumefaciens*, agent de la galle du collet des rosacées fruitières. *Agronomie* **16**: 517-522.

Bereswill S., Bugert P., Völksch B., Ullrich M., Bender C.L., Geider K., 1994. Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. *Applied and Environmental Microbiology* **60**: 2924-2930.

Bergsma-Vlami M., 2010. Development of molecular detection and identification methodologies on *Xanthomonas arboricola* pv. *pruni. COST 873 Meeting Applied genomics for genotyping and virulence analysis, Wädenswill 2010.* [http://www.cost873.ch/ 5_activites/meeting_detail.php?ID=25]

Bouzar A., 1994. Request for a judicial opinion concerning the type species of *Agrobacterium*. *International Journal of Systematic Bacteriology* **44**: 373-374.

Bradbury J.F., 1986. Guide to Plant Pathogenic Bacteria. CAB International, Slough, UK.

Braun Y., Smirnova A., Ullrich M., 2008. Impact of temperature on the regulation of coronatine biosynthesis in *Pseudomonas syringae*. In: Fatmi M'B., Collmer A., Iacobellis N.S., Mansfield J.W., Murillo J., Schaad N.W., Ullrich M. (eds). *Pseudomonas syringae* pathovars and related pathogens, pp. 159-165. Springer Science and Business Media B.V., Berlin, Germany.

Bultreys A., Gheysen I., 1999. Biological and molecular detection of toxic lipodepsipeptide-producing *Pseudomonas syringae* strains and PCR identification in plants. *Applied and Environmental Microbiology* **65**: 1904-1909.

Bultreys A., Gheysen I., de Hoffmann E., 2006. Yersiniabactin production by *Pseudomonas syringae* and *Escherichia coli*, and description of a second yersiniabactin locus evolutionary group. *Applied and Environmental Microbiology* **72**: 3814-3825.

Bultreys A., Gheysen I., 2008. Siderophore uses in *Pseudomonas syringae* identification. In: Fatmi M'B., Collmer A., Iacobellis N.S., Mansfield J.W., Murillo J., Schaad N.W., Ullrich M. (eds). *Pseudomonas syringae* pathovars and related pathogens, pp. 21-35. Springer Science and Business Media B.V., Berlin, Germany.

Bultreys A., Gheysen I., Planchon V., 2008. Characterization of *Pseudomonas syringae* strains isolated from diseased horse-chestnut trees in Belgium. In: Fatmi M'B., Collmer A., Iacobellis N.S., Mansfield J.W., Murillo J., Schaad N.W., Ullrich M. (eds). *Pseudomonas syringae* pathovars and related pathogens, pp. 283-293. Springer Science and Business Media, B.V. The Netherlands.

Bultreys A., Kaluzna M., 2010. Bacterial cankers caused by *Pseudomonas syringae* on stone fruit species with special emphasis on the pathovars *syringae* and *morsprunorum* race 1 and 2. *Journal of Plant Pathology* **92**: S1.21-33.

Burr T.J., Katz B., 1982. Evaluation of a selective medium for detecting *Pseudomonas syringae* pv. *papulans* and *Pseudomonas syringae* pv. *syringae* in apple orchards. *Phytopathology* **72**: 564-567.

Civerolo E.L., Sasser M., Heike C., Burbage D., 1982. Selective medium for *Xanthomonas campestris* pv. *pruni*. *Plant Disease* **66**: 39-43.

Civerolo E.L., Hatting M.J., 1993. *Xanthomonas campestris* pv. *pruni*: cause of *Prunus* bacterial spot. In: Swings J.G., Civerolo E.J. (eds). *Xanthomonas*, pp. 60-64. Chapman & Hall, London, UK.

Cubero J., Martínez M.C., Llop P., López M.M., 1999. A simple and efficient PCR method for the detection of *Agrobacterium tumefaciens* in plant tumors. *Journal of Applied Microbiology* **86**: 591-602.

Cubero J., van der Wolf J.M., van Beckhoven J.R.C.M., López M.M., 2002. An internal control for the diagnosis of crown gall by PCR. *Journal of Microbiological Methods* **51**: 387-392.

Cubero J., Lastra B., Salcedo C.I., Piquer J., López M.M., 2006. Systemic movement of *Agrobacterium tumefaciens* in several plant species. *Journal of Applied Microbiology* **101**: 412-421.

Dallai D., Parkinson N., Giovanardi D., Stefani E., 2009. Population studies of *Xanthomonas arboricola* pv. *pruni* and new strategies to its control in peach orchards. *Annual COST 873 Meeting, Cetara 2009*: 115.

Dhanvantari B.N., 1971. Overwintering sources of inoculum of bacterial spot of peach (*Xanthomonas pruni*) in Southwestern Ontario. *Proceedings of the Canadian Phytopathological Society* **37**: 21-30.

Gilbert V., Planchon V., Legros F., Maraite H., Bultreys A., 2010. Pathogenicity and aggressiveness in populatios of *Pseudomonas syringae* from Belgian fruit orchards. *European Journal of Plant pathology* **126**: 263-277.

Goodman C.A., Hattingh M.J., 1986. Transmission of *Xanthomonas campestris* pv. *prun*i in plum and apricot nursery trees by budding. *HortScience* **21**: 995-996.

Goodner B., Hinkle G., Gattung S., Miller N., Blanchard M., Qurollo B., Goldman B.S., Cao Y., Askenazi M., Halling C., Mullin L., Houmiel K., Gordon J., Vaudin M., Iartchouk O., Epp A., Liu F., Wollam C., Allinger M., Doughty D., Scott C., Lappas C., Markelz B., Flanagan C., Crowell C., Gurson J., Lomo C., Sear C., Strub G., Cielo C., Slater S., 2001. Genome complete sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**: 2323-2328.

Haas J.H., Moore L.W., Ream W., Manulis S., 1995. Universal PCR primers for detection of phytopathogenic *Agrobac-*

terium strains. *Applied and Environmental Microbiology* **61**: 2879-2884.

Hayward A.C., 1960. A method for characterizing *Pseudomonas solanacearum*. *Nature (London)* **186**: 405-406.

Hildebrand D.C., 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens. *Phytopathology* **61**: 1430-1436.

Janse J.D., 2006. Phytobacteriology. Principles and Practice. CABI Publishing, Wallingford, UK.

Janse J.D., 2010. Diagnostic methods for phytopathogenic bacteria of stone fruits and nuts in COST 873. *Bulletin OEPP/EPPO Bulletin* **40**: 68-85.

Kado C.I., Heskett M.G., 1970. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas* and *Xanthomonas. Phytopathology* **60**: 969-976.

King E.O., Ward M.K., Raney D.E., 1954. Two simple media for the demonstration of pyocianin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**: 301-307.

Lelliot R.A., Stead D.E., 1987. Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell, Oxford, UK.

López M.M., 2000. Enfermedades causadas por bacterias. In: Montesinos E., Melgarejo P., Cambra M.A., Pinochet J. (eds). Enfermedades de los Frutales de Pepita y de Hueso pp. 37-50. Ediciones Mundi-Prensa, Madrid, Spain.

López M.M., Bertolini E., Olmos A., Caruso P., Gorris M.T., Llop, P, Penyalver R., Cambra M., 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology* **6**: 233-243.

López M.M., Bertolini E., Marco-Noales E., Llop P., Cambra M., 2006. Update on molecular tools for detection of plant pathogenic bacteria and viruses. In: Rao J.R., Fleming C.C., Moore J.E. (eds). Molecular diagnostics. Current Technologies and Applications, pp. 1-46. Horizon Bioscience, Norfolk, UK.

López M.M., Llop P., Olmos A., Marco-Noales E., Cambra M., Bertolini E., 2009. Are the molecular tools solving the challenges posed by detection of plant pathogenic bacteria and viruses? *Current Issues in Molecular Biology* **11**: 13-46.

Luisetti J., Prunier J.P., Gardan L., 1972. Un milieu pour la mise en évidence de la production d'un pigment fluorescent par *Pseudomonas mors-prunorum* f. sp. *persicae. Annales de Phytopathologie* **4**: 295-296.

Luisetti J., 1988. *Pseudomonas syringae pv. persicae* (Prunier *et al.*) Young *et al*. In: Smith I. M., Dunez J., Lelliot R.A., Phillips D.H., Archer S.A. (eds). Manual de Enfermedades de las Plantas, pp. 181-183 Mundi-Prensa, Madrid, Spain.

Llop P., Caruso P., Cubero J., Morente C., López M.M., 1999. A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. *Journal of Microbiological Methods* **37**: 23-31.

Miyoshi T., Tachibana Y., 1994. A selective medium for isolation of *Pseudomonas syringae*, the pathogen of bacterial blossom blight of kiwifruit. *Annals of the Phytopathological Society of Japan* **60**: 729-734.

Mohan S.K., Schaad N.W., 1987. An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *phaseolicola* in contaminated bean seed. *Phytopathology* **77**: 1390-1395.

Moore L.W., Bouzar H., Burr T., 2001. *Agrobacterium*. In: Schaad N.W., Jones J.B., Chun W. (eds). Laboratory Guide for Identification of Plant Pathogenic Bacteria, pp. 15-35. APS Press, St. Paul, MN, USA.

Mougel C., Cournoyer B., Nesme X., 2001. Novel tellurite amended media and specific chromosomal and Ti plasmid probes for direct analysis of soil populations of *Agrobacterium* biovars 1 and 2. *Applied and Environmental Microbiology* **67**: 65-74.

Nesme X., Leclerc M.C., Bardin R., 1989. PCR detection of an original endosymbiont: the Ti plasmid of *Agrobacterium tumefaciens*. In: Nardon P., Gianinazzi-Peason V., Greines A.M., Margullis L., Smith C. (eds). Endocitobiology IV, pp. 47-50. INRA Editions, Versailles, France.

New P.B., Kerr A., 1971. A selective medium for *Agrobacterium radiobacter* biotype 2. *Journal of Applied Bacteriology* **34**: 233-236.

Ovod V.V., Knirel Y.A., Samson R., Krohn K.J., 1999. Immunochemical characterization and taxonomic evaluation of the O polysaccharides of the lipopolysaccharides of *Pseudomonas syringae* serogroup O1 strains. *Journal of Bacteriology* **181**: 6937-6947.

Pagani M.C., 2004. An ABC transporter protein and molecular diagnoses of *Xanthomonas arboricola* pv. *pruni* causing bacterial spot of stone fruits. Ph.D. Thesis. University of North Carolina State, Raleigh, NC, USA. Online [http://www.lib.ncsu.edu/theses/available/ etd-10042004-232356]

Palacio-Bielsa A., Cambra M.A., López M.M., 2009a. PCR detection and identification of plant-pathogenic bacteria: updated review of protocols (1989-2007). *Journal of Plant Pathology* **91**: 249-297.

Palacio-Bielsa A., Cubero J., López M.M., Collados R., Berruete I., Cambra M.A., 2009b. Sensitive real-time PCR detection of *Xanthomonas arboricola* pv. *pruni* in symptomatic and asymptomatic *Prunus* spp. *Annual COST 873 Meeting, Cetara 2009*: 31.

Palacio-Bielsa A., Roselló M., Cambra M.A., López M.M., 2010. First report of bacterial spot of stone fruits on almond caused by *Xanthomonas arboricola* pv. *pruni* in Europe. *Plant Disease* **94**: 786.

Park S.Y., Lee Y.S., Lim S.H., Jung J.S., 2008. Specific detection of *Xanthomonas arboricola* pv. *pruni* by PCR using primers based on *hrp* genes. *Journal of Plant Pathology* **90**: S2.306.

Pelludat C., Duffy B., Frey J.E., 2009. Design and development of a DNA microarray for rapid detection and identification of multiple European quarantine phytopathogenic bacteria. *European Journal of Plant Pathology* **125**: 413-423.

Peñalver J., Quesada J.M., Roselló M., Llop P., Morente M.C., López M.M., 2008. Evaluación de la sensibilidad y especificidad de un protocolo de PCR para el diagnóstico de *Xanthomonas arboricola* pv. *pruni. XIV Congreso de la Sociedad Española de Fitopatología, Lugo 2008*: 163.

Pothier J.F., Pagani M.C., Ferrante P., Pelludat C., Scortichini M., Ritchie D.F., Duffy B., 2009a. A duplex-PCR method for identification of *Xanthomonas arboricola* pv. *pruni* and detection in plant samples. EPPO *Conference on Diagnostics and Associated Workshops, York 2009*: 84.

Pothier J.F., Smits T.H.M., Vorhölter F.J., Szczepanowski R., Goesman A., Pühler A., Duffy B., 2009b. Complete genome sequence of the stone fruit quarantine pathogen *Xanthomonas arboricola* pv. *pruni*. *Annual COST 873 Meeting, Cetara 2009*: 107.

Pulawska J., Sobiczewski P., 2005. Development of a semi-nested PCR-based method for sensitive detection of tumourigenic *Agrobacterium* in soil. *Journal of Applied Microbiology* **98**: 710-721.

Pulawska J., Willems A., Sobiczewski P., 2006. Rapid and specific identification of four *Agrobacterium* species and biovars using multiplex PCR. *Systematic and Applied Microbiology* **29**: 470-479.

Puopolo G., Raio A., Zoina A., 2007. Early detection of *Agrobacterium tumefaciens* in symptomless artificially inoculated chrysanthemum and peach plants using PCR. *Journal of Plant Pathology* **89**: 185-190.

Ridé M., 1969. Bactéries phytopathogènes et maladies bactériennes des végétaux. In: Bourgin C.V.M. (ed.). Les Bactérioses et les Viroses des Arbres Fruitiers. Ponsot, Paris, France.

Saunier M., Malandrin L., Samson R., 1996. Distribution of *Pseudomonas syringae* pathovars into twentry-three O serogroups. *Applied and Environmental Microbiology* **62**: 2360-2374.

Sawada H., Ieki H., Oyaizu H., Matsumoto S., 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *A. radiobacter* and *A. rhizogenes*. *International Journal of Systematic Bacteriology* **43**: 694-702.

Sawada H., Ieki H., Masuda I., 1995. PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. *Applied and Environmental Microbiology* **61**: 828-831.

Sawada H., Yoshida K., Sawada H., 2004. Detection of tumorigenic *Agrobacterium* strains from infected apple seedlings by colony PCR with improved PCR primers. *Journal of General Plant Pathology* **70**: 342-347.

Schaad N.W., Stall R.E., 1988. *Xanthomonas*. In: Schaad N.W. (ed.). Laboratory guide for Identification of Plant Pathogenic Bacteria, 2nd ed., pp. 81-91. APS Press, St. Paul, MN, USA.

Schroth M.N., Thompson J.P., Hildebrand D.C., 1965. Isolation of *Agrobacterium tumefaciens-Agrobacterium radiobacter* group from soil. *Phytopathology* **55**: 645-647.

Scortichini M., 1995. Le malattie batteriche delle colture agrarie e delle specie forestali. Edizione agricole, Bologna, Italy.

Scortichini M., 2008. *Xanthomonas arboricola* pv. *pruni* detection. *Annual COST 873 Meeting, Athens 2008*: 14.

Shepard D.P., Zehr E.I., 1994. Epiphytic persistence of *Xanthomonas campestris* pv. *pruni* on peach and plum. *Plant Disease* **78**: 627-629.

Sorensen K.N., Kim K.-H., Takemoto J.Y., 1998. PCR detection of cyclic lipodepsinoapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Applied and Environmental Microbiology* **64**: 226-230.

Stolp H., Starr M.P., 1964. Bacteriophage reaction and speciation on phytopathogenic xanthomonads. *Phytopathologische Zeitschrift* **51**: 442-478.

Sudarshana P., McClean A., Klueper D.A., 2006. Development of a culture-independent real-time PCR assay for detection of *Agrobacterium tumefaciens* in soil. *Walnut Research Conference 2006*: 347-354.

Takanashi K., Shimizu K., 1989. *Pseudomonas syringae* pv. *castaneae*, pv. nov., causal agent of bacterial canker of chestnut (*Castanea crenata* Sieb. et Zucc.). *Annals of the Phytopathological Society of Japan* **55**: 397-403.

Vicente J.G., Alves J.P., Russell K., Roberts S.J., 2004. Identification and discrimination of *Pseudomonas syringae* isolates from wild cherry in England. *European Journal of Plant Pathology* **110**: 337-351.

Whitesides S.K., Spotts R.A., 1991. Frequency, distribution and characteristics of endophytic *Pseudomonas syringae* in pear trees. *Phytopathology* **81**: 453-457.

Wood D.W., Wood D.W., Setubal J.C., Kaul R., Monks D.E., Kitajima J.P., Okura V.K., Zhou Y., Chen L., Wood G.E., Almeida Jr. N.F., Woo L., Chen Y., Paulsen I.T., Eisen J.A., Karp P.D., Bovee Sr. D., Chapman P., Clendenning J., Deatherage G., Gillet W., Grant C., Kutyavin T., Levy R., Li M.-J., McClelland E., Palmieri A., Raymond C., Rouse G., Saenphimmachak C., Wu Z., Romero P., Gordon D., Zhang S., Yoo H., Tao Y., Biddle P., Jung M., Krespan W., Perry M., Gordon-Kamm B., Liao L., Kim S., Hendrick C., Zhao Z.-Y., Dolan M., Chumley F., Tingey S.V., Tomb J.-F., Gordon M.P., Olson M.V., Nester E.W., 2001. The genome of the natural genetic engineer *Agrobacterium* C58. *Science* **294**: 2317-2323.

Young J.M., 1977. First report of *Xanthomonas pruni* in almond in New Zealand. *New Zealand Journal of Agricultural Research* **20**: 105-107.

Zaccardelli M., Consiglio M.F., Mazzuchi U., 1995. Detection of *Xanthomonas campestris* pv. *pruni* in symptomless peach trees in winter. *Phytopathologia Mediterranea* **34**: 199-203.

Zaccardelli M., Malaguti S., Bazzi C., 1998. Biological and epidemiological aspects of *Xanthomonas arboricola* pv. *pruni* on peach in Italy. *Journal of Plant Pathology* **80**: 125-132.