UNIVERSITY OF BELGRADE FACULTY OF AGRICULTURE

Pseudomonas Pathogens of Stone Fruits and Nuts: Classical and Molecular Phytobacteriology

Laboratory handbook and presentations

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Predgovor

U proizvodnji koštičavih voćaka, pojava bolesti bakteriozne prirode je od izuzetnog značaja, pre svega zbog rasprostranjenosti i destruktivnosti patogena, kao i zbog nemogućnosti efikasnog suzbijanja. *Pseudomonas syringae* patogeni varijeteti *syringae* i *morsprunorum*, prouzrokovači bakterioznog izumiranja i raka, odavno su poznati patogeni koštičavih voćaka. Rezultati detaljnog proučavanja ovih bakterija su brojni u inostranoj literaturi, a bile su predmet istraživanja i domaćih autora. *Pseudomonas syringae* pv. *persicae*, prouzrokovač bakterioznog izumiranja bresve i nektarine, u Evropi je prisutan jedino u Francuskoj, a u našoj zemlji se nalazi na karantinskoj listi. Nedavno je na divljoj trešnji u Francuskoj opisan još jedan patogen, *P. syringae* pv. *avii*, a prouzrokuje simptome slične bakterioznom izumiranju. Rak-rane na granama trešnje, uz isticanje smole i nekrozu ksilema, mogu dovesti do potpunog izumiranja zaraženih stabala.

Obzirom da se radi nekoliko srodnih patogenih varijeteta jedne vrste bakterija, među kojima su jedni dobro poznati a drugi na karantinskoj listi i predstavljaju potencijalnu opasnost, namera autora ove publikacije je da olakšaju njihovu diferencijaciju i smanje mogućnost greške pri postavljanju dijagnoze. Ovaj laboratorijski priručnik je namenjen studentima doktorskih studija iz oblasti fitopatologije i fitopatolozima u dijagnostičkim laboratorijama.

Priručnik je nastao kao potreba naučne javnosti da se na jednom mestu sakupe metode i postupci laboratorijskog proučavanja patogenih varijeteta *Pseudomonas syringae* parazita koštičavih voćaka i na taj način olakša mladim fitobakteriolozima snalaženje u diferencijaciji srodnih bakterija koje ugrožavaju proizvodnju ovih biljaka. Inicijativa je proistekla iz projekta COST 873 „Bakterioze koštičavih i jezgrastih voćaka“, a u njenoj realizaciji su učestvovali vodeći fitobakteriolozi nekoliko evropskih zemalja i Novog Zelanda. Priručnik je dostupan u elektronskoj formi na sajtu Poljoprivrednog fakulteta, univerziteta u Beogradu i nije namenjen prodaji.

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TAXONOMY OF *PSEUDOMONAS SYRINGAE*

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ABSTRACT

The evolution of the classification, identification and nomenclature of *Pseudomonas syringae* is described. Originally referring to a pathogenic species of lilac, *P. syringae* came to represent more than 40 host specific pathogenic populations, as pathovars. DNA–DNA hybridization studies and recent multilocus sequence analyses (MLSA) indicate a '*P. syringae* complex' now encompasses up to nine *Pseudomonas* species and 60 pathovars of *P. syringae*. A revision of the complex would be likely to result in the distribution of these species and pathovars in a number of genomospecies. The future classification and nomenclature of these genomospecies is discussed.

INTRODUCTION

Biological discussion is dependent on reliable systems of names (nomenclature) provided by taxonomic studies. Bacterial taxonomy comprises two interdependent activities; classification and identification. These taxonomic activities cannot be conducted in isolation from one another. Classifications are based on comparative studies of authenticated strains and aim to give expression to natural relationships (Young *et al*., 1992). The outcome of classification is the development of names (nomenclature) to be applied to identified taxa. One expectation of modern systematics has been that genera and species will be precisely circumscribed and that methods would be available for allocation of isolates to those groups (Murray *et al*., 1990; Vandamme *et al.,* 1996; Stackebrandt *et al*., 2002; Tindall *et* al., 2010). If taxonomists do not provide such means there is no way to differentiate known from unknown taxa and to expand repositories of authentic strains for further study. Informative classifications that indicate bacterial relationships have been reported but, without giving practical means of identification of taxa, they are impractical because they cannot be applied.

An underlying theme of the history of bacteriology is of the uncertainty that has arisen when the three taxonomic elements have not been in synchrony. The taxonomy of plant pathogenic bacteria was subject to the same confusion, and within the genus *Pseudomonas*, a pathogenic population identified for part of its history as the '*P. syringae* complex', has given expression to many of the issues. This discussion briefly describes the history of the *P. syringae* complex to show how the problems were resolved, and to outline the principles that may guide taxonomy in future.

EARLY HISTORY OF THE GENUS *Pseudomonas* AND *P. syringae*

When it was first proposed by Migula (1894), the genus *Pseudomonas* was understood to comprise all bacteria that were Gram-negative rods, aerobic with chemorganotrophic metabolisms and motile by one or more polar flagella. Only recently was this definition refined largely using comparative analyses of 16S rDNA sequences. The 'fluorescent, poly-β-hydroxybutyrate negative pseudomonads' associated with the type species, *P. aeruginosa*, and including *P. syringae* and related species, are now included in the β-Proteobacteria. Most 'non-fluorescent, poly-β-hydroxybutyrate positive pseudomonads', *Acidovorax*, *Burkholderia* and *Ralstonia*, are now included in the γ-Proteobacteria.

Soon after the proposal of the genus, populations of plant pathogenic bacteria were reported as *Pseudomonas* species, beginning with *P. mori* (Boyer and Lambert 1893) Stevens 1913, followed by *P. syringae* van Hall 1902. Many plant pathogenic *Pseudomonas* spp. followed thereafter. Early on it was established that, with important exceptions, most pathogenic pseudomonads were specific to limited numbers of host taxa. *P. mori* was then, and still is, considered to be specific to *Morus* spp. By contrast, a small number of pathogens affected more than one unrelated host, most notably *P. syringae*, first isolated from lilac (see 'The pathogenic structure of *P. syringae*' below).

Until the 1960s, it was believed that a significant component of the physiology of pathogenic bacteria must be devoted to pathogenic activity (Burkholder and Starr, 1948) and it was further assumed that simple nutritional and cultural differences reflected deep-seated metabolic and genetic differences associated with pathogenicity. Early proposals of pathogenic species were based on small numbers of morphological, biochemical and nutritional tests, and colony appearance on different media, many of which are now known to be highly variable or unstable. This assumption, that specific ecological responses involved large components of cell metabolism was generally assumed by bacteriologists and resulted in a proliferation of species names as synonyms for the same pathogen. It was common for synonyms to be used in different parts of the world without understanding that these referred to the same pathogen and disease.

Species proposals were made without comprehensive descriptions, but especially without the deposition of type or other reference strains, making reinvestigation and revision impossible. It was only when adequate culture collections of authentic strains were made, allowing the systematic comparison of comprehensive numbers of strains of plant pathogenic bacteria under standardized conditions, that it became clear that many species, including many *Pseudomonas* spp. (Stanier *et al*., 1966), were not differentiated using any of the biochemical and nutritional tests or other methods then available.

Origins of the '*P. syringae* complex'. In a study of determinative tests considered to differentiate fluorescent plant pathogenic *Pseudomonas* spp., Lelliott *et al*. (1966) showed that five tests; production of levan, oxidase activity, capacity to rot potato, production of arginine dihydrolase, and hypersensitivity reaction in tobacco (LOPAT) differentiated five distinct pathogenic species groups. Species that gave negative reactions in the tests for oxidase activity, capacity to rot potato and production of arginine dihydrolase, and positive hypersensitivity reactions in tobacco were identified as LOPAT Group I pathogens.

Subsequently, the more extensive study of Sands *et al*. (1970) showed that many named species in LOPAT Group I could not be distinguished phenotypically and that the distinct pathogenic populations could not be differentiated using the biochemical and nutritional tests then available. Preliminary DNA-DNA hybridization studies (Palleroni et al., 1972; Pecknold and Grogan, 1973) indicated a genomic diversity within LOPAT Group 1, but t were inadequate as the basis for significant taxonomic conclusions. The idea evolved that there existed a '*P. syringae* complex' represented as a single species comprising pathogens capable of infecting limited ranges of hosts (Stolp *et al*., 1965; Doudoroff and Palleroni, 1974).

THE GENERAL REVISION OF BACTERIAL NOMENCLATURE

The realization that the nomenclature of plant pathogenic bacteria was in a confused state only paralleled the situation for bacteria in general. A survey (Buchanan *et al*., 1966) found that most bacterial names, of which there were 20,000–30,000, were illegitimate, were synonyms, or for which there was no record of authentic reference strains, making reinvestigation of taxa impossible. Recognition of the enormity of existing nomenclatural confusion and the lack of regulation of nomenclature led the International Committee on the Systematics of Bacteria (now the International Committee on the Systematics of Prokaryotes) to propose a complete revision of bacterial nomenclature, embodied in the1976 Revision of the International Code of Nomenclature of Bacteria (the Code; Lapage *et al*., 1975). This edition legislated a new start to bacterial nomenclature based upon an inventory of those bacteria published previously that met the criteria of the revised Code, and specified the requirements for the legitimate publication of correct names, discussed in Young (2008). Central to the revision was the development of the Approved Lists of Bacterial Names (Skerman *et al*., 1980), which were to include only names that conformed fully to the revised Code. When published, 1791 species in 290 genera were recorded. (Euzeby, 1997-2010). As a consequence of this revision, names of most important bacterial plant pathogenic species did not conform to the criteria for listing and would not be included as valid names in the Approved Lists, leaving them without standing in nomenclature. This lacuna led directly to the proposal (Young *et al*., 1978) and acceptance (Dye *et al*., 1980) of the International Standards for Naming Pathovars (the Standards) to regulate nomenclature to be applied to these plant pathogenic bacteria as infrasubspecies. The origins of pathovar nomenclature and a critique of it are detailed in Young (2008).

***P. syringae* pathovars.** Because most fluorescent plant pathogenic *Pseudomonas* spp. did not satisfy the criteria for recognition as distinct species, all LOPAT group I species were included in a single species, *P. syringae*, as pathovars. The first record (Young *et al*., 1978) was of 40 pathovars that included *P. mori* and *P. syringae*. Although the host specific species, *P. mori*, took priority over *P. syringae* because it was first published, the name, *P. syringae*, with its many hosts, was chosen as the species name because it would allow flexibility in subsequent refinement of classification of the associated diverse and heterogeneous pathogenic populations of that species without confusing changes in nomenclature.

Although *P. syringae* originally represented all bacteria circumscribed as group I in the LOPAT determinative scheme, subsequent studies have shown that the '*P. syringae* complex'

comprising the pathovars of *P. syringae* must be expanded to include a small number of closely related species (Table 1).

AFTER 1980

Following the introduction of the Approved Lists and the Standards, the classification and naming of plant pathogens in fluorescent *Pseudomonas* spp. followed in an orderly way, with the proposal of new species and pathovars (Table 1). Demonstrations that pathogens were members of *P. syringae* followed from identification using LOPAT and a small menu of other tests, and for the most part this approach stood the test of time. Occasionally, a more detailed investigation demonstrated heterogeneity within the complex, as when *P. syringae* pv. *avellanae* was shown to be a distinct species (Janse *et al*., 1996).

Implications of New Methods. Contemporaneously and coincidentally with development of the new nomenclature, new methods were increasingly applied to bacterial classification. Earlier classifications relied almost entirely on data provided by studies of morphology, metabolic reaction, nutrient utilization, pigment production etc. The development of chemotaxonomic tests, those that compared large components of the phenotype, such as cell wall composition, fatty acid and protein profiling, isoprenoid quinone and polyamine comparisons, as well as comparisons of DNA and RNA composition and sequences, offered the advantage in classification that large components of a phenotype could be compared directly. Such methods played an important role in circumscribing specific bacterial groups including plant pathogenic bacteria (Vandamme *et al*., 1996; Gillis *et al.,* 2005). A problem with most chemotaxonomic methods is that they use expensive supporting hard-ware, or they require levels of standardization that can only be met within a single laboratory and therefore are not portable. This increase in the numbers of such methods in the 1980s posed the need for some coordination if a multiplicity of alternative classifications generated by the diverse methods was to be avoided, especially in relation to novel molecular methods. Wayne *et al.* (1987) gave guidance to the practices associated with the proposal of new species. They suggested a quantitative definition of the bacterial species as the population whose strains share more than 70% DNA-DNA hybridization and have a T_m of less than 5^{o} C. This definition came to form the standard for species circumscriptions. Wayne *et al.,* (1987) also urged that hybridization data be supported by phenotypic data. In proposing this, they anticipated that phenotypic data would support the genomic framework; that there would be a congruence of systematics using different methods and that this would give expression to phylogenetic relationships. Murray *et al*. (1990) gave strong endorsement to the need for supporting evidence for species proposals based on DNA-DNA reassociation data and this principle has formed the basis of species classification to the present day (Tindall *et al*., 2010).

DNA–DNA Hybridization Studies and Genomospecies. In a comprehensive investigation of the *P. syringae* complex using DNA-DNA reassociation Gardan *et al*., (1999) identified nine genomospecies. Four of these represented the majority of species and pathovars of the

complex. These results have largely been confirmed by sequence studies (see below) and they imply a major revision of the *'P. syringae* complex'. For example, of the species identified in the complex, *P. amygdali, P. ficuserectae* and *P. meliae* were members of their genomospecies 2. If confirmed as an authentic species by further studies, then according to the Code it would be named *P. amygdali*, which is the earliest synonym, *P. ficuserectae* and *P. meliae* being pathovars of the species.

Because the genomospecies were not supported by ribotyping or comparisons of carbon source utilization using Biotype 100 (bioMerieux), Gardan *et al*. (1999) refrained from making formal species proposals. Had they successfully published formal species on the basis of their genomic data, then further taxonomic investigations of pseudomonad pathogens might have been paralysed in the way that occurred in *Xanthomonas* following the publication of the revision of Vauterin *et al*. (1995, Young et al., 2008)). By contrast, pathogens could be allocated to *P. syringae sensu lato* by phenotypic methods and about 13 pathovars have been reported since Gardan *et al*. (1999) (Table 1). Although the classification is understood to be over-simple, naming of pathogens has continued in the expectation of advances in methods of classification.

Gardan *et al*. (1992) re-established *P. savastanoi* (with the pathovars *savastanoi, glycinea* and *phaseolicola*) on the basis of DNA-DNA reassociation data but subsequently (Gardan *et al*., 1999) reported that *P. amygdali, P. ficuserectae* and *P. meliae* all shared high reassociation values with *P. savastanoi* and were all earlier synonyms, with *P. amygdali* as the earliest synonym. This highlights a common problem; that limited studies that do not take account of all relevant taxa may lead to classifications that are subsequently shown to be inadequate and new names that are confusing.

Polyphasic Studies. It is perhaps remarkable that, for a genus that has been studied in many ecological contexts, there is very little understanding of the basic physiology and metabolism of *Pseudomonas*; most studies have been based on *P. aeruginosa* and *P. fluorescens* (Palleroni, 2005). Several methods have been applied to differentiate *Pseudomonas* spp.; quinone systems, fatty acid, protein, polar lipid or polyamine profiles, but these only give satisfactory results when the species are clearly distinct (Peix et al., 2009). The interrelationships of polyphasic studies were illuminated by a multi-laboratory investigation of the genus *Pseudomonas* reported in *Systematic and Applied Microbiology* (1996). Many species, including plant pathogenic species, were examined by ribotyping (Brosch *et al*., 1996), fatty acid content of whole-cell hydrolysates and phospholipid fractions (Vancanneyt *et al.,* 1996a), SDS-PAGE of whole-cell protein (Vancanneyt *et al.,* 1996b), westprinting (Tesar *et al.,* 1996), and Biolog and BioMerieux API Biotype-100 systems (Grimont *et al.,* 1996). Using DNA-DNA reassociation as the basis for congruence, the notable feature of the accumulated data is the different relationships indicated between species by these different polyphasic methods as discussed in Young (2000). Few of these methods have been used to compare many plant pathogenic *Pseudomonas* spp. A review of studies of fatty acid profiles (Stead, 1990; 1992, Weller *et al*., 2000) shows that they do not correspond to the genomospecies. An investigation of the fatty acid profiles of almost all members of the *P. syringae* complex (Table 1) with the genomic groups indicated little or no correspondence between genomospecies and fatty acid groups (J. Elphinstone, pers. comm). This failure may be rooted in the character of genomospecies of the

P. syringae complex (Gardan *et al*., 1999). With few exceptions, they indicate measurable and high reassociation values; usually greater than 30% (Gardan et al., 1999). Such values indicate very high sequence similarities (Young *et al*., 1992) and this may be the reason why no differentiating phenotypic characters can be identified.

IDENTIFICATION OF *P. syringae* AND PATHOVARS

Immediately after 1980, the identification of plant pathogenic species could still be based on simple phenotypic tests because these formed the basis of species differentiation. With primary reliance for classification being increasingly orientated towards poly-phasic and molecular methods, novel species were proposed for which there were fewer simple or portable methods by which they could be identified, as noted for *Xanthomonas* above. For pathovars of *P. syringae*, the use of determinative tests was shown to be of limited value for the differentiation of pathovars (Young and Triggs, 1994; Palleroni, 2005), most of which could be identified by these means. However, it was usually possible to differentiate pathogens on an identified host, where the few individual pathovars are known (Young, 2000).

PCR primers offer a reliable method for the confirmation of identity of pathovars. Palacio-Bielsa *et al*. (2009) record a total of 246 papers describing primers for plant pathogenic bacteria. Of these, 30 describe primers for 19 members of the *P. syringae* complex: the species, *P. avellanae, P. cannabina* and *P. fuscovaginae,* and the pathovars, *actinidiae, alisalense, atropurpurea, coryli, glycinea, maculicola, morsprunorum, papulans, phaseolicola, pisi, savastanoi, sesami, syringae, tagetis, theae,* and *tomato*. Clearly a full inventory of primers for all pathovars is desirable. There is always a need for comprehensive studies to confirm specificity if false positive and false negative results are to be avoided (Bereswill *et al*., 1994).

SEQUENCING

It is unlikely that further comprehensive DNA-DNA reassociation studies of the *P. syringae* complex will be conducted; the expertise required and the time and cost making them almost prohibitive (Vauterin et al., 1997; Martens et al., 2008). One way forward will be by comparative sequencing analyses using appropriate genes. The criteria for such selection should be that they reflect classifications based on DNA-DNA reassociation in order to maintain continuity of nomenclature (Stackebtandt *et al*., 2002; Tindall et al., 2010). Because comparative analyses of genes in general often do not produce concordant relationships, a further criterion should be that genes selected should indicate relationships that correspond to that of overall genome and it is obvious that consideration of several genes is preferable to reliance on a single gene. A relatively small number of comparative sequence analyses have been made for the *P. syringae* complex. Those that have been made are incomplete. A comprehensive comparative analysis of 16S rDNA of *Pseudomonas* (Anzai *et al.* 2000) demonstrated a group comprising *P. amygdali, P. avellanae, P. caricapapayae, P. cichorii, P. ficuserectae, P. meliae, P. savastanoi, P. syringae* and *P. viridiflava.* Because 16S rDNA is so highly conserved, no discrimination

within the group was achieved. A study of *Pseudomonas* by Yamamoto et al., (2000) using concatenated *gyrB* and *rpoD* sequences demonstrated the grouping of a few species of the *P. syringae* complex in accord with the work of Gardan *et al*. (1999) (Table 1). Recently, Parkinson (pers. comm.) conducted a comprehensive comparative analysis of the *P. syringae* complex based on a partial sequence of the gene, *rpoD*, which gave good support to the genomospecies structure of the complex. In an ecological study, Sarkar and Guttman (2004) conducted a multilocus sequence analysis (MLSA) involving seven sequences from 21 pathovars. Although pathotype strains were not included as reference strains their results supported the four main genomospecies of Gardan *et al*. (1999).

THE PATHOGENIC STRUCTURE OF *P. SYRINGAE*

As conceived after 1980, *P. syringae* comprised a population composed of pathogenic strains, of which many sub-populations, as pathovars, had highly restricted host ranges, sometimes apparently confined to a single plant genus, e.g. *P. syringae* pv. *pisi*. Others had host ranges that comprised relatively limited but unrelated host taxa. *P. syringae* pv. *phaseolicola*, originally assumed to be specific to *Phaseolus*, has also been proved pathogenic to *Dolichos, Macroptilium, Pueraria, and Vigna* spp. Similarly, proved strains of *P. syringae* pv. *tabaci*, a pathogen also believed to be specific to *Tabacum*, were found on diseased *Desmodium, Glycine* and *Phaseolus*. A number of previous claims of very wide host ranges are probably due to misidentifications (Bradbury, 1986). The major exception is *P. syringae* pv. *syringae*. Bradbury (1986) identified 16 *Pseudomonas* species as synonyms of the pathovar. With the differentiation of the distinguishable pathovars, all other hosts that had previously been allocated to *P. syringae* were ascribed as hosts of *P. syringae* pv. *syringae* (Bradbury, 1986). The structure of the pathogenic populations of this pathovar is complex and not well understood. The pathogen, *P. syringae* pv. *syringae*, takes its name from the host from which it was first isolated, but strains proved pathogenic to lilac also infect more than 44 plant species and there are strains with the same determinative characteristics that do not attack lilac (Young, 1991). These latter may have significantly over-lapping host ranges with the pathogen from lilac and therefore be considered to be members of *P. syringae* pv. *syringae*. The capacity or not to infect a single host that is part of a host complex, even if it is the host after which the pathogen was named, should not justify formal differentiation. However, there are other strains that do not have the specific determinative characteristics of *P. syringae* pv. *syringae*, are not identified as pathovars to their host plant, and the identity of these is unknown. Perhaps they are peripheral members of *P. syringae* pv. *syringae* or perhaps they represent distinct specific pathovars. Parkinson (pers. comm.), in a comparative analysis of *rpo* sequences from a comprehensive range of strains from the *P. syringae* complex, indicates that strains from hosts associated with particular pathovars are distributed widely in the *P. syringae* complex. The significance of this in taxonomic or ecological terms is unclear.

Traditionally, the most reliable method of identification of pathogenic species was by proving specific pathogenicity to a suspect host and then applying the appropriate name. However, this method can fail, especially for pathovars of *P. syringae* because *P. syringae* pv.

syringae can produce symptoms identical to those of several pathovars. For instance, strains isolated from genera in the Cucurbitae and proved pathogenic to their original host have been identified as *P. syringae* pv. *lachrymans* (Bradbury, 1986). However, a comprehensive study of strains in the International Collection of Micro-organisms from Plants has shown that all strains from species other than those from *Cucumis sativus* were members of *P. syringae* pv. *syringae*, and that *P. syringae* pv. *lachrymans* appears to be specific to this species (unpublished data).

THE FUTURE

The direction of bacterial classification and the application of formal names is unclear. One possibility will see an insistence on the continuation of descriptions based on polyphasic classification (Vandamme et al., 1996; Gillis et al., 2005) with comprehensive phenotypic descriptions (Tindall et al., 2010). As noted, polyphasic classifications often do not give coherent support for species descriptions, the classification of genera and species being based on sequence data, sometimes a single sequence, 16S rDNA, alone. Furthermore, it may be that for some species, polyphasic, chemotaxonomic or other data may not be discoverable to support sequence interpretations, as is so far the case for the genomospecies of *P. syringae*. If this is so, then it is possible that the genomospecies of Gardan *et al*. (1999) or those indicated by sequencing data may never be translated into formal species that meet the criteria of the Code. An alternative approach is that of Lindström and Martínez-Romero (2005) who suggested that the characterization of species could be based on sequence data alone. Such an approach would be contrary to all previous taxonomic intention (Wayne *et al*., 1987; Murray *et al*., 1990; Stackebrandt *et al*., 2002; Gevers et al., 2005; Tindall *et al*., 2010) and, whether it has merit or not, it is unlikely to be adopted in the foreseeable future.

Formal Genomospecies? Rather than have taxonomic paralysis, an alternative might be to accept the present approach, requiring phenotypic and molecular data as the basis for formal species classifications and nomenclature, but to give greater formal support to the concept described by Ursing *et al*. (1995). They proposed that, where genomic groups could be delimited by DNA-DNA hybridization, but could not be differentiated by phenotypic means, they might best be referred to as genomovars, if only as a temporary measure. Extending on this proposal, where genomic groups are established by DNA-DNA hybridization or equivalent MLSA, or other genomic comparisons, then they could be named as genomospecies, with some recognition in the Code. This would provide a nomenclature giving expression to informative genotypic differences until such time as phenotypic characters could be identified that were correlated to the genomic differences. The term, '*Candidatus'*, is applied informally at present to unculturable bacteria for which phenotypic descriptions cannot be established and whose circumscriptions are based on molecular methods (Murray *et al*., 1995). It is intended to formulate specific rules in the Code to give clear guidance to their nomenclature (Stackebrandt *et al*., 2002). A similar formulation for 'genomospecies' could usefully provide a bridge in nomenclature between the absence of a nomenclature that illuminates genotypic classification, and the development of complete species circcumscriptions. However, it is possible that the highly conserved house-keeping genes will not correspond in a coherent way to measurable

phenotypic characters. In such an eventuality, robust guidance would be needed for a genomospecies nomenclature for such groups as those indicated in the *P. syringae* complex, in perpetuity.

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Table 1. The '*P. syringae* complex'. Column A. List of names of all *Pseudomonas* spp. and pathovars considered in the '*P. syringae*' complex; Column B. Allocation of these to genomospecies by Gardan *et al*. (1999). Column C. Allocation by Yamamato *et al*. (2000) using concatenated *gyrB* and *rpoD* genes.

A. Names	B. Genomospecies		C. *gyrB – rpoD*
P. amygdali	1	*P. syringae*	*P. syringae*
P. avellanae	1	*P. syr.* pv. *syringae*	*P. syr.* pv. *syringae*
P. cannabina	1	*P. syr.* pv. *aptata*	
P. caricapapayae	1	*P. syr.* pv. *atrofaciens*	
P. ficuserectae	1	*P. syr.* pv. *dysoxyli*[1]	
P. meliae	1	*P. syr.* pv. *lapsa*	
P. tremae	1	*P. syr. pv. panici*[2]	
P. viridiflava	1	*P. syr.* pv. *papulans*	
P. syringae	1	*P. syr.* pv. *pisi*	
P. syr. pv. *aceris*	2	*P. amygdali*	*P. amygdali*
P. syr. pv. *actinidiae*	2	*P. ficuserectae*	*P. ficuserectae*
P. syr. pv. *aesculi*	2	*P. meliae*	*P. syr. pv. savastanoi*[3]
P. syr. pv. *alisalensis*	2	*P. syr. pv. savastanoi*[3]	*P. syr.* pv. *glycinea*[3]
P. syr. pv. *antirrhini*	2	*P. syr.* pv. *aesculi*	*P. syr.* pv. *lachrymans*[4]
P. syr. pv. *apii*	2	*P. syr.* pv. *ciccaronei*	*P. syr.* pv. *morsprunorum*[5]
P. syr. pv. *aptata*	2	*P. syr.* pv. *dendropanacis*	*P. syr.* pv. *phaseolicola*[3]
P. syr. pv. *atrofaciens*	2	*P. syr.* pv. *eriobotryae*	
P. syr. pv. *atropurpurea*	2	*P. syr.* pv. *glycinea*[3]	
P. syr. pv. *berberidis*	2	*P. syr.* pv. *hibisci*	
P. syr. pv. *broussonetiae*	2	*P. syr.* pv. *lachrymans*[4]	
P. syr. pv. *castaneae*	2	*P. syr.* pv. *mellea*	
P. syr. pv. *cerasicola*	2	*P. syr.* pv. *mori*	
P. syr. pv. *ciccaronei*	2	*P. syr.* pv. *morsprunorum*[5]	
P. syr. pv. *coriandricola*	2	*P. syr.* pv. *myricae*	
P. syr. pv. *coronafaciens*	2	*P. syr.* pv. *phaseolicola*[3]	
P. syr. pv. *coryli*	2	*P. syr.* pv. *photiniae*	
P. syr. pv. *cunninghamiae*	2	*P. syr.* pv. *sesami*	
P. syr. pv. *daphniphylli*	2	*P. syr.* pv. *tabaci*	
P. syr. pv. *delphinii*	2	*P. syr.* pv. *ulmi*	
P. syr. pv. *dendropanacis*	3	*P. syr.* pv. *antirrhini*	*P. syr.* pv. *antirrhini*
P. syr. pv. *dysoxyli* [1]	3	*P. syr.* pv. *apii*	*P. syr.* pv. *maculicola*
P. syr. pv. *eriobotryae*	3	*P. syr.* pv. *berberidis*	
P. syr. pv. *fraxini*[3]	3	*P. syr.* pv. *delphinii*	
P. syr. pv. *garcae*	3	*P. syr.* pv. *maculicola*	
P. syr. pv. *glycinea*[3]	3	*P. syr.* pv. *passiflorae*	
P. syr. pv. *helianthi*	3	*P. syr.* pv. *persicae*	
P. syr. pv. *hibisci*	3	*P. syr.* pv. *philadelphi*	
P. syr. pv. *japonica*[6]	3	*P. syr.* pv. *primulae*[7]	
P. syr. pv. *lachrymans*	3	*P. syr.* pv. *ribicola*[8]	

P. syr. pv. *lapsa*	3	*P. syr.* pv. *tomato*	
P. syr. pv. *maculicola*	3	*P. syr.* pv. *viburni*	
P. syr. pv. *mellea*	4	*P. syr.* pv. *atropurpurea*	
P. syr. pv. *mori*	4	*P. syr.* pv. *coronafaciens*	
P. syr. pv. *morsprunorum* [5]	4	*P. syr.* pv. *garcae*	
P. syr. pv. *myricae*	4	*P. syr.* pv. *oryzae*	
P. syr. pv. *nerii*[3]	4	*P. syr.* pv. *porri*	
P. syr. pv. *oryzae*	4	*P. syr.* pv. *striafaciens*	
P. syr. pv. *panici*[2]	4	*P. syr.* pv. *zizaniae*	
P. syr. pv. *papulans*	5	*P. tremae*[9]	
P. syr. pv. *passiflorae*	6	*P. viridiflava*	*P. viridiflava*
P. syr. pv. *persicae*	7	*P. syr.* pv. *tagetis*	
P. syr. pv. *phaseolicola*[3]	7	*P. syr.* pv. *helianthi*	
P. syr. pv. *philadelphi*	8	*P. avellanae*	
P. syr. pv. *photiniae*	8	*P. syr.* pv. *theae*	
P. syr. pv. *pisi*	9	*P. cannabina*[9]	
P. syr. pv. *porri*	–[10]	*P. caricapapayae*	*P. syr.* pv. *coriandricola*
P. syr. pv. *raphiolepidis*	–	*P. syr.* pv. *actinidiae*	
P. syr. pv. *ribicola*	–	*P. syr.* pv. *alisalensis*	
P. syr. pv. *retacarpa*	–	*P. syr.* pv. *broussonetiae*	
P. syr. pv. *savastanoi*[3]	–	*P. syr.* pv. *castaneae*	
P. syr. pv. *sesami*	–	*P. syr.* pv. *cerasicola*	
P. syr. pv. *solidagae*	–	*P. syr.* pv. *coriandricola*	
P. syr. pv. *spinaceae*	–	*P. syr.* pv. *coryli*	
P. syr. pv. *striafaciens*	–	*P. syr.* pv. *cunninghamiae*	
P. syr. pv. *syringae*	–	*P. syr.* pv. *daphniphylli*	
P. syr. pv. *tabaci*	–	*P. syr.* pv. *fraxini*[3]	
P. syr. pv. *tagetis*	–	*P. syr.* pv. *japonica*[6]	
P. syr. pv. *theae*	–	*P. syr.* pv. *nerii*[3]	
P. syr. pv. *tomato*	–	*P. syr.* pv. *raphiolepidis*	
P. syr. pv. *ulmi*	–	*P. syr.* pv. *retacarpa*	
P. syr. pv. *viburni*	–	*P. syr.* pv. *solidagae*	
P. syr. pv. *zizaniae*	–	*P. syr.* pv. *spinaceae*	

[1]The pathotype strain is not an authentic strain of the pathogen of *Dysoxylum*, which is proposed as *Xanthomonas dyei* pv. *dysoxyli* (Young *et al*., 2010).

[2]*P. syringae* pv. *panici* is a doubtful name (Young and Fletcher, 1994).

[3]Based on their DNA-DNA hybridization studies, Gardan *et al*. (1992) proposed the new species, *P. savastanoi*, to which these pathovars were allocated. It is now understood that these proposals did not take account of earlier synonyms (Gardan *et al*., 1999), of which *P. amygdali* takes priority.

[4]The pathotype strain of *P. syringae* pv. *lachrymans* was known not to be representative of the pathovar (Young *et al*., 1996). Gardan *et al*., 1999 showed that this strain was a member of Genomospecies 3. A proved pathogenic strain (CFBP 1664) is a member of Genomospecies 2.

[5]The pathotype strain of *P. syringae* pv. *morsprunorum* was known not to be representative of the pathovar (Young *et al*., 1996). Gardan *et al*., 1999 showed that this strain was a member of Genomospecies 3. A proved pathogenic strain (CFBP 2116) is a member of Genomospecies 2.

[6]*P. syringae* pv. *japonica* is a later synonym of *P. syringae* pv. *syringae* (Young, 1992).

[7]Gardan *et al*. (1999) reported that the pathotype strain of *P. syringae* pv. *primulae* is a member of *P. viridiflava* and that a proved pathogenic strain (CFBP 11007) is a member of Genomospecies 3.
[8]Gardan *et al*. (1999) reported that the pathotype strain of *P. syringae* pv. *ribicola* is a member of *P. viridiflava* and that a proved pathogenic strain (CFBP 10971) is a member of Genomospecies 3.
[9]New species proposed in the study of Gardan *et al*. (1999).
[10]Not investigated by Gardan *et al*. (1999).

ISOLATION AND IDENTIFICATION OF PLANT PATHOGENIC BACTERIA

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Introduction

Diagnosis, whether as part of research or to serve growers, crop industries or government quarantine should be conducted efficiently, and therefore the quickest and simplest processes leading to satisfactory outcomes are essential. To be done well, diagnosis requires experience of relevant pathogens and their diseases and facility with a range of laboratory methods. Ideally, diagnosis would follow a bifurcating set of tests leading to a single conclusive identification. However, because characters are not unique to particular bacterial taxa, and because few if any characters are expressed in all strains of a taxon, this is not possible. A significant complication is that determinative tests were often confirmed in the past by investigation of a particular group of bacterial taxa. Subsequent taxonomic revisions resulting from division, amalgamation or expansion of a taxon may mean that a determinative test is no longer specific or reliable. However, where the host is known, diagnosis can be successful by conducting several indicative tests that progressively increase the probability of identity until a relatively small numbers of confirmatory tests can be employed to prove identity.

Specimens

Successful diagnosis of bacterial pathogens depends on processing plant specimens from which the pathogen can be obtained in pure culture. This requires that specimens be received in the laboratory in a fresh condition and are processed expeditiously. Specimens received from individuals who are unaware of requirements commonly exhibit over-developed symptoms from which isolations are difficult or impossible. Success is most likely from specimens in which there are the earliest indications of infection. If specimens are received by mail, then they should have been packed in damp paper to maintain evaporative cooling during transport.

Preliminary examination

Close inspection of specimens and recording symptoms may in some cases give a reliable identification though, in cases such as quarantine control, formal confirmation may still be necessary.

Preliminary confirmation of the presence of bacteria can be done by examining a wet mount of the tissue using a phase contrast microscope at about $\times 200$. If left for 15–20 minutes prior to examination, very large numbers of bacteria will usually be seen merging from the exudation at the cut edge of the lesion. Motility can be stimulated by mounting in nutrient broth.

Tissue

Leaves, stems and fruit

Lesions are usually visible and selection should be of those with oily, water-soaked margins if present. Isolations should always be from the smallest lesions, or lesion margins, minimizing inclusion of brown necrotic tissue.

Secondary woody tissue

Sampling from woody tissue is complicated because the active margin is not usually externally visible. By the time visible symptoms of disease are expressed, as sunken tissue over the lesion, as cankers, or as wilt, the disease may have fully developed, tissue become heavily necrotized and populations of the pathogen have declined. Pathogen activity must be discovered by dissection. Pathogens expressing wilt symptoms are usually present in vasculature in advance of visible necrosis and are best sampled from that tissue.

Root tissue

Prior to sampling, root tissue should be surface-sterilized by soaking washed, soil-free, excised tissue in 5–10 % commercial hypochlorite (3% active chlorine) for 15 minutes or more as necessary, followed by rinsing briefly in clean or sterile water.

Isolation

Isolation is made by excising pieces of tissue and crushing them in a drop of clean or sterile water. A flat-ended glass rod will crush the most slippery tissue. The tissue suspension is spread, using a glass spreader, on surface-dried agar plates that are then incubated at 25–27^0 C. King's medium B is useful because some differentiation at the generic level can be made from the colony forms that develop. Sugar-containing media favour fungal contamination and should be avoided. Incubation is at 25-27^0C. Some pathogens only grow below 20^0C.

Notes on isolation

1. Effective spreaders can be made from soda-glass pipettes using a gas lighter or match. The tip is sealed and a 'hockey-stick' made that has a 1–1.5 cm spreading edge. These can be made easily, spread well, do not damage the agar surface and cool quickly.
2. Plates can be dried on the bench or in a laminar flow cabinet. The time required depends on temperature and humidity. Hours or overnight on the bench; 30-60 minutes in a cabinet. Dried plates should not be so 'dry' that suspension does not 'stick' to the surface. Nor should they be so 'wet' that the suspension does not dry immediately on the plate. Remedial steps, for 'dry' plates is to add a drop of water to the inoculum on the plate and continue spreading and, for 'wet' plates is to dry more or to leaves inoculated plates open to complete drying.

Examination of Plates

First examination of plates should be made after 24 h. At this stage, visible colonies are almost invariably saprobes because pathogenic bacteria, with the possible exception of some soft-rotting bacteria, are slower growing. Colonies of pathogens first appear after 36–48h. The presence of large numbers of saprobes, or the presence of more than 3–4 colony types, is a warning that the tissue section has been invaded by secondary organisms, that pathogenic populations are in decline, and that identification of pathogens may be extremely difficult if not impossible. Incubation is continued until a population becomes visible as an almost unmixed population on the agar surface. Depending upon the pathogen involved, this can take up to 10 days.

As soon as colonies of putative pathogens are distinguished, representative single colonies should be restreaked to confirm purity and sub-cultured onto storage slopes.

Diagnosis

Diagnosis to generic level can now be initiated using a relatively small number of tests, based on the information available. It is commonly recommended that the first step in diagnosis should be the test for the Gram reaction. However, because most common pathogens are Gram negative, this test does not usually reduce possible candidates significantly. A more direct method is to consider colony form. At every stage in the diagnosis, symptoms and host range should be reconsidered as indicating likely target pathogens.

Preliminary Interpretation

Because of their different growth rates, pathogens first appear on plates in the following order:

2 days; *Agrobacterium*, the enterobactera, non-fluorescent pathogents

2 days (late); fluorescent *Pseudomonas*

3 days; *Xanthomonas*

>5 days; the coryneform Gram-positive genera, *Clavibacter*, *Curtobacterium, Nocardia*, *Rathayibacter* and *Rhodococcus*

Knowledge of the host and careful consideration of colony form, reduces the probable pathogens to a small number that can be reduced even further, sometimes to a single pathogen, using selected determinative tests. As a guide:

1. The oxidation/fermentation test distinguises the enterobacterial genera (*Brenneria, Dickeya, Enterobacteria, Erwinia, Pantoea, Pectobacterium* and *Samsonia*) from all others. Refer to host list for species ID.

2. The test for poly-β-hydroxybutyrate distinguishes *Acidovorax, Burkholderia* and *Ralstonia* spp. from all others. Refer to host list for species ID.
3. Production of fluorescent pigment under UV light (less than 260 nm) distinguishes *P. agarici*, *P. asplenii*, *P. avellanae*, *P. cannabina, P. cichorii,* ***P. costantinii*****,** *P. caricapapayae*, *P. cichorii*, *P. fuscovaginae*, *P.* (*marginalis*) *fluorescens*, ***P. salomonii*****,** *P. savastanoi, P. syringae*, *P. tolaasii*, *P. tremae* or *P. viridiflava*. from all others. Follow with LOPAT tests and refer to host list.
4. Production of mucoid (xanthan) gum on glucose-containing media and inhibition in triphenyl tetrazolium chloride containing media distinguish *Xanthomonas*. Most *Xanthomonas* produce a distinct pale yellow pigment (xanthomonadin), which must be carefully distinguished from the darker yellow pigment produced by the common saprobe, *Pantoea agglomerans*. The 20 species and 150 pathovars of *Xanthomonas* cannot be distinguished by non-molecular methods. Refer to host list for species and pathovar IDs.
5. The Gram reaction confirms the identity of Gram-positive species. Refer to host list for species ID.
6. *Note on non-pigmented strains*

Some pathogenic species and pathovars in *Pseudomonas* and *Xanthomonas* do not produce expected pigments (e.g., *P. tremae*, *P. syr.* pv. *persicae*, *X. dyei*, *X*. '*campestris'* pv. *viticola*). It is by prior and specific knowledge of the characteristics of pathogens from the relevant host that further steps towards correct identification can be made.

Media

Most traditional media were developed for clinical studies and contain nutrient levels that can be inhibitory to environmental organisms. Peptone in media can profitably be reduced to 10 g. Some media such as King's medium B do not maintain viability as well as low nutrient concentration media, such as R2A and YPA, which support viability for many months. Growth rates are usually slow on artificial media when bacteria are first isolated from plant tissue but increase when they have adapted to them.

King's medium B agar

Proteose peptone No. 3 (Difco), 20g; K_2HPO_4, 1.5g; $MgSO_4.7H_2O$, 1.5g; glycerol, 10 ml; agar, 15g; SDW; 1l.

Yeast-extract Dextrose Carbonate (YDC) Agar

Yeast-extract, 10g; glucose, 5g; $CaCO_3$, 20g, SDW, agar, 15g; 1 litre.

YPA

NH_4Cl, 0.5 g; KCl, 0.2 g; $MgSO_4.7H_20$, 0.2 g; K_2HPO_4, 1.0 g; yeast extract (Difco), 3.0 g; agar (Danisco, NZ) 12 g; de-ionized water (DW), 1 L.

Triphenyl Tetrazolium chloride

YPA or NA plates containing 0.0, 1.0, 2.0 or 5.0 g/L TTC are inoculated and incubated at 25^{o}C for 7 days to test for growth (Lovrekovich & Klement, 1960).

Tests

Gram Reaction (KOH Method)

An alternative to staining methods to differentiate cell wall structure is based their solubility in 3% potassium hydroxide KOH. A copious loopful of fresh colony growth is suspended in a drop of KOH on a slide and the loop is then lifted from the suspension. A viscous thread (of DNA) drawn out by the loop indicates that the bacteria is Gram negative.

Poly-β-hydroxybutryate inclusion test

Cultures are grown in a medium with a high carbon-nitrogen ratio. Bacterial suspension is fixed on a slide as described for the Gram stain and flooded with Sudan black, leaving for 15 min. Sudan black solution is prepared as a 0.3% solution in 70% ethanol, shaken and allowed to stand overnight. Drain off excess stain and allow to dry then rinse in water and allow to dry. Counterstain with safranine and examine with a microscope at x200 using oil immersion. Poly-β-hydroxybutryate inclusions are stained blue-grey to black. Reactions are variable; strains of *Ralstonia* giving strong reactions, while those of *Acidovorax* and *Burkholderia* may be weaker. Therefore reference strains should be examined until complete confidence with the method is obtained.

Light microscopy

Motility is quickly established by examining bacterial suspensions in any nutrient medium using a phase contrast microscope at x200 magnification. An indication of flagella insertion can be gained by the nature of the bacterial movement. Bacteria with peritrichous flagella tend to torpedo through the suspending medium, while those with polar insertion have a lolloping motion in which the cell rotates around its longitudinal axis. The motion of cells with few peritrichous flagella may resemble that of polar flagellate cells.

Electron microscopy

Diluted bacterial suspensions from the synaeresis fluid associated with 24 h cultures incubated on YPA or other low nutrient, non-carbohydrate containing medium at 25^{o}C are suspended on formvar-carbon-coated 400 mesh grids and stained with 5 g/L aqueous uranyl acetate.

Flagella stain

Rhodes (1958) is an easy and reliable method.

Rhodes, M.E., 1958. The cytology of *Pseudomonas* spp. as revealed by a silver-plating staining method. *Journal of General Microbiology* **18**, 639-648.

References

Schaad, N. W., Jones, J. B., Chun, W. (eds) 2001. Laboratory guide for the identification of plant pathogenic bacteria. American Phytopathological Society (APS Press).

NOTES ON PATHOGENICITY TESTING

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Pathogenicity tests are an essential, but not routine, requirement for identification. From plant preparation to inoculation and completion of recording may take many weeks or months. Success is not assured and failure and repetition could cause very serious delays. The general conditions resulting in bacterial diseases are known, but specific requirements are obscure so that the conditions under which they are conducted may be sub-optimal.

With very few exceptions, bacterial pathogens only affect actively growing plant tissue. Therefore young plants, recently struck cuttings, or new growth on mature plants must be used. The same cultivar as that from which isolations were made, or known susceptible cultivars should be selected. Bacteria only reach infection sites and infect naturally in free water, which is therefore essential on surfaces. Warm temperatures are desirable for post infection development, but some pathogens do not cause disease in permanently high temperatures (e.g. *P. syr.* pv. *syringae* above ~ 22 ^{0}C).

Inoculum

Bacterial suspensions in water at >10^{6} – 10<^{8} cfu/ml from bacterial growth on plates of suitable agar is sprayed onto plant surfaces. Concentrations approaching 10^{8} cfu/ml may cause a hypersensitive reaction by bacteria not pathogenic to the host.

Inoculation

Inoculation of foliage must be conducted either in a mist cabinet, or by covering the plants in plastic bags. It is common practice to pre-treat plants in high humidity but efficacy has not been proved. Because natural infection is not guaranteed, a pattern of needle-pricks should be made on stems, petioles and leaves. Prior to inoculation, the top-soil of pots should be watered. Inoculum is sprayed onto all surfaces, paying special attention to the under-surfaces of leaves. Plant tissue should not be infiltrated because bacteria not pathogenic to the host may grow in the fluid, also resulting in a confusing hypersensitive reaction. Pumps delivering 30-40 KPa are suitable. A simple atomizer can be made by bending the tip of a disposable syringe needle at an angle to create a mist at manual syringe pressures. Simple bulb atomizers will also serve. Immediately after, plants should be transferred to a mist cabinet, or covered with internally wetted plastic bags. These should be sealed around pots or plants part to maintain high humidity and plants should not be exposed to full sunlight. Bags are removed after 24 – 48h. Extended incubation of some plants (e.g. *Pisum*) results in anomalous symptoms.

Inoculation of stems to induce galls, knots, or cankers can be done by pricking stems and petioles with a hypodermic needle charged with inoculum. Inoculations to induce wilts should puncture the cambium. No further treatment is necessary.

Negative controls are plans treated identically but without bacterial inoculum. Positive controls are proved pathogenic strains of suspect organisms. Quarantine regulations may prevent inclusion.

Incubation at temperatures fluctuating between ~12–22^{0}C are probably most suitable. Lower temperatures result in slow symptom development. Continuous high temperatures may result in anomalous symptoms.

Recording

The absence of any progressive necrosis at puncture points indicates that strains are not pathogenic or the plants are not susceptible. Progressive necrosis compared with absence at controls indicates that tissue is susceptible, but that inoculation conditions do not favour natural infection. Severity of natural infections many vary considerably for no identifiable reason. Plants should be observed for at least four weeks or until symptoms are fully mature, keeping a complete record. A detailed photographic record may be invaluable later.

SOME BIOCHEMICAL AND PHYSIOLOGICAL TESTS FOR IDENTIFICATION OF PSEUDOMONAS SYRINGAE AND RELATED SPECIES

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Below is an **Overview of Pseudomonas spp. associated with stone fruits and nuts** as described in: Janse J D 2010. Diagnostic methods for phytopathogenic bacteria of stone fruits and nuts in COST873. EPPO Bulletin 40: 68 – 85. **References mentioned can be also found in this article**, which will be distributed as a PDF for the course

The Pseudomonas syringae *complex on stone fruits (excluding the quarantine pathogen P.s. pv. persicae, see below)*

Pseudomonas syringae pv. avii - Canker of wild cherry

Notes

- Reported from France in 2003 (Ménard *et al.*, 2003). Very much related to *Pss* and also pathogenic to cultivated cherry.
- Strains from *Psa*, *Pss* and *Pmp* studied in Belgium (A. Bultreys, M. Steenackers, CWRA, Gembloux, Belgium).

Pseudomonas syringae pv. *syringae* - Apical necrosis of mango (*Mangifera indica*)

Notes:

- *Pss* was described on mango for the first time in 1999 from Australia, causing shoot necrosis, it was not found on fruits (Kennelly, 2007; Young, 2008), see Young, 2008.
- This disease-pathogen combination is not (yet) studied in COST873 although there is mango production e.g. in S. Spain and S. Sicily, Italy, Portugal and Israel and problems have been already reported from Spain, Portugal and Israel and the Emirates (Cazorla *et al.*1998; Gagnevin & Pruvost, 2001). Fatmi *et al.*, 2008 reported characterization of strains using rep-PCR and amplified ribosomal DNA restriction analysis (ARDRA).

***Pseudomonas syringae pv. morsprunorum* - Bacterial canker of stone fruits, blossom blast, gummosis, leaf spot of stone fruits**

Notes:

- This pathogen can cause diverse symptoms on leaves, buds, flowers and branches. The canker phase on shoots, branches and limbs most important. Hail storms favor the leaf spot phase.
- Two races have been described based on phage typing that show some host specificity (Freigouns & Crosse, 1975, Gilbert et al, 2009), and can be discriminated using some biochemical tests (see Table 2) and with rep-PCR. Their ecological significance is unclear.

Pseudomonas syringae pv. syringae - Bacterial canker or blast of stone and pome fruit, apoplexy of apricot, dead bud of cherry, twig dieback in hazelnut

Notes:

- *Pss* is a very diverse pathovar (may be even collection of pathovars) with many hosts. Many strains show some host specificity (Scortichini *et al.*, 2003).
- A severe outbreak of Pss on new apricot cultivars was reported by Scortichini (2006) and a fruit scab on nectarine by Scortichini & Janse in 2008.
- Apricot apoplexy caused by the related *P. viridiflava* was described by Scortichini & Morone (2008).

Methods **:**

Methods presented can be used for discrimination between different pathovars of *P. syringae* and were mainly developed by CWRA, Gembloux, Belgium (A. Bultreys), also see Young & Triggs, 1994.

- Good semi-selective media are Hildebrands pectate medium, Kings medium B and KBC with boric acid and fungicides (Schaad *et al.*, 2001). Medium MS3 (Vicente *et al.*, 2004) is used with success in the Latvian lab.
- Determination of ice nucleation activity (Lindow, 1990).
- Biochemical tests useful in discrimination between Pss, Pmp and Psa are the so-called GATTa tests, see Table 2 (Latorre & Jones, 1979) and between fluorescent pseudomonads the so-called LOPAT tests, see Schaad *et al.*, 2001 and Table 5. Toxin (lipodepsipeptides) detection via a biological test on PDA the yeast *Geotrichum candidum* or better on peptone-glucose-NaCl agar with the yeast *Rhodotorula pilimanae* and for coronatine on potato disks(Bultreys & Gheysen, 1999).
- Toxin detection with a molecular test (Table 4):
    1. PCR with primers (1,040-bp fragment) for the *syrD* gene (syringomycin and syringopeptin secretion according to Bultreys & Gheysen (1999).
    2. PCR for coronatine toxin production (Fig. 9b), developed by Bereswill *et al.* (1994).
    3. PCR for yersiniabactin with primers PSYE2/PSYE2R according to Bultreys *et al.* 2006.

- Pyoverdins are siderophores that are important for the fitness of fluorescent *Pseudomonas* spp. and differ between them and can therefore be used for identification. The different pyoverdins can be identified visually or by HPLC according to Bultreys *et al.*, 2001 and 2003 (Table 6) and used for differentiation of *Pseudomonas* strains
- A very suitable pathogenicity test on cherry fruitlets was developed by the Institute of Pomology, Skierniewice, PL (Sulikowska *et al.*, 2009). Others are mentioned in Thomides *et al.*, 2005. Bean pods (Schaad et al., 2001) can also be used to this purpose.

Pseudomonas syringae pv. persicae **(EPPO Quarantine List A2) - Dieback/canker on nectarine and peach**

Notes:

- The disease caused by *P. s*. pv. *persicae* was first observed in France 1967 and proved to be present also in New-Zealand in 1988, where also *Prunus salicina* (Japanese plum) was infected. It was once isolated in the UK from *P. cerasifera.*
- *P. s*. pv. *persicae* produces persicomycins (phytotoxic compounds) that may yield a tool for PCR detection (Barzic, 1999).

Methods:

- For diagnosis there is EPPO standard PM 7/43, biochemical tests useful in discrimination from other *Pss* pathovars are mentioned in Table 3.
- A pathogenicity test can be performed in dormant 1 year-old shoots of young trees from mid September to the end of January.

1. Methods that can be used for identification (biochemical, physiological, molecular and pathogenicity)

1.1. Below is **Tabular overview of discriminative tests for Pseudomonas spp.** associated with stone fruits and nuts. More information can be found from: Janse J D 2010. Diagnostic methods for phytopathogenic bacteria of stone fruits and nuts in COST873. EPPO Bulletin 40: 68 – 85.

Table 1 – Tests for discrimination between *Pseudomonas avellanae* and some related *Pseudomonas syringae* pathovars

Test	*P avellanae*	*P.s pv coryli*	*P.s* pv. *syringae*	*P.s. pv. morsprunorum*
Ice nucleation	-	V	+	-
Utilization of: adonitol	-	-	+	-
sorbitol	-	+	+	+
erythritol	-	-	+	V
L+tartrate	-	-	-	+
L-lactate	-	-	+	-
Gelatine liquefaction	-	-	+	-
Arbutin hydrolysis	-	+	+	-
Aesculin hydrolysis	-	+	+	V
syrB gene	-	-	+	-

- = negative; + = positive; V = variable

Table 2 – GATTa and some additional tests for discrimination between *Pseudomonas syringae* pv. *syringae*, *P.s.* pv. *morsprunorum* and *P.s.* pv. *avii*

Test	P.s. pv. *syringae*	*P.s.* pv. *morsprunorum* race 1	*P.s.* pv. *morsprunorum* race 2	P.s. pv. *avii*
Gelatin hydrolysis	+	-	-	-
Aesculin hydrolysis	-/+	-	+	-
Tyrosinase activity	-	+	-	-
Utilization of Tartaric acid	-/+	+	+	+
Utilization of Lactic acid		+	+/-	-
Fluorescence on KB	+	+/-	+/-	-
Colour after growth in Nutrient Sucrose broth	Y	W	Y/W	?

- = negative; + = positive; +/- = variable/weak; Y= yellow; W=white

Table 3 – Biochemical tests useful in discrimination of the quarantine bacterium *P.s.* pv. *persicae* from *P.s.* pv. *syringae* and *P.s.* pv. *morsprunorum*

Test	*P.s.* pv *persicae*	*P.s.* pv. *syringae*	*P.s.* pv. *morsprunorum*
Fluorescence on KB	-	+	+
Utilization of inositol	-	+	+
sorbitol	-	+	+
erythritol	-	-	V
L+tartrate	-	-	+

- = negative; + = positive; V = variable

Table 4 – Differential PCR's for races of *Pseudomonas syringae* pv. *morsprunorum*, *P. s.* pv. *syringae* and *P. s.* pv. *avii*

Bacterium	PCR *cfl* coronatine	PCR *syr B* or *syrD*	PCR irp1 yersiniabac
P.s. pv. *morsprunorum* race 1	+, few -	-	-
P.s. pv. *morsprunorum* race 2	-	-	+
P.s. pv. *syringae*	-	+, few -	-
P.s. pv. *avii*	-	-	+

- = negative; + = positive

Table 5 – LOPAT tests for differentiation of fluorescent *Pseudomonas* spp.

	Levan	Oxidase	Potato rot	Arginine dihydrolase	Tobacco HR
P. syringae	+	-	-	-	+
P. viridiflava	-/+	-	+	-	+
P.cichorii	-	+	-	-	+
P. marginalis	-/+	+	+	+	-
P.delphinii group	-	-	-	-	+
P. tolaassii group	-	+	-	+	-

- = negative; + = positive; +/- = variable

Table 6 – Differentiation of some *Pseudomonas* spp. On the basis of pioverdin production, oxidase and potato rot

	Pyoverdin	Oxidase	Potato rot
P. syringae	Pa	-	-
P. viridiflava	Pa	-	+
P.cichorii	Pa	+	-
Other *Pseudomonas* spp.	Pt	+/-	+/-

- = negative; + = positive; +/- = variable;
Pa = atypical pyoverdin; Pt = typical pyoverdin, see Bultreys et al., 2003

1.2. Biochemical tests

Composition of media

King' s medium B (King *et al*. 1954) per litre:

Proteose peptone (Difco No. 3/Oxoid L46)	20 g
$K_2HPO_4.3H_2O$	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15.0 g
Glycerol	15.0 mL

supplemented with 200 mg cycloheximide (Sigma) after autoclaving. The medium has pH 7-7.2. Autoclaving is performed at 121°C for 15 min. (The medium is commercially available as Pseudomonas Agar F, Difco).

Nutrient sucrose agar or NSA:
5% sucrose w/v added to Difco or Oxoid nutrient agar.
Adjust pH to 7.2. Autoclaving is performed at 121^0 C for 15 min

Nutrient Agar (NA)

Nutrient Agar (Difco)	23,0 g
Distilled water	1,0 L

Dissolve ingredients and sterilise by autoclaving at 121 °C for 15 min.

Yeast Peptone Glucose Agar (YPGA)

Yeast extract (Difco)	5,0 g
Bacto-Peptone (Difco)	5,0 g
D(+) Glucose (monohydrate)	10,0 g
Bacto-Agar (Difco)	15,0 g
Distilled water	1,0 L

Dissolve ingredients and sterilise by autoclaving at 121 °C for 15 minutes. pH should be 7-7.2

Ayers et al., 1919 mineral salts medium for carbon compound utilization

NH4H2PO4	1.o g
KCl	0.2 g
MgSO4.7H2O	0.2 g
Bromthymolblue (1.6% v/v in 95% alcohol)	1 ml
Agar	12 g

Dissolve and adjust pH to 7.2 and autoclave for 15 min at 121
Carbon compounds 0.1% can be added after filter sterilisation to autoclaved and cooled (50 C) medium
Observe at 3, 7 and 14 days incubation at 27-28C

Gelatin liquefaction

Bacto yeast extract (Difco) 3 g;
Oxoid peptone , 5 g;
Gelatin (B.D.H.), 120 g;
distilled water, 1 litre.
Dissolve ingredients and sterilise by autoclaving at 121 °C for 15 minutes. pH should be 7-7.2

Incubate for 48-72 h at 27-28C and place tubes at ***c.* 4º**for **30** min before recording results Liquefaction is only scored as positive when the medium flows readily when tilted; a very viscid type of liquefaction which flows slowly should be ignored.

Reduction of nitrate

(8 ml of medium/tube) were made in a medium containing:

KNO_3	1 g
Oxoid peptone	*5* g;
yeast extract (Difco)	*3* g;
Oxoid agar No. *3*	*3* g;
distilled water,	1 litre

Dissolve ingredients and sterilize by autoclaving at 121 °C for 15 minutes. pH should be 7-7.2

Stab inoculate and incubate for 48-72h at 27-28C. Tubes of 8 ml of medium are examined for evidence of gas production and tests for nitrite are made by adding the following solutions (modified Griess Ilosvay's reagents) :1 ml of a 0.6% (v/v) solution of dimethyl-a-naphthylamine and 1 ml of a 0.8% (w/v) solution of sulphanilic acid, both in 5 *N* acetic acid (development of a red colour is positive). If no distinct red color develops in ***1*** h, add zinc dust: when red color develops no complete denitrification occurred.

Aesculin and arbutin hydrolysis

Peptone	10g
Aesculin or arbutin	1g
Sodium citrate	1g
Ferric citrate	0.05 g

Adjust pH to 7.0 Autoclave for 15 min at 121 ^{o}C
Incubate for 3-4 days at 27-28 and observe development of brown color (Sneath, 1979)

Arginin hydrolysis (Thornley's medium 2A)

Peptone	1.g
NaCl	5 g
K2HPO4	0.3 g
Agar	3 g
Phenol red	1 mg
Arginine HCl	10.0 g

Adjust pH to faint pink colour (pH 7.2)
Stab inoculate tubes and seal with 1-2 ml sterile paraffin oil. Incubate at 27-28C for 3-4 days and observe for development of red colour
Schaad et al. 2001

Oxidase test

Tetramethyl-p-phenylenediamine dihydrochloride 1% w/v in sterile distilled water
Rub a small loopful of growth from a 24-48h pure culture on Nutrient glucose agar (Nutrient agar (Difco) with 0.1 % glucose) on a filterpaper impregnated/wetted with the above mentioned solution. Positive when a purple colour develops within 10 seconds, delayed positive when purple between 10 and 60 seconds and negative when purple after 60 sec or not purple at all. Use a platinum or plastic loop, to avoid false positives (oxidation by iron particles from other type of loops)

Potato soft rot
For this test, well washed potato tubers are surface sterilized with 70% ethanol and then peeled under microbe-free conditions. With a flamed scalpel cut slices about 0.7 cm thick. Place slices in a sterile Petri dish containing sterile water to a depth of 3 mm, or place the slices on filter paper moistened with sterile water. Place a loopful of inoculum in the middle of each slice and incubate for 24-48 h at 27ºC. Soft rotting bacteria produce massive rot in 24 h. However, many other species, including saprophytic pseudomonads and *Bacillus* spp.*,* also show pectolytic activity, although it is usually not very intense. *Pseudomonas syringae* tests negative.

Hypersensitive reaction on tobacco leaves
Infiltrate bacterial suspension (approximately 10^8 CFU/ml) with a hypodermic syringe into the intercellular space of a healthy actively growing tobacco (Nicotiana tabacum, e.g. cv. White Burley or Samson) leaf. Reaction is positive if the injected tissue turns necrotic within 24 h. Non-pathogenic bacteria do not cause necrotic symptoms, though occasionally some chlorosis may develop a few days after inoculation. Although this test indicate that a strain is phytopathogenic, it is not a substitute for the pathogenicity test on a susceptible host.

KOH test
Mix a loopful of a 24-48h culture on NA or NSA with 2 drops of 3% KOH on a microscope slide. When touching the suspension with a loop strands (sticky threads) can be drawn from Gram-negative bacteria and not from gram-positive bacteria

1.3 Some other culture media and test media and their preparation

Nutrient Agar (NA)

Commercially available nutrient agar should be used (Difco, Oxoid, etc.)

Yeast-Peptone-Glucose Agar (YPGA)

Yeast extract (Difco)	5 g
Bacto peptone (Difco)	5 g
D(+)glucose(monohydrate)	10 g
Bacto agar (Difco)	15 g
Distilled water	1 litre

Dissolve ingredients in water. Sterilize during 15 min at 115-121°C. Cool to 50°C and pour plates (c. 15 ml/plate)

Phosphate buffered saline (PBS), 0.01M, pH 7.2

$Na_2HPO_4.12H_2O$	2.7 g
$NaH_2PO_4.2H_2O$	0.4 g
NaCl	8 g
Distilled water	1 litre

Dissolve ingredients and check pH. Sterilize during 15 min at 115-121 °C

Nutrient broth and nutrient broth 5% NaCl

Use a nutrient broth commercially available (Difco, Oxoid, etc.) according to instructions (8 g per litre distilled water).

Dissolve and sterilize for 15 min at 115-121°C

For Growth in NaCl test: add 5% (or other concentration desired) to Nutrient broth, dissolve and sterilize.

Minimal medium according to Hugh & Leifson for carbon compound testing

KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
$NH_4H_2PO_4$	1.0 g
Difco Bacto peptone	1.0 g
Difco Bacto Agar	3.0 g
carbon source	**10 g**
Bromothymolblue	0.03 g
Distilled water	1 litre

Dissolve ingredients and adjust pH to 7.0-7.2 with 1N KOH. Dispense in 5 ml quantities in 10 ml screw cap tubes. Sterilize for 10 min at 115°C.

Note: In this medium you add 1% Na-malonate, trehalose, maltose, α-methylglucoside, glucose (O-tube, 5 ml and F-tube, 10 ml)

Cellulose medium

Na-taurocholate	5 g
$NH_4H_2PO_4$	1 g
$MgSO_4.7H_2O$	0.2 g
KCl	0.2 g
Bromothymolblue	0.05 g
$CaCO_3$	3 g
Carboxymethylcellulose (Sigma)	80 g

Kovacs reagent

a) p-dimethylamino-benzaldehyde	5 g
b) amyl-alcohol	75 ml
c) concentrated HCl **(caution!)**	25 ml

Dissolve a) in b) by gently warming in water bath (50°C).Cool and add c to solution a+b.
Protect from light and store refrigerated

Gram-stain dyes

Crystal violet - dissolve 2 g in 20 ml 96% ethanol. Dissolve also 0.8 g ammonium oxalate in 8o ml distilled water. Mix the two solutions

Lugol's iodine - Dissolve 1 g iodine and 2 g potassium iodide under slight warming **(under a chemical hood: iodide vapours are toxic!)** in 300 ml distilled water

Safranin O - Dissolve 2.5 g Safranine O in 100 ml alcohol 96%. Dilute this stock solution to 1:10 to obtain the working dilution

1.4 Experiment 1 - Inoculation of different *Pseudomonas* and *Erwinia* strains in tubes for oxidative/fermentative and acidification of carbohydrate tests

Principle

The above mentioned *Pseudomonas* bacteria can be discriminated by different biochemical tests, for which Tables can be found in this document and in literature. The most useful ones have been selected for this course. A further selection of these tests are shown and explained in Table 7 (here only *Erwinia* (*Pectobacterium*) strains (*Erwinia atroseptica* = *Eca, E. carotovora* subsp. *carotovorum* = Ecc and *E. chrysanthemi*= *Echr*) are presented, but the principle remains the same for *Pseudomonas* spp.) and will be partly practiced (O & F test, acid production from carbon compounds). This Table also gives the results of the discriminative tests. For a description of the tests see under **Experiment 2 - procedure**..

Table 7 - Identification tests [1] for three different *Pectobacterium* (*Erwinia*) bacteria

Test	Reaction of					
	Eca		***Echr***		***Ecc***	
Acid from:		[2]		[2]		[2]
D-trehalose	**-**		**+**		**-**	
α-methylglucoside	**+**		**-**		**-**	
maltose	**+**		**-**		**-**	
Alkali						
Na-malonate	**-**		**+**		**-**	
Indole production	**-**		**+**		**(+)**	
H_2S production	**+**		**+**		**+**	
Hydrolysis of cellulose	**-**		**+**		**-**	
Oxidative/fermentative metabolism of glucose (O and F tube)	**+/+**		**+/+**		**+/+**	
Growth in 5% NaCl	**+**		**-**		**+**	
Reducing substances from sucrose	**+**		**-**		**-(+)**	

+ = positive; - = negative; (+) = positive in some strains

[1] Not all discriminative tests included in this practical study

[2] Column for noting results obtained

Tests which will be performed, marked yellow

Oxidative/fermentative metabolism of glucose (O&F test) and acid/alkali production from carbon compounds

Procedure

- Sterilize a loop in the flame and cool in alcohol
- Pick up a little growth from the NA plate of the pure culture of the *Erwinia* and/or Pseudomonas strain that will be tested
- Transfer to a tube with 2 ml sterile distilled water in an **aseptic way (working near the flame, opening tube and plate as short as possible)**
- Suspend the bacterial growth with the loop in the tube (light milky suspension, c.10^7 cells.ml^{-1}
- Inoculate each test tube with one loopful of bacterial suspension **(sterilize and cool loop between each test tube!)**
- Put on top of the medium in the **F-tube** c. 0.5 ml of **sterile paraffin in an aseptic way - do not flame tube after adding paraffin**
- Put a **H_2S filter paper strip in the indole tube** by clamping it between tube and screw cap as shown in the drawing, in an aseptic way.
- Put all inoculated tubes in a rack in an incubator at 27°C. Incubate at least for 2 days at 27ºC, not longer! **Note name and date on tubes or rack.**

1.5 Experiment 2 - Performance and judgement of identification tests

Principle

Acid formation from carbon-sources:

Tubes contain a minimal medium (low nutrient content, with only one carbon source added, in this case D-trehalose, maltose, α-methylglucoside and glucose). When bacteria are able to decompose these C-sources, acid degradation products will be formed. The medium becomes therefore more acid and the pH indicator present in the medium (bromthymolblue, green at pH7) will change from green to yellow. When a tube with glucose is sealed from air by sterile paraffin, only organisms with (facultative) anaerobic metabolism will be able to decompose glucose. All *Erwinia* bacteria are facultative anaerobic, *Pseudomonas* is strictly aerobic.

Alkali formation from carbon sources:

The same principle as for acid formation as described above, but now the bacteria form alkaline products following decomposition of the carbon compound (Na-malonate in this case). The indicator dye will change from green to blue when the pH is raised.

Hydrolysis of cellulose:

Erwinia chrysanthemi is able to hydrolyze carboxymethylcellulose, while other Erwinia's are not. A thick syrup of cellulose in a minimal medium has a high viscosity. The viscosity will be lowered after hydrolytic enzymes (cellulases) of *Echr* have decomposed the molecule as shown in the drawing.

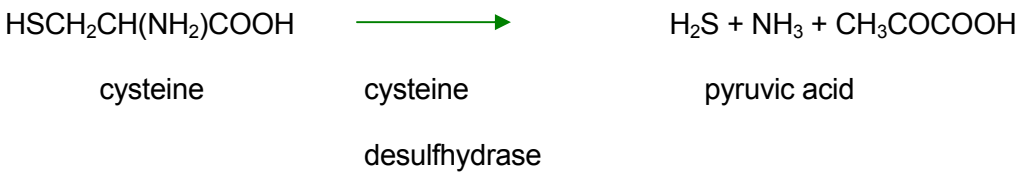
Indole formation:

Echr and some strains of *Ecc* produce indole from the amino acid tryptophane by means of tryptophanase. The other *Erwinia* strains do not produce indole. The production of indole can be verified by first extraction it by ether from the growth medium and subsequently staining it with p-Dimethylaminobenzaldehyde in concentrated HCl (Kovac's reagent). The latter compound reacts with indole to form the red coloured rosindole dye.

Growth in 5% NaCl:
Certain bacteria will grow at high salt concentrations and withstand high osmotic forces, others will not.

H_2S production:

All *Erwinia* bacteria produce H_2S from the amino acid cysteine by the following reaction:



Heavy metals can be used for the detection of H_2S (lead, bismuth, iron). When filter paper is impregnated with lead acetate and exposed to volatile H_2S, the H_2S reacts with lead acetate to form a black precipitate on the filter paper, viz. lead sulphide.

Reducing substances from sucrose:

Upon decomposition of sucrose reducing compounds may be formed by bacteria. The reaction is used to differentiate *Eca* fro other Erwinia's. Reducing substances can be detected by adding Benedict's Reagent. The cupric sulphate in the Benedict's reagent will be reduced to an orange Cu_2O precipitate after boiling.

Procedure:

Acid formation from D-trehalose, maltose, α-methylglucoside and glucose (O-tube):

Check for yellow color, score green as negative (-), faint green as doubtful (d), a little yellow in the top layer as weak positive (+/-)and a strong yellow color as positive (+).

Fermentative metabolism of glucose (F-tube):

Check for a yellow color under the paraffin. Green is negative (-); a little yellow discoloration just under the paraffin is doubtful (d); yellow is positive (+).

Alkali formation from NA-malonate:

Check for a blue color, score green or yellowish as negative (-), dark green as doubtful (d), a little blue in the top layer as weak positive (+/-) and a strong blue colour as positive (+).

Hydrolysis of cellulose:

- Check cultures for a yellowish or light green color, indicative of degradation of cellulose. However, not always cellulose decomposition is followed by a color change in the medium!

- Check viscosity in comparison with a non-inoculated control tube. Do this by running the fluid in the two tubes simultaneously slowly to the top of the tube by tilting. If the fluid runs at the same speed the test is negative, if the fluid of the inoculated tube runs faster, the test is positive. More accurate measurements can be made in a viscosimeter.

H_2S formation:

Check if the filter paper in the indole/H_2S tube turned black. A black colour, even when only the rim of the paper, is a positive reaction.

Indole formation

- **Remove first the H_2S filter paper strip**
- Add carefully **(stay away from open fire, do not spoil!)** 1 ml of di-ethylether to the indole/H_2S tube
- Shake the tube vigorously, place in rack
- Wait for 1 minute
- Add 0.5 Kovac's indole reagent **slowly along the wall of the tube**, in order to get a ring of reagent between growth medium and the ether layer **(Do not spoil: strong acid!).** Check for a red color of the Kovac's reagent. Wait for a few minutes. Score yellow as negative (-), red as positive (+).

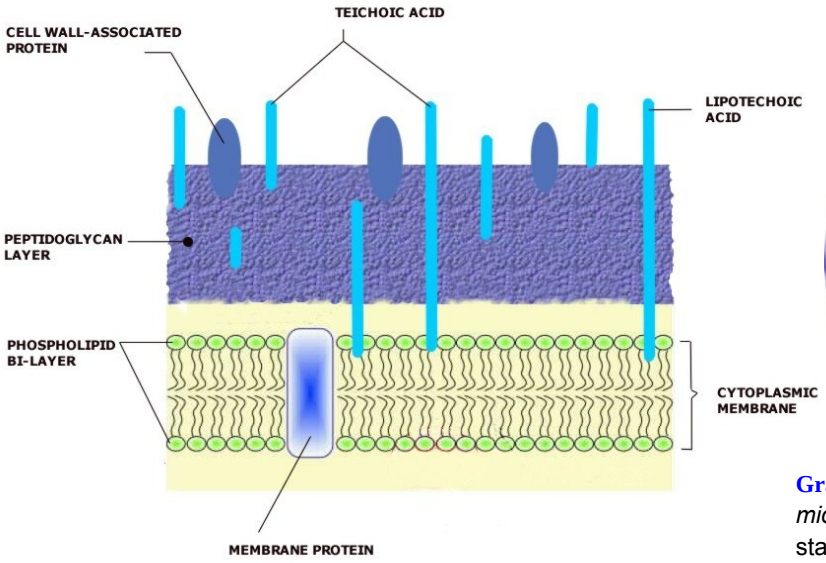
Growth in 5% NaCl

Check for growth by shaking the tube, score positive if a cloud of growth is seen after shaking or if the tube is turbid. Score negative when the medium is still clear.

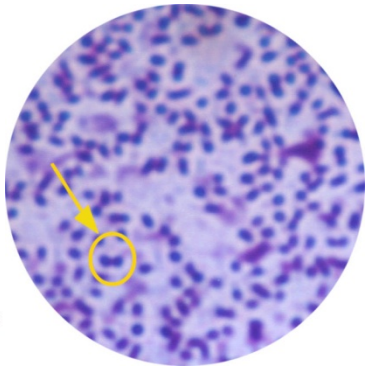
Reducing substances from sucrose:

- **Check first if a water bath with boiling water is available**
- Add 5 ml blue Benedicts reagent to the BS tube
- Tightly close the tube
- Boil tube for 10 min in a boiling water bath
- Check for change in color. Score orange precipitate as positive; dark brown discoloration with an orange precipitate as positive; dark green as doubtful and blue as negative.

DIFFERENCES IN CELL WALL STRUCTURE AND COMPOSITION BETWEEN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

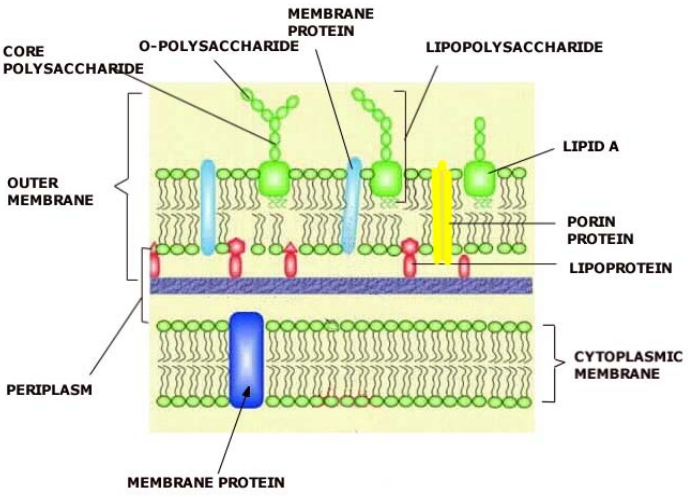


GRAM-POSITIVE CELL WALL

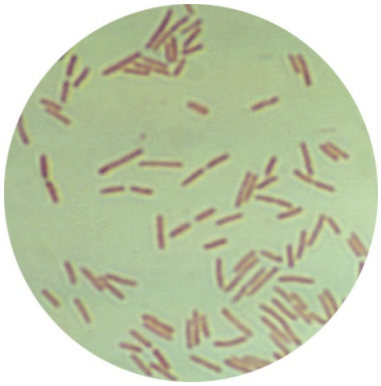


Gram-positive cells of *Clavibacter michiganensis* subsp. *sepedonicus,* staining blue with the primary dye crystal violet in the Gram stain.

Arrow: cells in typical, so-called



GRAM-NEGATIVE CELL WALL



Gram-negative cells of *Pseudomonas savastanoi* pv. *fraxini*, staining red with the counter stain using safranine in the Gram stain.

Fig. 1
Structure of bacterial cell walls and reaction in Gram stain. For description of Gram stain, see text.
From: J.D. Janse 2006. Phytobacteriology, Principles and Practice

1.6 Experiment 3 - Gram stain

Principle:

The Gram stain is important for classification and differentiation of bacteria. The specificity is determined by the difference in cell wall structure and composition of so-called Gram-positive and Gram negative bacteria (**Fig. 9**). Gram-positive means that the bacterium retains a complex of crystal-violet and iodine even after decolouration action with alcohol. Gram-negative bacteria do not retain crystal violet and are stained red with the counter stain safranin. Pseudomonas syringae will be used as an example of a Gram-negative bacterium and *Rhodococcus fascians* as an example of a Gram-positive bacterium.
A simple reaction to demonstrate the presence of gram-positive or negative cell wall is the KOH test (see above)

Procedure

- Place two drops of tap water on a slide
- Sterilize a loop in the flame and cool in alcohol
- Pick up a little growth of *P. syringae* and disperse completely in a drop of water on the slide. **The drop should be very light milky - otherwise there are too many cells present**
- Repeat the procedure for *R. fascians*
- Dry the smears in the air
- Fix the dry smear in the flame by passing the slide **(bacteria up!)** with forceps two or three times through the flame (with the speed of handling a cheese slicer)
- Cool the slide for 1 min on the table
- **Wear preferably disposable gloves - Gram-stain dyes are toxic!**
- Flood the slide with **crystal violet** for *one minute*
- Wash in tap water, shake off excessive water
- Flood with **Lugol's iodine** for *one minute*
- Replace iodine by **alcohol 96%** or 100% for *25 seconds, not more (risk of too much destaining, false results)*
- Wash by rinsing in tap water
- Flood the slide with **safranin** for *30 seconds*
- Wash by shortly rinsing in tap water
- Blot dry with filter paper
- Examine with a drop of immersion oil and a 100:1 oil immersion objective of a light microscope.

Expected result

***P. syringae* cells** are visible at 1000x magnification under immersion oil as red, short rod-shaped cells. If the decolouration with alcohol was not sufficient the cells are still purple.
***R. fascians* cells** are visible as purple, long and irregular rod-shaped cells. Also see **Fig. 1**

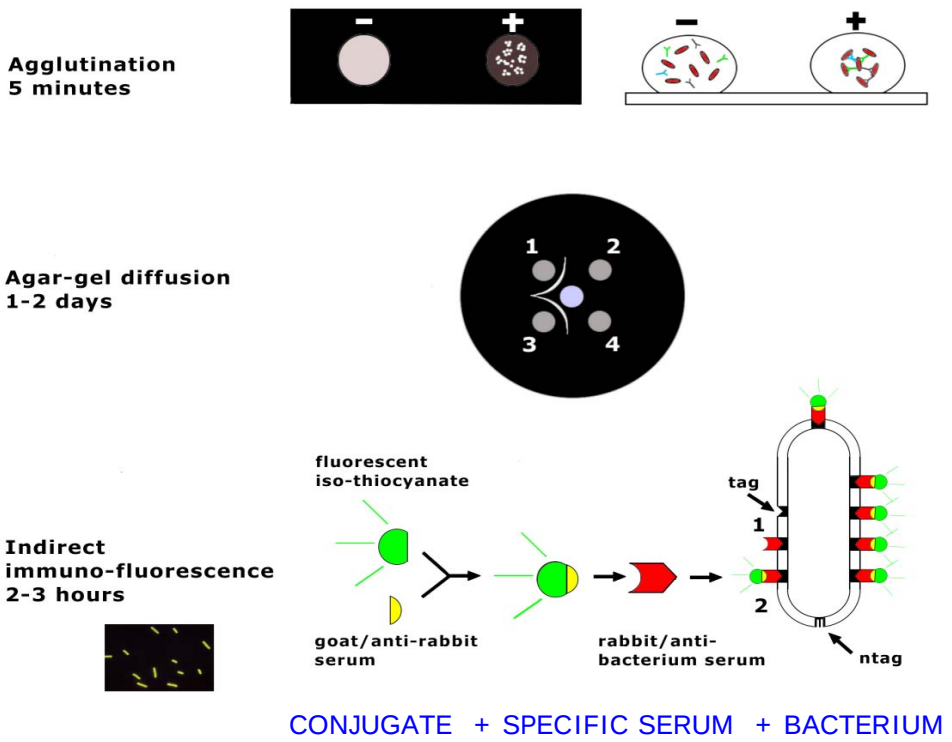
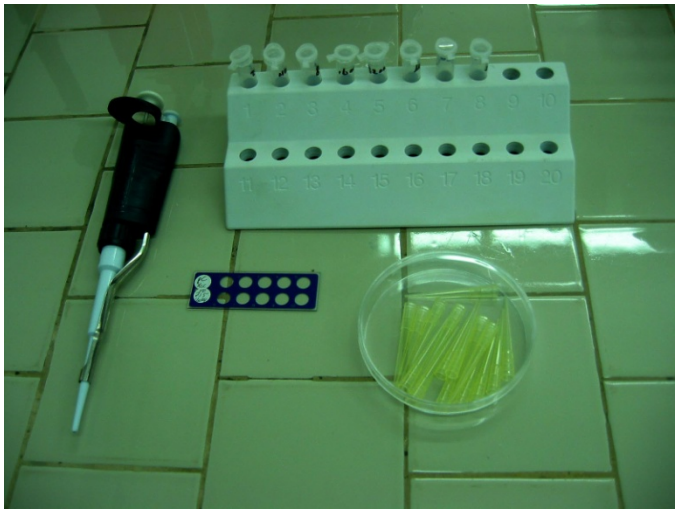


Fig. 2

a) Serological techniques used in detection and identification of bacteria.
tag = target antigen; ntag = non-target antigen. Also see text.
From: J.D. Janse 2006. Phytobacteriology, Principles and Practice.

b) Dilution series of antiserum in so-called eppendorf tubes, pipette and disposable tips and IF microscope slide.



1.7 Experiment 4 - Immuno-fluorescence test with *Pseudomonas syringae* pv. *syringae*

Principle

Immunofluorescence (IF) is a very sensitive and robust serological test where the primary reaction of antigen and antibody is made visible. Binding reactions can be observed at very high titres (**titre = highest dilution of the antiserum, where a clear reaction is still visible**) of antiserum. In the IF test **antibodies** are **marked with a chemical dye that is fluorescing in blue light**, mostly fluorescein isothiocyanate (FITC). For IF a light-microscope fitted for epi-fluorescent light is necessary with the suitable excitation and barrier filters for FITC (**Fig. 12** *bottom left*). In so called **indirect IF** (**Fig. 10a**) the bacteria are first treated with a pathogen-specific rabbit or mouse antiserum (against the target bacterium). After incubation and washing, a second, labelled **anti-rabbit or anti-mouse** serum, prepared in another animal (e.g. goat), is applied. This anti-rabbit or anti-mouse serum is called **conjugate**. Only the antibodies bound to the bacteria will fluoresce, the others are removed by washing.

Procedure

- You will receive a blue, ten-well microscope slide, where a suspension of 10^6 cells.ml^{-1} has been heat-fixed on the wells of the slide (ready for use).
- You will find a row of eppendorf vials with a dilution series of an antiserum specific for *Pseudomonas syringae* pv. *syringae*. The serum has been diluted in PBS (0.01 M) and is ready to use
- Apply 20 µl (with a micro pipette, using disposable tips) serum) of the highest dilution (**1:25600) on the last well (no.10) of the slide**. Apply a drop of the forelast dilution (**1:12800**) on well **no. 9**, etc., until well no.2. **Well no.1 does not receive antiserum (negative control)**. See **Fig. 3a** and **b**
- Place the slide very carefully (the drops should not mix!) on a wet filter paper and incubate for 25 min (+ or – 5 min) at room temperature (RT) under a cover (to avoid evaporation).
- Wash slides for 2 min in PBS Tween (will be done with 10 slides in a tray), remove PBS Tween and place the tray in fresh PBS, wash again for 2 min. Blot slides dry with filter paper or tissue (**blot gently and do not wipe!**).
- You will find an eppendorf vial with ready to use (1:100 diluted in PBS) –rabbit, FITC labeled conjugate.
- Apply to each well (also the first, which is the negative control well, to check eventual cross-reactivity of the conjugate) 20 µl (with a micro pipette, using disposable tips) of the conjugate solution.
- Place the slide very carefully (the drops should not mix!) on a wet filter paper and incubate for 25 min (+ or – 5 min) at room temperature (RT) under a dark cover (to avoid evaporation and influence of light on the light sensitive conjugate).

- Wash slides for 2 min in PBS Tween (will be done with 10 slides in a tray), remove PBS Tween and place the tray in fresh PBS, wash again for 2 min. Blot slides dry with filter paper or tissue (**blot gently and do not wipe!**).
- You will find an eppendorf vial with ready to use glycerine embedding buffer.
- Place **small droplets of glycerine buffer** with a micro pipette on each well, **without touching the slide with the pipette**.
- Place a long cover slip on the IF slide so that all windows are covered.
-
- Apply a small drop of immersion oil on the cover slip in the centre of each well.
- Observe under epi-fluorescent light with a fluorescence microscope (40ximmersion, 63x immersion or 100ximmersion objective)
- Determine the titre (last dilution of antiserum where cells show a bright fluorescence) and chek the negative control well no. 1 (where no bacterial cells should be visible)

Expected result

Cells of *Pss* fluoresce green-yellow on a black background (**Fig. 12** *bottom right*). Titre of the serum is determined and compared to titre as determined in earlier tests.

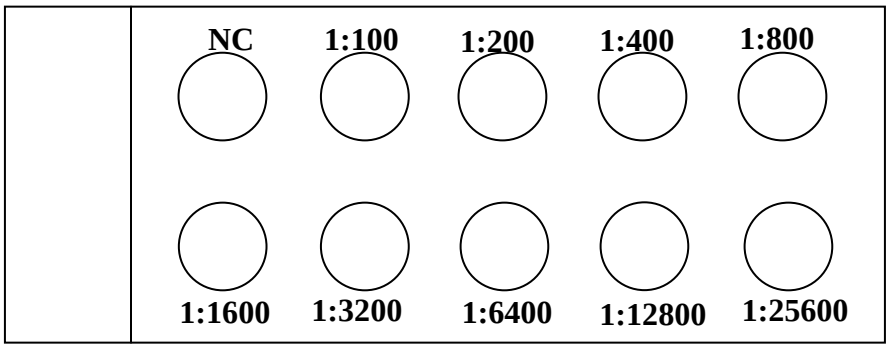
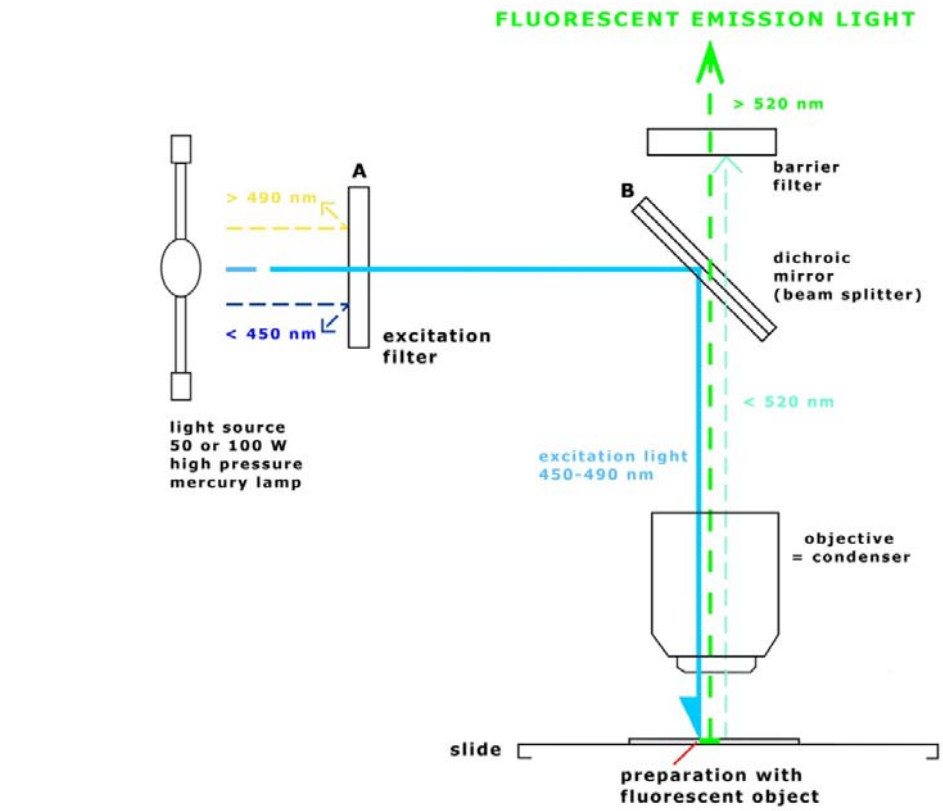
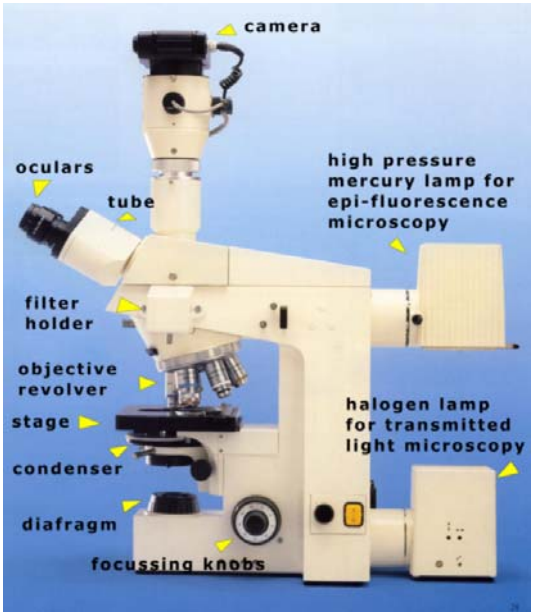


Fig. 3a Scheme of dilution of antiserum, see text

Fig. 3b
Application of antiserum to bacteria (or plant extract containing bacteria) fixed to wells on a microscopic slide for use in the **indirect immunofluorescence (IF) test**. After incubation with a specific antibody, slides are washed and incubated with a second antibody labeled with a fluorescent dye (called conjugate). **Positive reaction** between antibodies and bacteria are made visible **under a fluorescence microscope (Fig. 12)**

From: J.D. Janse 2006. Phytobacteriology, Principles and Practice.





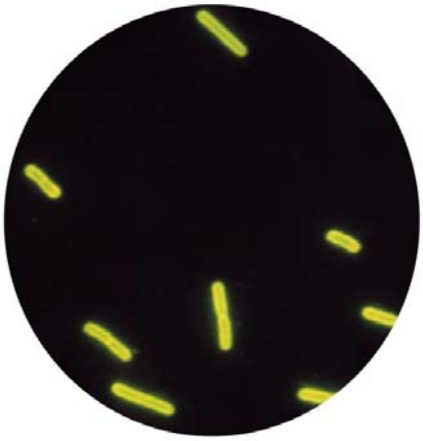


Fig. 4

Top: **Path of light in an (epi-) fluorescent microscope**. A fluorescent compound like FITC as used in the IF-test needs a specific **excitation** wavelength (**blue light of 450-490 nm**), from a powerful (mercury) lamp to start its fluorescence and also a specific fluorescence **emission** wavelength (**yellow-green light of 520-550 nm**). Through a combination of filters the correct wavelengths are allowed to enter the preparation and eyepiece, resulting in the picture as shown *below, right*

Below left: **Modern fluorescence microscope** (Zeiss Axio).

Below right: View of **IF-positive cells** of *Erwinia chrysanthemi* under the fluorescence microscope.

From: J.D. Janse 2006. Phytobacteriology, Principles and Practice.

IF-Buffer (10 mM phosphate buffered saline (PBS), pH 7.2)

This buffer is used for dilution of antibodies
Na2HPO4.12H2O 2,7 g
NaH2PO4.2H2O 0,4 g
NaCL 8,0 g
Distilled water 1,0 l
Dissolve ingredients, check pH and sterilise by autoclaving at 121 °C for 15 minutes.

IF-buffer-Tween

This buffer is used to wash slides.
Add 0,1 % Tween 20 to the IF buffer.

Phosphate buffered glycerol, pH 7,6

This buffer is used as a mountant fluid on the windows of IF slides to enhance fluorescence.
Na2HPO4.12H2O 3,2 g
NaH2PO4.2H2O 0,15 g
Glycerol 50 ml
Distilled water 100 ml

Anti-fading mountant solutions are commercially available e.g. Vectashield® (Vector Laboratories) or Citifluor® (Leica).

Determination of contamination level in IF and FISH tests

1. Count the mean number of typical fluorescent cells per field of view (c).
2. Calculate the number of typical fluorescent cells per microscope slide window (C).
   $C = c \times S/s$
   where S = surface area of window of multispot slide
   and s = surface area of objective field
   $s = \pi i2/4G2K2$ where i = field coefficient (varies from 8 to 24 depending upon ocular type)
   K = tube coefficient (1 or 1,25)
   G = magnification of objective (100 ×, 40 × etc.).
3. Calculate the number of typical fluorescent cells per ml of re-suspended pellet (N).
   $N = C \times 1\,000/y \times F$
   where y = volume of re-suspended pellet on each window
   and F = dilution factor of re-suspended pellet.

1.8 BOX PCR Protocol

(based on EPPO standard, developed by J.D. Janse that will soon be published)

Isolation of DNA

1. Suspend 1/3 loopful of bacteria from a 48-72h culture on NA or YPGA in 100 µl R/DNAse free water in a 1.5 mL eppendorf vial (c. 10^9 cells/mL). Other suitable non selective media could be used.
2. Vortex to homogeneous suspension

3. Lyze bacteria and extract DNA by simply heating for 15 min at 95ºC, or with a DNA extraction kit, such as according to Roche HighPure PCR Template Preparation Kit (cat. no. 1-796-828, 100 isolations) a follows:

a) Principle

- Bacterial cells are lyzed during a short incubation with lysozyme in proteinase K and all nucleases are inactivated by guanidine-HCl.
- Nucleic acids bind selectively to glass fibres in the High Pure Purification filter tube.
- Bound nucleic acids are washed with Inhibitor Removal Buffer in order to remove PCR-inhibitory components.
- Bound nucleic acids are washed with Wash Buffer to remove salts, proteins and other cellular contaminants.
- Purified nucleic acids are recovered from the glass fibre using a low salt Elution Buffer.
- Purified DNA can subsequently be used for (rep)PCR, restriction digestion or AFLP.

Equipment and consumables

- Pipettes (P10, P20, P100, P200, P1000)
- Microcentrifuge (e.g. Eppendorf)
- Thermomixer of heating block (e.g. Eppendorf 5436)
- Vortex (e.g. IKA MS2 minishaker)
- Tray with ice
- Racks for eppendorf vials (1.5 ml)
- DNase en RNase free reaction tubes (1.5 ml)
- DNase en RNase free collection tubes (1.5 ml) without lid (included in kit)
- Filtertips SSNC 10 µl, 20 µl, 200 µl en 1000 µl
- Latex disposable gloves, powder free

c) Buffers, etc.

- Roche High Pure PCR Template Preparation Kit (Catalog no. 1 796 828)
- Lysozyme solution (10 mg/mL lysozyme in 10 mM Tris-HCl, pH 8.0)

- Isopropanol
- R/DNase free water

Procedure

REMARK: To avoid 1) cross-contamination of samples with pathogens and/or DNA of pathogens, 2) PCR-inhibitors or 3) skin contact with irritating and/or toxic compounds, latex disposable gloves, powder free should be used.

Note: Pre-warm elution buffer to 70˚C

1. Pipette 200 µl extract (of bacterial suspension in R/DNase free water in a 1.5 mL vial.
2. Add 5 µL Lysozyme solution (10 mg/mL lysozyme in 10 mM Tris-HCl, pH 8.0) and incubate 15 min at 37ºC.
3. Add 200 µL Binding Buffer and 40 µl Proteinase K, mix immediatly and incubate 10 min at 70ºC.
4. Add 100 µl isopropanol and mix well.
5. Pipette the sample into the upper reservoir of a combined Filter Tube-Collection Tube assembly
6. Centrifuge at 8000 rpm for 1 min in a microcentrifuge.
7. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube and add 500 µl Inhibitor Removal Buffer.
8. Centrifuge at 8000 rpm for 1 min.
9. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube and add 500 µl Wash Buffer.
10. Centrifuge at 8000 rpm for 1 min.
11. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube and add 500 µl Wash Buffer.
12. Centrifuge at 8000 rpm for 1 min.
13. Discard the collection tube with flowthrough. Combine the filter tube with a new collection tube
14. Centrifuge at 14000 rpm for 10 sec to remove residual Wash Buffer.
15. Insert the filter tube into a clean 1.5 mL reaction tube.
16. Add 200 µl Elution Buffer that has been pre-warmed to 70ºC.
17. Centrifuge at 8000 rpm for 1 min.
18. Discard the filter tube. The flowthrough in the reaction tube contains the DNA.
19. The DNA solution can be used directly or stored in a freezer at -20ºor -80ºC

a) Ergonomic aspects

All activities are under the SOP safe handling of chemicals and SOP Safe handling of Quarantine organisms.

- Binding Buffer and Inhibitor Removal Buffer contain the toxic and irritating guanidine hydrochlorid. Avoid contact and inhalation using standard safety precautions and equipment.
- Lysozyme: avoid contact and inhalation of the powder when preparing the stock solution using standard safety precautions and equipment.

- Lysozyme stock solution: avoid skin contact and wear gloves.

b) Environmental aspects
All waste is disposed according to SOP safe handling of chemicals

c) Remarks

- Vials with Lysozyme solution and Proteinase K should be kept on ice to avoid diminution of enzyme activity.
- Lysozyme solution is stored in 1-time use portions at -20ºC. Non-used solutions are discarded.

PCR

According to the excel sheet, example given for performance of BOX-PCR on ABI Prism 7000 real-time PCR machine. Can also be performed on classical PCR machines or other Real-time PCR machines.

BOX PCR		
Code: xxxxxx	Version: 2	Date of entry: 14-02-2008

Date: "dd-mm-yyyy" **Technician:** "Name" **PCR cycler:** "Nr."

Primers:

Reference: Smith et al. (2001) European Journal of Plant Pathology **107**: 739-748, 2001

Forward: BOX-A1R CTA.CGG.CAA.GGC.GAC.GCT.GAC.G

Reverse: Forward = Reverse

Product: 100 - 3500 bp

Program: Blanco Fingerprint BOX on ABI Prism 7000 (30 cycli). **Reporter:** Classic PCR

95°C 7:00 min	94°C 1:00 min 53°C 1:00 min 65°C 8:00 min	30 x	65°C 16:00 min	4°C ∞

Duration PCR: 6:04 hours

Mastermix:

For reaction volume of 25 µL:	**Per reaction**	**1Reaction**	**Endconc.**
R/DNAse-free water	18.05µL	18.05µL	

Reaction Buffer (10 x, Invitrogen)	2.50µL	2.50µL	1x
$MgCl_2$ (50 mM, Invitrogen)	0.75µL	0.75µL	1.5 mM
dNTP mix (10 mM each, Promega)	0.50µL	0.50µL	0.2 mM
BOX AIR (20 µM)	1.00µL	1.00µL	0.8 µM
PlatinumTaq (5 U/µL, Invitrogen)	0.20µL	0.20µL	1 U
DNA extract	2.00µL	- -	
Total	25.00µL	23.00µL	

Samples:

Nr.	Sample	Strain
1		
2		

Nr.	Sample	Strain
25		
26		

Electrophoresis

1. Prepare a 2% agarose gel of minimum 20 cm in length
2. Add 6 gram agarose to 300 mL 1xTBE buffer in a 0.5 l to 1l flask. Melt the agarose in a microwave. to complete dissolving of agarose
3. Place the gel tray in the casting system and choose appropriate combs
4. Cool dissolved agarose under running tap water to hand warm.
5. Poor agarose solution in the 15 cm gel tray, remove air bubbles with a disposable pipette tip and place the combs.
6. Clean the flask/Erlenmeyer immediately with hot water to remove residual agarose.
7. Leave agarose to solidify (minimum 20 min)
8. Remove the combs when gel has been formed. Clean combs carefully with hot water.
9. Submerge the gel in electrophoresis unit in 1x TBE buffer (c 1 litre for Biorad Sub-Cell GT)
10. Mix a 6 µl PCR sample with 1.2 µl loading buffer on a piece of Parafilm and load the gel; load the 1kb ladder (diluted Invitrogen 2 µL ladder and 4 µL water) in a similar amount.
11. Run the gel in the cold room at 90 V (for c. 2.5 hours), constant voltage. This corresponds to 6 V/cm, measured ad the distance between the electrodes.
    Note: **Be careful and <u>wear gloves</u> at all times when you might touch the agarose gel. Ethidium bromide is a very powerful mutagen.**
12. Stain the gel for 40 min in an ethidium bromide solution of 0.6 mg/mL in 0.5 x TBE (60 (l of a 10 mg/mL stock solution in 1 litre 0.5 x TBE, and destain for 30 min in distilled water in 0.5 x TB E (for less background).
13. Visualise the bands on the gel under UV light

Main sources of information:

Lelliot & Stead 1987. Methods for the diagnosis of bacterial diseases of plants (Edit. T.F. Preece). Blackwell Scientific Publications

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TOXINS OF *PSEUDOMONAS SYRINGAE* AND THEIR USE IN DIFFERENTIATION OF STONE FRUIT ASSOCIATED PATHOVARS

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Toxins are the metabolic byproducts of some plant pathogenic pseudomonads that are excreted directly into the host plant and express themselves by causing visible symptoms in their host. Several toxins have been identified as products of a number of pathovars of *Pseudomonas syringae* (Bender et al., 1999). They are only expressed by a relatively small number of pathovars. They are not specific because the same toxin can be found in different pathovars. Phytotoxins are not essential for pathogenicity in *P. syringae* pathovars but they function as virulence factors, and their production results in increased disease severity. Although they may greatly increase damage to plants, it is not clear that they always benefit the pathogenic capability of the bacterium. For example, *P. syringae* pv. *phaseolicola* multiplies optimally in bean at temperatures above 24^{o}C without producing the chlorotic haloes associated with phaseolotoxin production.

Identified toxins include extracellular polysaccharides, chlorosis- and necrosis- inducing compounds, cell wall-degrading enzymes, and phytohormones.

Phytotoxins produced *in planta* are also usually produced *in vitro*. *P. syringae* is reported to induce a wide variety of symptoms on plants, including blights (rapid death of tissue), leaf spots, and galls. Some of them appear as a consequence of particular toxin production, such as chlorosis (coronatine, phaseolotoxin, and tabtoxin) or necrosis (syringomycin and syringopeptin) (Bender et al., 1999). Phytotoxins generally lack specificity and express activity in a wider range of plants than the host range of their pathogen.

Pathovars of *P. syringae* associated with stone fruits and nuts produce several well-characterized phytotoxic compounds (Table 1) which can be used for the pathovar differentiation. The presence of a fatty acid complex ‘persicomycin’ in cultures of *P. syringae* pv. *persicae* and in infected peach is the basis for a claim that these compounds are toxins, but nothing more is known. In this protocol we will focus on laboratory procedures for detection of *P. syringae* pvs *syringae* and *morsprunorum* toxins routinely used in phytobacteriological

laboratories, such as toxin bioassays or molecular protocols for detection of phytotoxins and toxin synthesis genes.

Table 1. Phytotoxins produced by *Pseudomonas* spp. associated with stone fruits

Toxin	Producing organism	Chemical class or biosynthetic origin
Coronatine	*P. syringae* pv. *morsprunorum*	Polyketide
Syringomycins[a]	*P. syringae* pv. *syringae*	Lipodepsinonapeptide

[a]Includes the related toxins syringotoxin, syringostatin, and pseudomycin.

1. SYRINGOMYCIN

Syringomycin is a low molecular weight non-specific toxin, representative of the cyclic lipodepsinonapeptide class of phytotoxins. It is produced by *P. syringae* pvs. *aptata, atrofaciens,* and *syringae*. It induces necrosis in plant tissues, and early studies of its mode of action established that the plasma membrane of host cells is the primary target. Syringomycin represents the first example of a virulence factor from a plant-pathogenic bacterium that targets host plasma membranes to form ion channels in lipid bilayers and causes cytolysis.

Syringomycin apppears to be generally toxic to eukaryotes and some bacteria. Therefore, it can most easily be detected in bioassays using sensitive fungi. The fungus, *Geotrichum candidum*, or the yeast, *Rhodotorula pilimanae*, can be used as indicator organisms in bioassays for this phytotoxin.

1.1. *Syringomycin bioassay*

The test bacterium is point inoculated onto a suitable medium (potato dextrose agar or similar but not malt extract agar which has antibacterial factors) and grown for 4 days at 25-28^{o}C. Meanwhile, a culture of syringomycin sensitive organism is also cultivated to obtain suitable growth which, for *G. candidum*, is followed by washing the growth with sterile water, filtering through layers of cheesecloth and collecting the resulting suspension in the container of hand-held atomizer. The surface of the medium, containing 4-day-old bacterial culture, is then

sprayed with a suspension of indicator organism (*G. candidum* or *R. pilimanae*). After further incubation for 1-2 days, clear zones of fungus inhibition are observed around bacterial colonies as an indication of syringomycin production (Figure 1; Xu and Gross, 1988). The bioassay does not distinguish between syringomycin and syringotoxin.

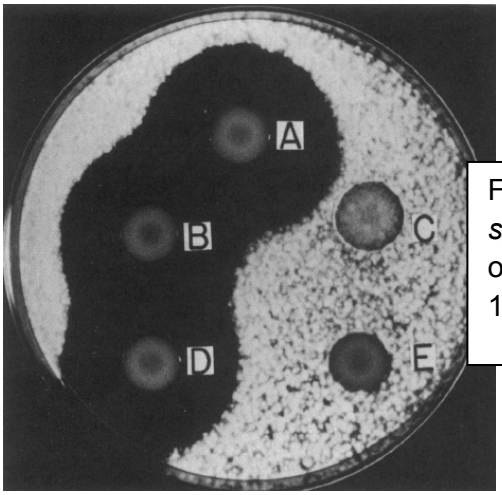

Fig. 1. Bioassay for syringomycin production by *P. syringae* pv. *syringae* on PDA as shown by a zone of inhibition of *G. candidum* growth (Xu and Gross, 1988).

1.2. *Molecular detection of syringomycin related genes*

The sequences of four genes putatively involved in syringomycin regulation (*syrP*), synthesis (*syrB* and *syrC*), and secretion (*syrD*) are known (Bultreys and Gheysen, 1999). PCR amplification of the 752-bp *syrB* fragment offers rapid and accurate detection of cyclic lipodepsinonapeptide-producing strains, and its sequence provides some predictive capabilities for identifying syringotoxin and syringostatin producers.

For *syrB*, the oligonucleotide sequences are:

primer B1: 5'-CTTTCCGTGGTCTTGATGAGG-3'

primer B2: 5'-TCGATTTTGCCGTGATGAGTC-3'

Either whole-cell suspensions of bacteria grown overnight or purified genomic DNA can be used (Schaad et al., 2001)

PCR amplification is performed in a total volume of 100 µl. Each reaction mixture contains 1 X PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), a 0.5 µM concentration of each primer, a 200 µM concentration of each dNTP, 1.5 mM $MgCl_2$, 0.025 U of *Taq* DNA polymerase per µl, 100 to 200 ng of genomic DNA, and 30 µl of mineral oil to prevent evaporation. The PCR runs for 35 cycles. Each cycle consists of template denaturation at 94°C for 1.5 min, primer annealing at 60°C for 1.5 min, and DNA extension for 3.0 min at 72°C. After the cycling period is finished, an additional extension of 10 min at 72°C is included. Small aliquots (5 µl) of the PCR products are analyzed on 1% agarose gels (Sorensen et al., 1998).

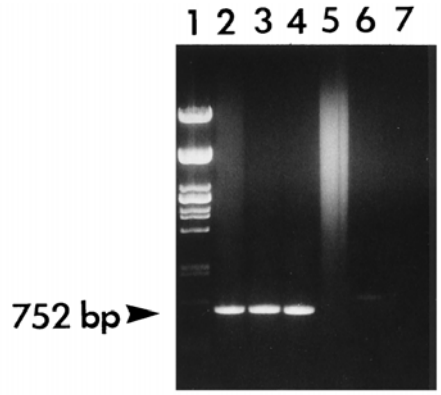

Fig. 1. PCR amplification of the 752-bp fragment of *syrB* (Sorensen et al., 1998)

2. CORONATINE

This chlorosis-inducing toxin is produced by the stone fruit pathogen *P. syringae* pv. *morsprunorum* and also by pvs *atropurpurea, glycinea, maculicola,* and *tomato.* In addition to chlorosis, coronatine induces stunting and hypertrophy of plant tissue and is important for virulence of the pathovars that produce it (Bereswill et al., 1994).

2.1. *Coronatine bioassay*

Coronatine is not antimicrobial and it is detected by its ability to induce chlorosis in a variety of plants. However, this assay is qualitative rather than quantitative. Völksch et al. (1989) have described a sensitive semiquantitative bioassay for coronatine based on production of a

hypertrophic reaction on potato tuber tissue. Variability can occur depending on the potato cultivar and the age of tuber tissue.

2.2. *Molecular detection of coronatine*

The strong conservation of the coronatine biosynthetic gene cluster between pathovars suggested that the amplification of conserved regions of the cluster by PCR might be used for specific detection of coronatine-producing bacteria. PCR amplification with primers 1 and 2 resulted in the specific detection of a 0.65-kb fragment in coronatine-producing strains (Bereswill et al., 1994).

The standard protocol is based on Bereswill et al. (1994), and is recommended by Schaad et al. (2001). The protocol (below) used in Laboratory for Phytobacteriology, Faculty of Agriculture, Belgrade, uses a reduced reaction mixture of 25 µl.

For *cfl* gene the oligonucleotide sequences are:

Primer 1: 5`-GGC GCT CCC TCG CAC TT-3`
Primer 2: 5`-GGT ATT GGC GGG GGT GC-3`

PCR Mix	**Final conc.**	**Quantity per reaction (µl)**
Water (molecular grade)		16.7
PCR Buffer with KCl and $MgCl_2$ (10X)	1X	2.5
dNTP (10mM)	0.2mM	0.5
Primer 1 (10µM)		2
Primer 2 (10µM)		2
Taq Polymerase (5U/µl)	1.5U	0.3
[a]Sample		1
Total volume		25 µl

[a]Use a standard procedure to isolate genomic DNA or use whole-cells of the strain

Amplification program

Temperature	**Time**	**No. of cycles**
93°C denaturation	2 min	1
93°C denaturation 67°C annealing 72°C elongation	2 min 1 min 2 min	37
4°C	∞	∞

PCR product (5 µl) is analyzed by electrophoresis in 1.5% agarose gel to determine if 650 bp amplification product is present.

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DIFFERENTIATION PROCEDURES FOR *PSEUDOMONAS SYRINGAE* PVS *SYRINGAE*, *MORSPRUNORUM* AND *PERSICAE*

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Pseudomonas syringae pvs *syringae* and *morsprunorum* are wide spread and well known pathogens of stone fruits, compared to pv. *persicae* which is only recorded on nectarine and peach in France, nectarine, peach and Japanese plum (*Prunus salicina*) in New Zealand, and *Prunus cerasifera* in the UK (EPPO PM 7/43). Being locally present in the EPPO region, pv. *persicae* is listed on A2 list of organisms recommended for regulation as quarantine pests. Due to overlapping host range, similar symptomatology, and many characteristics they have in common, *P. syringae* pvs *syringae, morsprunorum* and *persicae* can easily be misidentified. This may have significant consequences considering quarantine status of pv. *persicae.* Thus, in order to differentiate those pathovars it is essential to use tests that efficiently discriminate them from each other.

Biochemical tests

P. s. pv. *persicae* belongs, like *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum*, to LOPAT Group Ia (Lelliott et al., 1966). It can be distinguished from the other two stone fruits attacking pathovars by using classical and molecular techniques. Detailed differences in biochemical characters between the three stone fruits associated pathovars of *P. syringae* are shown in Table 1, as well as in EPPO diagnostic procedure PM 7/43 and Table 3 of J. D. Janse's protocols (see this manual).

After isolation and subsequent cloning of single colonies, growth of bacteria on King's medium B can be indicative for the pathovar designation. *P. s*. pv. *persicae* grows more slowly on King's medium B than the other two pathovars (Figure 1). Also, it does not produce green fluorescent pigment on this medium. However, this characteristic is not discriminative since nonfluorescent pv. *morsprunorum* strains were recorded.

Table 1. Biochemical characters of *Pseudomonas syringae* pv. *persicae* in comparison with pathovars *syringae* and *morsprunorum* (based on EPPO document PM 7/43).

Test[1]	*Pseudomonas syringae*		
	pv. *syringae*	pv. *morsprunorum*	pv. *persicae*
Fluorescence on King's medium B	+	+ or -	-
Fluorescence on CSGM[2]	+	+	+
Levan production	+	+	+
Gelatine hydrolysis	+	+	-
Aesculin hydrolysis	+	+	-
Acid production from:			
Inositol	+	+	-
Sorbitol	+	+	+
Erytritol	+ or -	+ or -	-
Utilisation of:			
DL lactate	+ or -	-	-
D(–) tartrate	+ or -	-	-
L(+) tartrate	-	+	-
Ice nucleation	+	-	+

[1]Fluorescence – appearance of green or blue pigment which diffuses into medium visible under UV-light; levan production – occurrence of mucoid colonies on sucrose-rich medium; gelatin hydrolysis – liquefaction of solid medium; aesculin hydrolysis – dark brown discoloration of the medium; remaining tests – yellow discoloration of medium. For preparation of media and performance of tests (see Lelliott & Stead , 1987; Schaad et al., 2001).
[2]Casamino-sucrose-gelatin medium (Lelliott & Stead, 1987).

Although all three patovars are known as levan-producing bacteria, *P. s*. pv. *persicae* produces less growth and smaller colonies on nutrient agar sucrose (NAS) medium after 72h of incubation at 26^{o}C (Figure 2). They also can be differentiated according to their vitality on NAS medium and catalase test, where pv. *morsprunorum* loses its vitality and consequently shows negative catalase reaction after 4-day-growth on NAS medium (Garrett et al., 1966).

Hydrolysis of gelatin and aesculine could be helpful tests in further differentiation. Unlike pv. *persicae*, pvs *syringae* and *morsprunorum* produce positive results (Table 1; Figure 3). In addition to this, acid production from inositol and erytritol separates these two from pv. *persicae*. However, disadvantage of these tests is that they are laborious and time consuming.

Ice nucleation test produces quick results (Lindow, 1990; Schaad et al., 2001). Pv. *morsprunorum* does not show ice nucleation activity (Table 1; Figure 4).

Pathovars *syringae*, *morsprunorum* and *persicae* can be also efficiently differentiated based on toxin (i.e. syringomycin, coronatine) production either using bioassay or molecular tests (see Obradović and Young protocol for toxin detection in this manual). *P. s*. pv. *persicae* produces

persicomycins as phytotoxic compounds (Barzic, 1999), but this is not yet utilized for development of PCR based tool for pv. *persicae* detection.

Molecular tests

PCR based procedures have been utilized in differentiation of pseudomonads associated with stone fruits. The protocols are mainly based on detection of toxin encoding genes, such as syringomycin regulation (*syrP*), synthesis (*syrB* and *syrC*), and secretion (*syrD*) genes; coronatine toxin production *cfl* gene; or yersiniabactin gene (see Table 4 in J.D. Janse's protocols and Obradović and Young protocol in this manual). More information can be also found in publication of Janse (2010). PCR based on conserved repeated DNA sequences, referred to as rep-PCR offered a highly sensitive level of analysis, suitable for species and in some cases pathovar genomic fingerprinting and identification (Figure 5). BOX PCR protocol, based on EPPO standards, will soon be published also by J.D. Janse (see protocols in this manual).

Pathogenicity tests

Hypersensitive reaction (HR) on tobacco (cv. White Burley, Xanthi, Samsun) leaves is reliable indication of pathogenicity of tested bacterium. However, it is not a substitute for testing of pathogenicity by inoculating susceptible host plant. In case of these pvs, reproduction of typical symptoms on woody tissue can take more than a month. Beside tobacco HR, reaction of unripe nectarine fruits as well as lemon fruits and string bean pods, which can be observed within few days, can indicate pathogenicity of tested strains (Obradović, unpublished). Usually, *P. syringae* pv. *syringae* strains can be distinguished from the other two pvs by severity of symptoms they induce in these plant organs (Figure 6).

In general, in order to achieve accurate identification it is necessary to combine several different approaches and tests before making any conclusions.

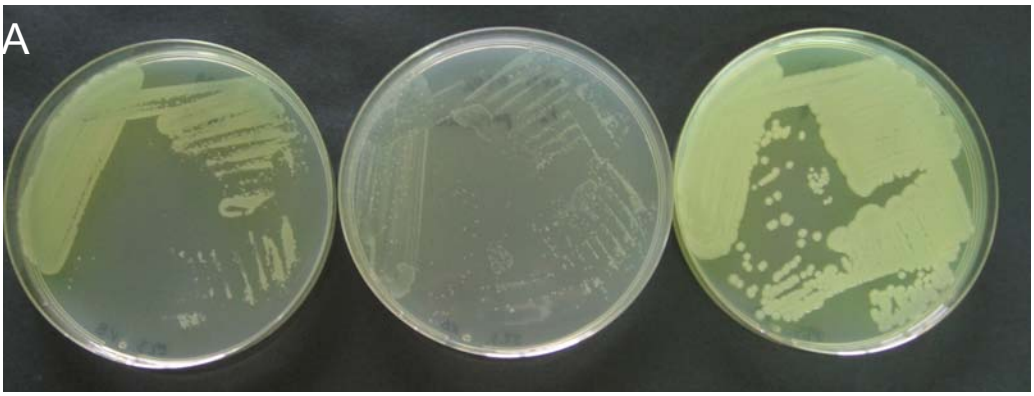

Figure 1. Growth on King's B medium. A - *P. s.* pv. *mors-prunorum*, B - *P. s* .pv. *persicae*, C - *P. s.* pv. *syringae.*

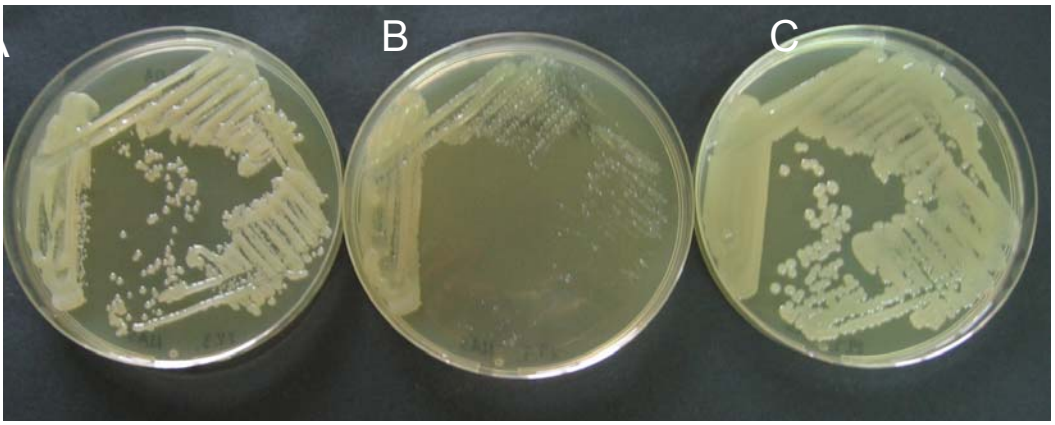

Figure 2. Levan production on NAS medium. A - *P. s.* pv. *mors-prunorum*, B - *P. s* .pv. *persicae*, C - *P. s.* pv. *syringae.*

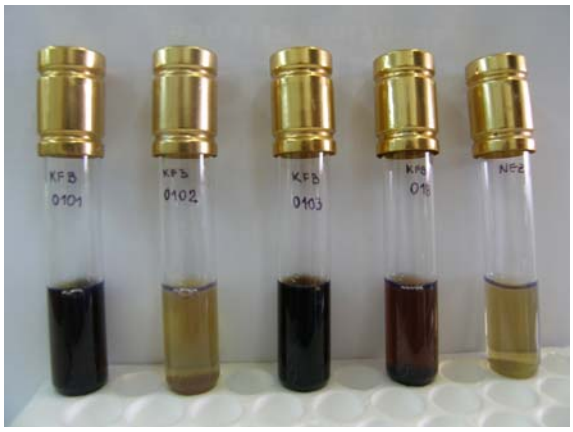

Figure 3. Aesculin hydrolysis. Dark brown discoloration of the medium = positive reaction: KFB 0101 - *P. s.* pv. *mors-prunorum*; KFB 0102 - *P. s.* pv. *persicae*; KFB 0103 and KFB 018 - *P. s.* pv. *syringae*; NEZ – Uninoculated control.

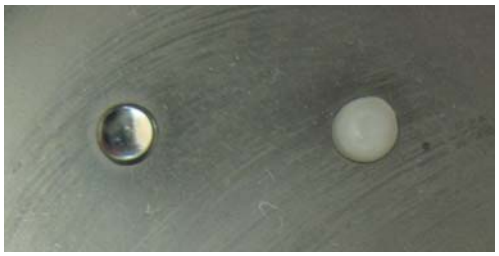

Figure 4. Ice nucleation activity. Left – negative; right – positive

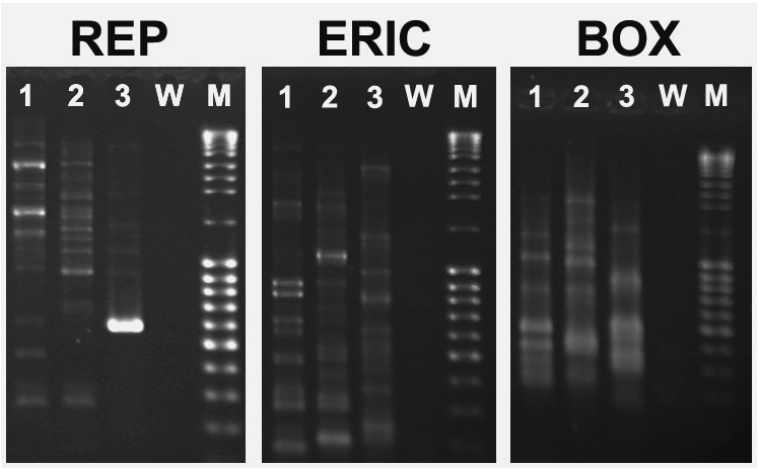

Figure 5. Rep-PCR genomic fingerprinting

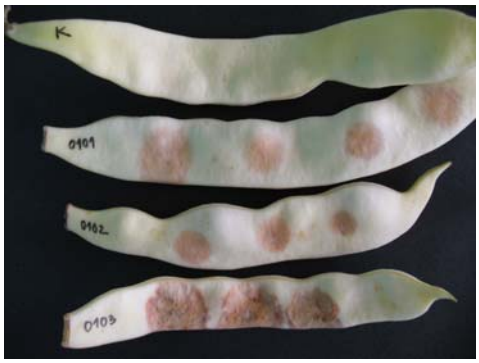

Figure 6. Reaction of string bean pods: K – sterile water; 0101 - *P. s.* pv. *morsprunorum*; 0102 - *P. s.* pv. *persicae*; 0103 – *P. s.* pv. *syringae*.

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