Taxonomic revision of *Xanthomonas axonopodis* pv. *dieffenbachiae* strains and pathogenicity on Araceae plants

Elena Corina Constantin

Promoters

Em. Prof. Dr. Paul De Vos
Dr. Martine Maes
Prof. Dr. Anne Willems

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Examination committee

Prof. Dr. Savvas Savvides (Chairman)
VIB Inflammation Research Center (IRC) Ghent University
Laboratory for Protein Biochemistry and Biomolecular Engineering (L-ProBE)
Faculty of Sciences, Ghent University, Belgium

Em. Prof. Dr. Paul De Vos (Promoter)
Laboratory for Microbiology
Faculty of Sciences, Ghent University, Belgium

Dr. Martine Maes (Promoter)
Plant Sciences Unit – Plant Health
Flanders research institute for agriculture, fisheries and food (ILVO), Merelbeke, Belgium

Prof. Dr. Anne Willems (Promoter)
Laboratory for Microbiology
Faculty of Sciences, Ghent University, Belgium

Prof. Dr. Aurélien Carlier (Secretary)
Laboratory for Microbiology
Faculty of Sciences, Ghent University, Belgium

Prof. Dr. Monica Höfte
Laboratory of Phytopathology, Department of Crop Protection
Faculty of Bioscience Engineering, Ghent University, Belgium

Dr. Danny Vereecke
Laboratory of Applied In vitro Plant Biotechnology, Department of Applied Biosciences
Faculty of Bioscience Engineering, Ghent University, Belgium

Dr. Bart Cottyn
Plant Sciences Unit – Plant Health
Flanders research institute for agriculture, fisheries and food (ILVO), Merelbeke, Belgium

Dr. Joël Pothier
Environmental Genomics and Systems Biology Research Group
Institute for Natural Resource Sciences, Zurich University of Applied Sciences (ZHAW), Wädenswil, Switzerland

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List of abbreviations

AAI	Average Amino acid Identity
ANI	Average Nucleotide Identity
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment search Tool
bp	base pair
CCRC	Complex Carbohydrate Research Center
CCUG	Culture Collection University of Göteborg
CECT	Colección Española de Cultivos Tipo
CFBP	French Collection of Plant Associated Bacteria
CIRM-CFBP	International Centre for Microbial Resources-French Collection of Plant Associated Bacteria
CWDE	Cell Wall Degrading Enzyme
DDH	DNA-DNA Hybridization
DSF	Diffusible Signaling Factor
DSM	Deutsche Sammlung von Mikroorganismen
EPPO	European and Mediterranean Plant Protection Organization
EPS	Extracellular Polysaccharide
FAME	Fatty Acid Methyl Ester
FAO	Food and Agriculture Organization of the United Nations
hrp	Hypersensitive Response and Pathogenicity
JCM	Japan Collection of Microorganisms
KACC	Korean Agricultural Culture Collection
LMG	Laboratory for Microbiology Ghent
LPS	Lipopolysaccharide
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
NCPPB	National Collection of Plant Pathogenic Bacteria
OD	Optical Density
PATRIC	Pathosystems Resource Integration Center
PB	Phosphate Buffer
PCR	Polymerase Chain Reaction
PD	Culture Collection of Plant Pathogenic Bacteria
PDDCC	Plant Diseases Division Culture Collection

PE	Paired-ended
QBOL	Quarantine Barcoding of Life
RAST	Rapid Annotations using Subsystems Technology
rpf	regulation of pathogenicity factors
T2SS	Type II Secretion System
T3E	Type III Secretion Effector
T3SS	Type III Secretion System
T4SS	Type IV Secretion System
T6SS	Type VI Secretion System
TSB	Trypticase Soy Broth
WGS	Whole Genome Sequence
Xad	*Xanthomonas axonopodis* pv. *dieffenbachiae*
Xca	*Xanthomonas citri* pv. *aracearum*
Xe	*Xanthomonas euvesicatoria*
Xpd	*Xanthomonas phaseoli* pv. *dieffenbachiae*
Xps	*Xanthomonas phaseoli* pv. *syngonii*

Problem statement and outline

The Araceae plant family, commonly known as aroids, encompasses ca. 125 genera and over 3700 species that are extraordinary diverse, with their attractive foliage being the most widely recognized feature as ornamentals, but others known as important staple food in the tropics where they originally reside. Many genera have been cultivated as ornamentals and are commercially among the important foliage plants used for interiorscaping.

Xanthomonas axonopodis pv. *dieffenbachiae* (Xad) is known as the causal agent of bacterial blight on Araceae. It is the most destructive disease of ornamental aroid plants worldwide and is an ongoing threat to commercial production. It first emerged in The United States in 1939 and has since spread globally. Since its introduction in the Netherlands in 1997, probably mainly through the import of infected plant material including tissue culture plants of *Anthurium* spp., Xad has been a source of concern. Its systemic nature and long-term persistence in symptomless *Anthurium* made Xad difficult to intercept, and the fear of qualitative and quantitative losses is high. Moreover Xad was known to be heterogeneous genetically as well as in terms of virulence, which challenges they being one biological group of pathogens and their designation as a single pathovar.

The PhD-study was initiated as part of the EU-FP7 project QBOL ('Development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health'). In this QBOL project, ILVO (M. Maes) and UGent-Laboratory of Microbiology (P. De Vos) have been coordinator for the barcoding of the regulated plant bacterial pathogens. The objective was to develop a barcoding strategy to target bacteria from this quarantine list. Several Q-bacteria are described as species, but most as pathovars, which is a special purpose classification on the basis of a pathological feature. Moreover, in contrast to higher biota, there is no single gene in bacteria with the appropriate resolution level for barcode identification up to this pathovar level. More than one barcode region is needed, and a barcode decision scheme has been proposed based on core and accessory genes. This QBOL barcoding strategy for the bacteria is now a tool available on the Q-bank website (http://www.q-bank.eu and http://www.q-bank.eu/Bacteria/DefaultInfo.aspx?Page=MolecularDS).

The barcode decision scheme uses the following steps:

- 16S rRNA gene sequencing is used as the first exploratory test in case there is no preliminary idea on the genus of bacterium that has been isolated from a plant commodity.
- In the next decision step, one core gene locus is used to discriminate bacterial species within the genus. The result can eventually be confirmed with a second barcode region.
- To further discriminate at the pathovar level, extra barcodes located in other core or accessory gene regions are being identified.

The sequencing results revealed the heterogeneity within specific pathogens, as was the case for the *Xanthomonas* pathogens on aroids, which are up till now named as *Xanthomonas axonopodis* pv.

dieffenbachiae (Xad). Indeed, the first need was to revisit the taxonomy and the plant-associated specialization of these bacterial pathogens of high economic importance.

The selection of Xad in this PhD research has been made taking into account the Q-relevance for the EU and the expected variability and complexity within the taxa. The heterogeneity of the pathogen genetically as well as in terms of virulence raised many questions which we tried to answer in this study.

The first objective of this dissertation was to clarify the taxonomic position of strains identified as *Xanthomonas axonopodis* pv. *dieffenbachiae.* This led to the taxonomic revision of the *X. axonopodis* species complex, the allocation of the Xad strains into three different *Xanthomonas* species, and a better understanding of the relationships between strains belonging to these species. Clarification was also needed in support of the phytosanitary policy which recommends Xad for quarantine regulation. As a consequence, the second objective was to investigate whether the different taxonomic groups that we identified within Xad have a comparable pathogenic capacity on aroids and should therefore still be regarded as quarantine on aroids. This aspect has been studied by testing the pathogenic reactions in bio-assays on aroid plant genera and by whole genome sequencing, comparing an arsenal of pathogenicity-related genes between representative Xad strains. This led to conclusions on the diversity of the Xad strains regarding host range and aggressiveness, and can also be regarded as a contribution to bacterial phytopathology in general.

This thesis starts with a general introduction (**chapter 1**), which contains i) a description of the biology, history, economic importance and cultivation of aroids, ii) a brief outline of the history and current taxonomy of the genus *Xanthomonas*, iii) an overview of the available scientific literature on Xad and bacterial blight. The next two chapters presents the study performed in the frame of the present PhD. In **Chapter 2**, the taxonomic relatedness between a collection of Xad strains and phylogenetically related *Xanthomonas* species and pathovars was investigated using MLSA, DDH, ANI calculation and biochemical analyses. **Chapter 3** combines, research on the pathogenic abilities of Xad strains tested on Araceae plants from six different genera and the pathogenicity-related gene content of four of these Xad strains by means of whole genome sequence analysis. The aim was to evaluate whether the phylogenetic heterogeneity of Xad strains as concluded from Chapter 2 is also reflected in a pathogenic heterogeneity, leading to conclusions on the importance of the different taxa within Xad as pathogens of aroids, and with possible implications for regulation as quarantine pathogens. New pathovar names are indeed proposed for the groups that clearly exhibit pathogenicity on the aroids.

Finally, the major findings of this work are summarized and the future research perspectives are discussed in **chapter 4**.

Summary

Aroids are monocots that in our region and Europe in general are mostly known for their ornamental values as cut flowers, pot and landscape plants. Production of aroids occurs worldwide and is threatened by bacterial blight, which is caused by *Xanthomonas axonopodis* pv. *dieffenbachiae*. Because of its virulence and its broad host range within the Araceae family, *Xanthomonas axonopodis* pv. *dieffenbachiae* (Xad) is considered to be the most serious bacterial disease causing extensive crop losses in commercial foliage plant production. Thus it is a regulated pest in several countries and is included in the A2 list of the European and Mediterranean Plant Protection Organization (EPPO). For practical diagnosis, and plant health regulation, it is important that quarantine organisms can be unambiguously identified. Therefore, the aim of this study was to resolve the taxonomic and pathogenic identity of this aroid pathogen in order to avoid further confusion.

Main objectives of this study were i) to clarify the taxonomic position of the diverse strains identified as Xad, ii) an attempt to resolve the complex taxonomic situation of *X. axonopodis sensu* Vauterin *et al*. (1995), and iii) to investigate whether the different taxonomic Xad groups have an important pathogenic capacity on aroids and should therefore be regarded as quarantine on aroids.

The first study (Chapter 2) aimed at accurately assess the taxonomic position of this Xad pathogen. It led to the taxonomic revision of the *X. axonopodis* species complex and to a better understanding of the relationships between strains belonging to this and related species. We used MLSA on a broad collection of strains, and defined then a relevant subset of strains to be studied in DDH experiments, ANI calculations and biochemical analyses. By means of MLSA, the 109 strains were allocated into four phylogenetic groups, PG I to PG IV. These four groups belonged to four different species: *X. axonopodis*, *X. citri*, *X. euvesicatoria* and *X. phaseoli*. Interestingly, Xad strains belonged to *X. citri*, *X. euvesicatoria* and *X. phaseoli* but not to *X. axonopodis*. Besides the new taxonomic subdivision and classification of the Xad strains, several important taxonomic proposals were made within the *Xanthomonas* genus: reclassification of *X. perforans* and *X. alfalfae* as *X. euvesicatoria*, and reclassification of *X. fuscans* as *X. citri*.

The second study (Chapter 3) focused on the pathogenic capacity of strains previously named as Xad associated with six aroid hosts. Two inoculation methods were used to evaluate the capability of eleven strains to cause either a local infection and/or further progression of the infection into the plant system. Several conclusions could be made based on these *in planta* tests. i) Xad strains belonging to *X. phaseoli* and *X. citri* are infective for the aroid species. ii) Xad strains originally isolated from *Syngonium* plants belong to *X. phaseoli* and have a host range restricted to its original host *Syngonium*. Therefore we proposed to classify them as the separate *X. phaseoli* pv. *syngonii*. iii) On the contrary, the Xad strains originally isolated from *Philodendron* are classified into *X. euvesicatoria*, but their

pathogenicity on the tested aroids is weak and doubtful and therefore we disregard them as specific pathogens of Araceae. Finally, iv) within the new taxonomic groups, the virulence of the different Xad strains varies to a certain extend.

Furthermore, to improve the understanding about their different pathogenic capacity, we explored whole genome sequences for sets of pathogenicity-related genes present in four representative strains. Our findings revealed that also the *X. euvesicatoria* strain had the characteristics for being a plant pathogen, the majority of known virulence factors are present. Consequently, *X. euvesicatoria* strains may have another yet undefined host range and their pathogenicity is either not expressed or is repressed in aroid plants.

In conclusion, this research showed that the previously reported phylogenetic heterogeneity of Xad strains (Chapter 2) was also reflected in a pathogenic heterogeneity and thus justifying the installation of three pathovars for the pathogens in aroids: *X. phaseoli* pv. *dieffenbachiae* comb. nov., *X. phaseoli* pv. *syngonii* comb. nov., and *X. citri* pv. *aracearum* comb. nov. In contrast, based on our study, the *X. euvesicatoria* strains isolated from *Philodendron* and formally also regarded as Xad strains do not represent “real pathogens” or quarantine organisms for aroids. At this time we consider them as under-studied plant pathogenic strains of unknown plant health related relevance. Further studies are needed to determine whether they should be designated as a separate pathovar of other hosts.

Samenvatting

De araceeën of aronskelkfamilie zijn eenzaadlobbigen (monocotylen) die in onze regio en algemeen in Europa best gekend zijn om hun sierwaarde als snijbloemen en als pot- en tuinplanten. De productie van araceeën wordt wereldwijd bedreigd door een bladvlekkenziekte, veroorzaakt door *Xanthomonas axonopodis* pv. *dieffenbachiae* (Xad). Wegens zijn virulentie en breed gastheerbereik in de aronskelkfamilie, wordt Xad beschouwd als de meest ernstige bacteriële ziekte die grote verliezen veroorzaakt bij de commerciële productie van de decoratieve bladplanten. De pathogeen wordt daarom ook sterk gecontroleerd in meerdere landen en staat ook op de A2 lijst van de European and Mediterranean Plant Protection Organization (EPPO). Voor praktische diagnose-doeleinden en in het kader van de regulering is het belangrijk dat quarantaine-organismen ondubbelzinnig identificeerbaar zijn. Daarom was het doel van deze studie om de taxonomie en pathogene eigenschappen van deze pathogeen op araceeën op te helderen.

De hoofddoelstellingen van deze studie waren i) de taxonomische plaatsing te verduidelijken van de diverse stammen die eerder als Xad geïdentificeerd werden, ii) te trachten de complexe taxonomie van *X. axonopodis sensu* Vauterin *et al.* (1995) op te helderen, en iii) te onderzoeken of de taxonomisch verschillende Xad groepen, zoals geïdentificeerd onder i), belangrijke en verschillende pathogeniciteit vertonen tegen araceeën en dus ook dienen te worden beschouwd als quarantaine organismen voor deze plantenfamilie.

De eerste studie (Hoofdstuk 2) had tot doel de taxonomische plaats van Xad stammen te bepalen. Dit resulteerde in de taxonomische revisie van het *X. axonopodis* species complex en een beter inzicht in de verwantschappen tussen stammen van deze soort en naburige soorten. We gebruikten multilocus sequentie analyse (MLSA) op een brede verzameling van stammen en selecteerden daarna een relevante subset van stammen om in te sluiten bij DNA-DNA hybridizaties (DDH), ANI berekeningen en biochemische testen. Met MLSA werden 109 stammen in vier fylogenetische groepen ingedeeld, PG I tot PG IV. Deze vier groepen behoren tot vier verschillende soorten: *X. axonopodis*, *X. citri*, *X. euvesicatoria* and *X. phaseoli*. Opvallend was dat Xad stammen tot *X. citri*, *X. euvesicatoria* en *X. phaseoli* behoorden, maar niet tot *X. axonopodis*. Naast de nieuwe taxonomische indeling en classificering van de Xad stammen, werden enkele belangrijke taxonomische voorstellen gedaan in het genus *Xanthomonas*: reclassering van *X. perforans* en *X. alfalfa* als *X. euvesicatoria*, en reclassering van *X. fuscans* als *X. citri*.

De tweede studie (Hoofdstuk 3) focuste op het pathogeen vermogen van Xad stammen die nu nieuw toegewezen zijn aan de drie *Xanthomonas* species op zes soorten van araceeën. Twee inoculatiemethoden werden gebruikt om de pathogeniciteit te evalueren van elf Xad stammen. Hiermee evalueerden we enerzijds lokale infectie van het blad en anderzijds een verdere progressie

van de infectie in de hele plant. Verschillende besluiten werden getrokken uit deze *in planta* testen. i) Xad stammen die behoren tot *X. phaseoli* en *X. citri* zijn pathogeen op araceeën. ii) Xad stammen oorspronkelijk van *Syngonium* planten afkomstig, behoren tot *X. phaseoli*, zijn dus ook pathogeen, maar met een waardplantbereik dat beperkt is tot de originele waardplant *Syngonium.* Daarom stelden we voor om ze te klasseren in de afzonderlijke pathovar, *X. phaseoli* pv. *syngonii.* iii) Daarentegen werden de Xad stammen oorspronkelijk afkomstig van *Philodendron* geklasseerd in *X. euvesicatoria*, en de pathogeniciteit van deze stammen op de geteste araceeën was zwak en twijfelachtig en daarom beschouwen we deze niet als specifieke pathogenen van araceeën. Ten slotte, iv) de virulentie van de diverse Xad stammen binnen de nieuwe taxonomische groepen vertoont een zekere variatie.

Om een beter inzicht te bekomen in hun divers pathogeen vermogen, hebben we bovendien het voorkomen van groepen pathogeniciteitsgenen onderzocht in de volledige genoomsequenties van vier representatieve stammen. Onze resultaten toonden aan dat ook de *X. euvesicatoria* stam de kenmerken heeft van een plantpathogeen en de meeste gekende virulentiefactoren in zich draagt. Dit wijst erop dat *X. euvesicatoria* stammen een ander, mogelijk nog niet gekend waardplantbereik kan hebben en dat hun pathogeniciteit niet tot expressie komt of onderdrukt is in araceeën.

Tot besluit, dit onderzoek toonde aan dat de eerder gerapporteerde fylogenetische heterogeniteit van Xad stammen (Hoofdstuk 2) ook weerspiegeld wordt in een heterogene pathogeniciteit die de creatie van drie pathovars rechtvaardigt voor deze pathogenen op araceeën: *X. phaseoli* pv. *dieffenbachiae* comb. nov., *X. phaseoli* pv. *syngonii* comb. nov., en *X. citri* pv. *aracearum* comb. nov. Onze studie toont verder aan dat *X. euvesicatoria* stammen geïsoleerd uit *Philodendron*, die vroeger als Xad stammen werden beschouwd, geen “echte pathogenen” zijn voor araceeën en we stellen dus voor van deze niet op nemen voor regulering en EPPO A2 quarantaine lijst. Momenteel beschouwen we hen als te weinig onderzochte pathogene stammen van onbekend belang voor plantengezondheid. Verdere studies zijn nodig om te bepalen of zij zouden moeten toegewezen worden aan een andere pathovar op andere waardplanten.

Chapter 1:

General introduction

1.1 The family Araceae

1.1.1 Introduction

The Araceae (commonly known as aroids) are a widely distributed monocotyledonous family. They are commonly used as ornamentals (cut flowers and pot plants) or for landscaping in most (sub) tropical areas (Chen *et al*., 2005). Some species such as *Alocasia macrorrhizos*, *Amorphophallus paeoniifolius*, *Colocasia esculenta* and *Xanthosoma sagittifolium* are utilized for food in the tropics and subtropics. In this first part of the general introduction, the biology, history, economic importance and cultivation techniques will be briefly outlined.

Kingdom: Plantae
Subkingdom: Tracheobionta
Super division: Spermatophyta
Division: Magnoliophyta
Class: Liliopsida
Subclass: Arecidae
Order: Arales
Family: Araceae

1.1.2 Biology of Araceae plants

The high biodiversity of Araceae with ca. 125 genera and over 3700 species (Mayo *et al*., 1997), reflects their ability to occupy a wide range of environments. Most species of Araceae are ornamental plants and the most important genera are *Anthurium* Schott, *Philodendron* Schott and *Dieffenbachia* Schott (Pedralli, 2002) (Figure 1.1). Among these genera *Anthurium* grew in importance as a flowering pot crop due to development of dwarf cultivars (Henny, 1995). Although members of the Araceae family can be found in almost every climatic region except deserts and polar regions, most aroids are tropical and subtropical species. Species adapted for areas with cool or dry periods are characterized by dormancy of their corms, underground rhizomes or seeds, which allows them to survive unfavorable periods. This family also displays a notable diversity of life forms, including geophytes, climbers, epiphytes, helophytes, and free floating aquatic species (Bown, 2000; Croat, 1990).

Figure 1.1. *Anthurium*, *Philodendron* and *Dieffenbachia*, the most important ornamental plants of Araceae.

Their vegetative parts are extremely varied; for instance, stems can be creeping or climbing and form rhizomes or distinct tubers; leaves range from simple to complexly divided, flowers range from very small to very big. For example, *Amorphophallus titanium* has the largest inflorescence in the word (approximately 2 meters), while the inflorescence of *Homalomena minutisima* measure few millimeters and is very difficult to be seen with the naked eye (Bown, 2000). A common characteristic of all aroids species is the spathe and spadix type of inflorescence (Figure 1.2). Flowers may be bisexual (monoclinous, hermaphrodite) or unisexual (diclinous). Unisexual flowers usually are born in separate female and male zones of the spadix, which often has a sterile apical appendix (Mayo *et al*., 1997; Judd *et al*., 1999). Because many aroids are monoecious there can be a high likelihood of self-pollination which leads to less genetic variety and less ability to adapt to environmental changes. The plant needs cross pollination to ensure a high variation. The aroid family is characterized by protogyny in which the female flowers ripen first and then later the male flowers produce pollen. The stages are easily observed in anthuriums, which have bisexual flowers (Bown, 2000). Protogyny is closely associated with cross pollination (Lebot, 2009). Araceae inflorescences are specifically adapted to insect pollination, although "wind tunnel" pollination has also been proposed (Mayo *et al*., 1997). Many plants in this family are heat-producing (thermogenesis) (Seymour & Schultze-Motel, 1997). Their flowers can reach up to 45°C even when the air temperature is much lower. In this way, the plants attract the insects to pollinate the plant and also prevent tissue damage in cold regions (Chauveau & Lance, 1982). The fruits of Araceae are typically juicy berries and most commonly red or orange (Mayo *et al*., 1997).

Chromosome numbers are available for 862 species (26% of the family), ranging from 2n=10 to 2n=168 and suggesting an ancestral haploid chromosome number of n=16 or n=18 (Cusimano *et al*., 2012). More recently, the phylogenetic differences (Cusimano *et al*., 2011) were highlighted in a study on the cytogenetic differences among six genera within Araceae (Lakshmanan *et al*., 2015).

Figure 1.2. Aroid inflorescence. The spathe-and-spadix inflorescence is the main distinguishing feature of aroids. It display great plasticity in size and shape. Original figure taken from Bown, 2000.

1.1.3 History and economic importance

The word “aroid” is derived from the Latin word “arum”, which means lily and individual species of the Araceae have been recorded since those ancient times. The origin and the evolution of the Araceae is still poorly known. New fossil discoveries have been made in the Araceae (Friis *et al*., 2004). These have pushed back the history of the family more than 120 milion years ago to the early Cretaceous. Over 800 species of Araceae are of economic importance (ornamental, medicinal, edible). In our regions, the economically most important ornamental aroids belong to the *Anthurium* genus. The production value per ha for *Anthurium* cut flowers and pot plants in 2014 was approximately 398 and 977 thousand euros, respectively and the total production value approximately 33 and 82 million euros, respectively (Van der Gaag & Bergsma-Vlami, 2015). Other than their use as ornamentals, the

family also offers many other benefits. Plowman (1969) documented the economic and commercial uses of aroids such as production of essential oil from *Acorus*, leaf extracts of *Caladium* for treatment of cancerous ulcers, dyes from *Dieffenbachia seguine*, and a powder used as an antidote for snakebite from *Dracontium asperum*. In the Amazon region, many species of Araceae are used for medicinal purposes, including the treatment of malaria and associated fevers and the most important species are from the genus *Philodendron* Schott (Kvist *et al*., 2006). The aroids also play an important role in the food security of millions of people in the tropics (Jackson *et al*., 2006). The Food and Agriculture Organization of the United Nations (FAO) estimates that around half a billion people in the (sub)tropics and developing world are involved in aroid cultivation, consumption and commerce. *Colocasia esculenta* (taro) and *Xanthosoma sagittifolium* (tannia) are the world's oldest cultivated food plants and the most widely distributed and consumed aroids (Opara, 2002). Most aroid plant parts are edible and have good nutritional qualities. The roots and tubers are rich in carbohydrates and the leaves and stalks are an important source of protein, vitamins and minerals. Although highly nutritious, aroids also contain anti-nutrients, particularly oxalic acid, which can cause irritation of the skin, therefore appropriate preparation methods are required before they can be used as food (Mayo *et al*., 1997).

1.1.4 Aroids breeding and cultivation

For our regions, breeding objectives are mainly focused on developing new cultivars with improvement of traits related to ornamental value and stress resistance. Because the value of aroid plants lies in the esthetic qualities, the improvement of ornamental traits, such as plant form, color, leaf shape, texture, as well as growth rate has always been important to any breeding program.

Aroid plants are predominantly cross-pollinating species. Elite parents are selected for intercrossing each generation and commercially interesting offspring are sexually or asexually propagated. The parents used in aroid plant hybridization are usually not derived from inbred, single-seed descent because inbreeding depression limits development of inbred lines in most foliage plant genera (Henny & Chen, 2004). Traditional breeding through hybridization has focused on heterozygosity. Intercrossing distinct clones with desirable characters, the populations created can be utilized for selection of new clones. When the parent clones are heterozygous, each seedling is a potential new cultivar and can be fixed by vegetative propagation (Henny & Chen, 2004). Depending on crossing parents, *intraspecific* (within a single species), *interspecific* (between different species) and *intergeneric* (between different genera) hybridization can be distinguished. Interspecific hybridization is the most common practice in producing hybrid cultivars in aroid plant breeding though the success of the method using traditional breeding depends on how closely the parental species are genetically relates (Lakshmanan *et al*., 2015). In Araceae, there is no report yet on successful intergeneric hybrid production. Interspecific hybridization is well investigated in *Anthurium* (Kamemoto & Kuehnle, 1996; Henny, 1999) and

Philodendron with the production of *P. corsinianum*, a hybrid between *P. lucidum* and *P. cariaceum* (Wilfret & Sheehan, 1981). Interspecific hybridization may also produced hybrids in *Aglaonema* and *Alocasia* through conventional breeding (Henny & Chen, 2004). Other efforts were done to improve adventitious shoot regeneration for transformation or mutant selection in *Anthurium*, *Dieffenbachia* and *Spathiphyllum* (Orlikowska *et al*., 1995). Selection of varieties for resistant breeding is also prominent in aroids (Anaïs *et al*., 2000; Snijder *at al*., 2004; Goktepe *et al*., 2007; Seijo *et al*., 2010). Cultivar resistance to bacterial blight disease can be used to produce commercial resistant varieties (Anaïs *et al*., 2000). Until recently, very little molecular cytogenetic information was known for Araceae. Cusimano *et al*. (2011) performed a phylogenetic study to infer Araceae chromosome evolution based on molecular data compared with morphological and anatomical data analyses. Meanwhile, the clear cytogenetic differences were highlighted for six economically important species within Araceae (*Anthurium andraeanum*, *Philodendron scandens*, *Syngonium auritum*, *Monstera deliciosa*, *Spathiphyllum wallisii and Zantedeschia elliottiana*) (Lakshmanan *et al*., 2015). These molecular cytogenetic information and chromosome data are useful in further aroid breeding programmes.

The aroids display such a diversity of ecological types that it is difficult to provide general information regarding cultivation that is applicable for all species. Many diverse cultivation systems are used around the world, depending on the climate, species and market situation of each producing region. For example, *Anthurium* species are relatively easy to grow, they require high humidity and minimum temperature of 18-20°C while *Alocasia* species needs more care because they are only suitable for warm conditions (high temperature, high humidity and shade) (Mayo *et al*., 1997). Also the genera with tubers or rhizomes demands special care, e.g. *Amorphophallus*, *Caladium*, *Taccarum*, *Xanthosoma*. These plants must be kept dry during their dormant period and they are best grown in pots in order to control the soil humidity, requiring abundant water during the growing period.

1.2 The genus *Xanthomonas*

1.2.1 Introduction

Xanthomonas is one of 22 currently acknowledged genera (http://www.bacterio.net) within the family of *Xanthomonadaceae*, order *Xanthomonadales* of the class of *Gammaproteobacteria* (Garrity *et al*., 2005). The type species is *X. campestris*, and the type strain is *X. campestris* pv. *campestris* LMG 568 (equivalent strain numbers: ATCC 33913, CCRC 12846, CCUG 47691, CECT 97, CFBP 5241, DSM 3586, JCM 13371, KACC 10913, NCPPB 528, PDDCC13). The genus *Xanthomonas* comprises 27 plant-associated bacterial species and although most members of the genus are thought to have a narrow host range, *Xanthomonas* as a genus is able to infect a broad range of plants, covering at least 124 monocotyledonous and 268 dicotyledonous species (Leyns *et al*., 1984). The genus was first proposed by Dowson (1939), who described 60 species. Although several efforts were made by different research groups to reclassify members of *Xanthomonas* (De Vos & De Ley, 1983; Swings *et al*., 1983; Van Den Mooter & Swings 1990; Yang *et al*., 1993; Vauterin *et al*., 1995), the taxonomy and classification in the genus is still undergoing revision because of phytopathogenic diversity (Vauterin *et al*., 2000; Rademaker *et al*., 2005; Schaad *et al*., 2005) and more recent in depth genomic characterization. *Xanthomonas* taxonomy continues to be controversial.

1.2.2 Bacterial taxonomy

Taxonomy (from Greek: *taxis*, "arrangement" and *nomia,* "method") is the biological discipline of defining groups of organisms based on their shared characteristics, and giving names to the different groups. Cowan stated in 1968 (Cowan, 1968) that taxonomy is divided in three parts: (1) classification, arranging the organisms into groups based on similarity; (2) nomenclature, labeling the groups defined

by classification; (3) identification, assigning an unknown organism to an known taxonomic group. The interaction between these items are represented in a flow diagram in Figure 1.3. Modern taxonomy also includes phylogeny and population genetics as an integral part of the classification process (Vandamme *et al*., 1996).

Prokaryote classification is the most recent among the different classifications of all living organisms. The taxonomic classification system (also called the Linnaean system after its inventor Carl Linnaeus, a Swedish botanist, zoologist and physician) uses a hierarchical model. There, the basic unit of biological classification, the species, was named according to the Linnaean *binomial* system consisting of two parts: a noun (substantive) in the nominative case, which correspond to the "genus", followed by the epithet (adjective) that indicates the "species" in that genus. Together these form the scientific name that identifies the species. This binominal system was applied both to plants and animals. In 1786, the Danish naturalist Friedrich Müller described several bacterial species and attempted the first bacterial classification.

Initially, bacterial species were defined according to the damage they produce. Plant pathologists assigned a new specific epithet to bacteria causing diseases on plants from which bacterial plant pathogens had not previously been isolated and/or diseases that looked different than other bacterial diseases on a particular host. This common practice was reflected in the "new host – new species" concept by Starr (1981). The number of species resulting from this practice grew rapidly, resulting in complex genera consisting of hundreds of species.

The initially bacterial taxonomy evolved into a more objective one, after the release of the canonical *Bergey's Manual for Determinative Bacteriology* in 1923, which represented a modern identification key for bacteria. At that time there was no common agreement on prokaryotic classification (Staley & Krieg, 1989), this manual and the later editions became the reference work on bacterial classification. These publications provided formal description of all bacterial taxa and keys for the identification of new isolates (Murray & Holt, 2005). Meanwhile, more flexible approaches such as numerical taxonomy and chemotaxonomy aimed to sort individual strains into species, genera and higher groupings (Rosselló-Mora & Amann, 2001). The need for a more formalized bacterial taxonomy led to the formation of an International Committee on Systematic Bacteriology (ICSB), now known as the International Committee on Systematics of Prokaryotes (ICSP). Between 1970 and 1980, the ICSB adopted the International Code of Nomenclature of Bacteria (Bacteriological Code; 1990 Revision (Lapage *et al*., 1992) and an Approved Lists of Bacterial Names (Skerman *et al*., 1980). The decision to recognize species as valid only if they were represented by a legitimate name, a species description and a type strain required pathologists to admit that many species differed only in host range, a character not considered to form part of a species description in terms of the Bacteriological Code. With these restrictions, many pathogens were considered to be members of the same species. This

problem was solved by recognizing the infrasubspecific term "pathovar" for populations of pathogens within species and by creating the International Standards for Naming Pathovars of Phytopathogenic Bacteria (The Standards (Dye *et al*., 1980), and the adaptation of its own "Comprehensive List of Names of Plant Pathogenic Bacteria" (Bull *et al*., 2010).

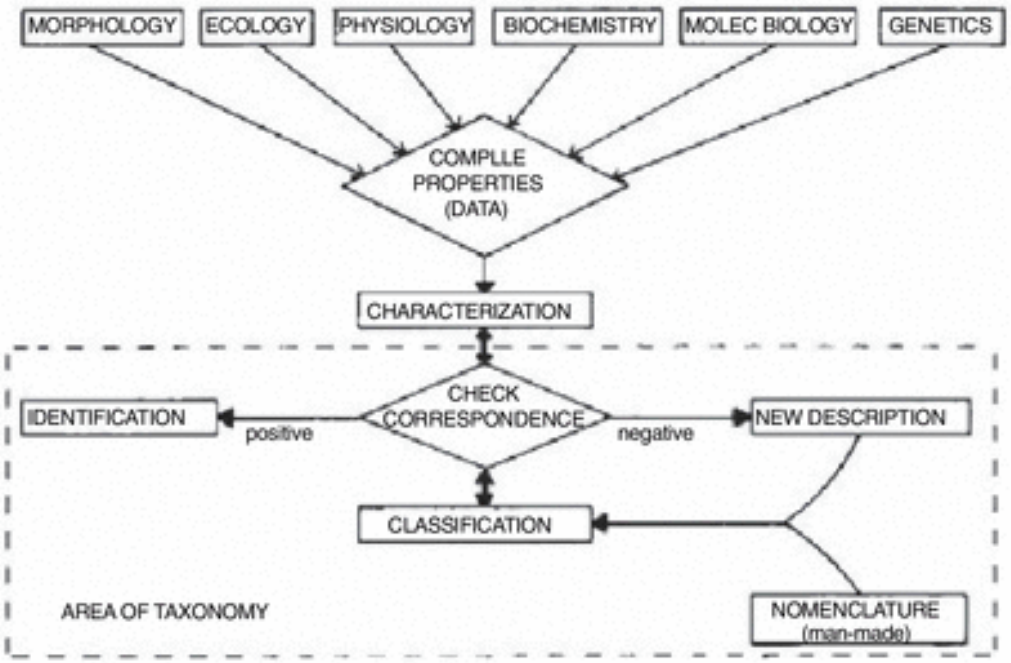

Figure 1.3. Infomation flow diagram indicating the relationships between the characterization and the classification of a bacterial strain. Original figure taken from Trüper & Krämer, 1981.

The Gold Standard of DNA-DNA homology. A new DNA homology based species concept was created in 1987, defining it as a group of strains, including the type strain, sharing 70% or greater DNA-DNA relatedness with 5°C or less ΔT_m (difference in melting temperature in degrees Celsius between the homologous and heterologous hybrids under standard conditions (Wayne *et al*., 1987). Although this concept was considered the "gold standard" (Stackebrandt & Goebel, 1994) several practical problems existed because DNA-DNA hybridization (DDH) was time-consuming and because different methods were used to determine the level of DDH and these did not always show the same results (Gevers *et al*., 2005). Therefore the value of 70% DNA relatedness was considered indicative rather than absolute. A first alternative solution for this problem was provided with the appearance of 16S ribosomal RNA gene (16S rRNA gene) sequencing (Woese, 1987). Since the 16S rRNA is present in all bacteria, is functionally constant and is composed of conserved and variable regions, it has consistently served as a good taxonomic marker for deriving taxonomic relationships (Vandamme *et al*., 1996). Therefore it was suggested that strains sharing at least 97% 16S rRNA gene sequence identity, should be considered members of the same species (Stackebrandt & Goebel, 1994). However, the resolution of 16S rRNA gene was often insufficient to elucidate affiliations between closely related species (Fox *et al*., 1992;

Gevers *et al*., 2005) and sometimes it was impossible to draw a conclusion based on this threshold of 97% sequence identity (Figure 1.4.). Stackebrandt and Ebers (2006) proposed to shift this 97% value to the new 98.7-99%, but this has not yet been widely adopted and was only recently started to be used (Yarza *et al*., 2014).

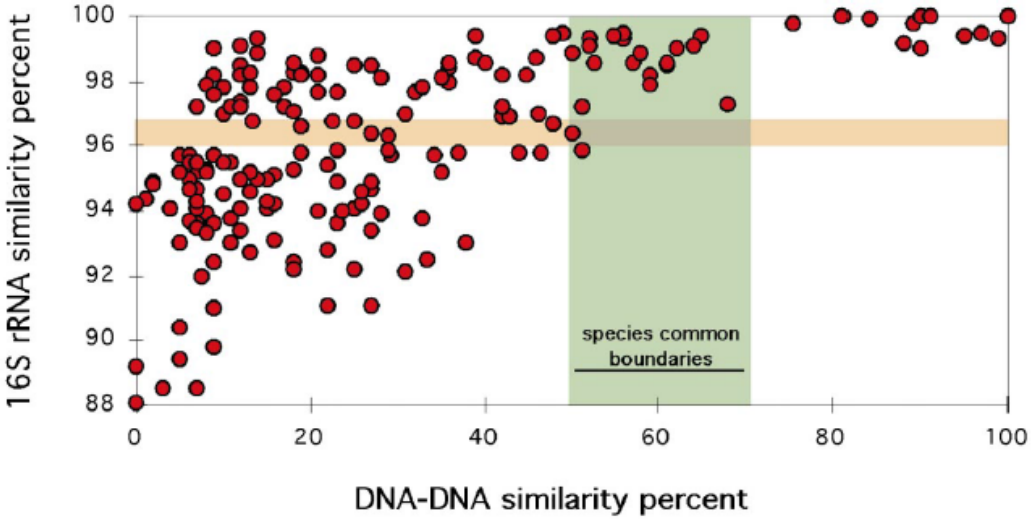

Figure 1.4. Comparison of DNA-DNA and 16S rRNA similarities. 180 values from 27 independent articles of the IJSB vol. 49 (1999) are represented. These data combine intrageneric values obtained for members of *Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* and Gram positives of high GC phyla. Original figure taken from Rosselló-Mora & Amman, 2001.

The current consensus in bacterial taxonomy is to use a polyphasic approach for characterizing and classifying bacteria. A range of genotypic and phenotypic techniques are applied to characterize a bacterial species in the most comprehensive way possible (Vandamme *et al*., 1996; Moore *et al*., 2010). In practice, a species is defined as "a group of strains characterized by a certain degree of phenotypic consistency, by a significant degree (50 to 70%) of whole genome DNA relatedness and over 97% 16S ribosomal RNA gene sequence identity" (Coenye *et al*., 2005). In general, phenotypic techniques are very useful in characterizing an organism and chemotaxomic methods might help in drawing a picture of high-level taxonomy. But phylogeny mostly cannot be determined based on phenotype alone. Therefore, genotypic methods, such as 16S rRNA gene sequencing and rRNA homology are mostly applied. However, the current species concept is criticized by some researchers as being too conservative, leading to an underestimation of the real diversity (Rosselló-Mora & Amman, 2001).

Genomics based taxonomy. Currently, focus in bacterial taxonomy is on whole-genome sequencing (WGS), which might contribute to unravel evolutionary relationships between prokaryotes and to result in a workable, satisfying species concept (Coenye *et al*., 2005; Gevers *et al*., 2005; Konstantinidis & Tiedje, 2005). As stated earlier, 16S rRNA gene sequencing suffers from lack of resolution for closely

related strains, and alternatives were found in the analysis of housekeeping genes. Such approach is known as Multi Locus Sequence Typing (MLST) or Multi Locus Sequence Analysis (MLSA). MLSA schemes often provide higher resolution than 16S rRNA gene sequencing, allowing differentiation at the species level (Moore *et al*., 2010). Two of the most recent methods to delineate bacterial species are Average Nucleotide Identity (ANI) and Average Amino acid Identity (AAI) (Richter & Rosselló-Mora, 2009). Both parameters rely on the pairwise comparison of whole genome sequences to determine a set of orthologous genes conserved among both genomes. Preliminary results have shown that ANI and AAI-values correlated extremely well with experimentally determined DDH-values, and the comparison between these techniques resulted in a threshold value of 95% ANI and 95-96% AAI for species delineation, comparable to the 70% DNA-relatedness value. Therefore it has been suggested that ANI could be a more practical replacement for DDH within the current species concept (Konstantinidis & Tiedje, 2005; Goris *et al*., 2007). However, it has also been observed that strains with a 95% ANI value could still have up to 20% difference in gene content, leading to a stricter ANI cut-off (98-99%) to obtain a higher predictive value for species delineation than the 70% DDH (Konstantinidis *et al*., 2006).

1.2.3 Taxonomy of the genus *Xanthomonas*

The genus *Xanthomonas* has been subject of numerous taxonomic and phylogenetic studies because of its phytopathogenic diversity. The 16S rRNA gene sequence homogeneity within *Xanthomonas* is very high with just three phylogenetic lineages being detected (Hauben *et al*., 1997). The largest lineage includes 15 *Xanthomonas* species, and the high degree of conservation of the 16S rRNA has limited the study of interspecific relationships within the genus (Figure 1.5.).

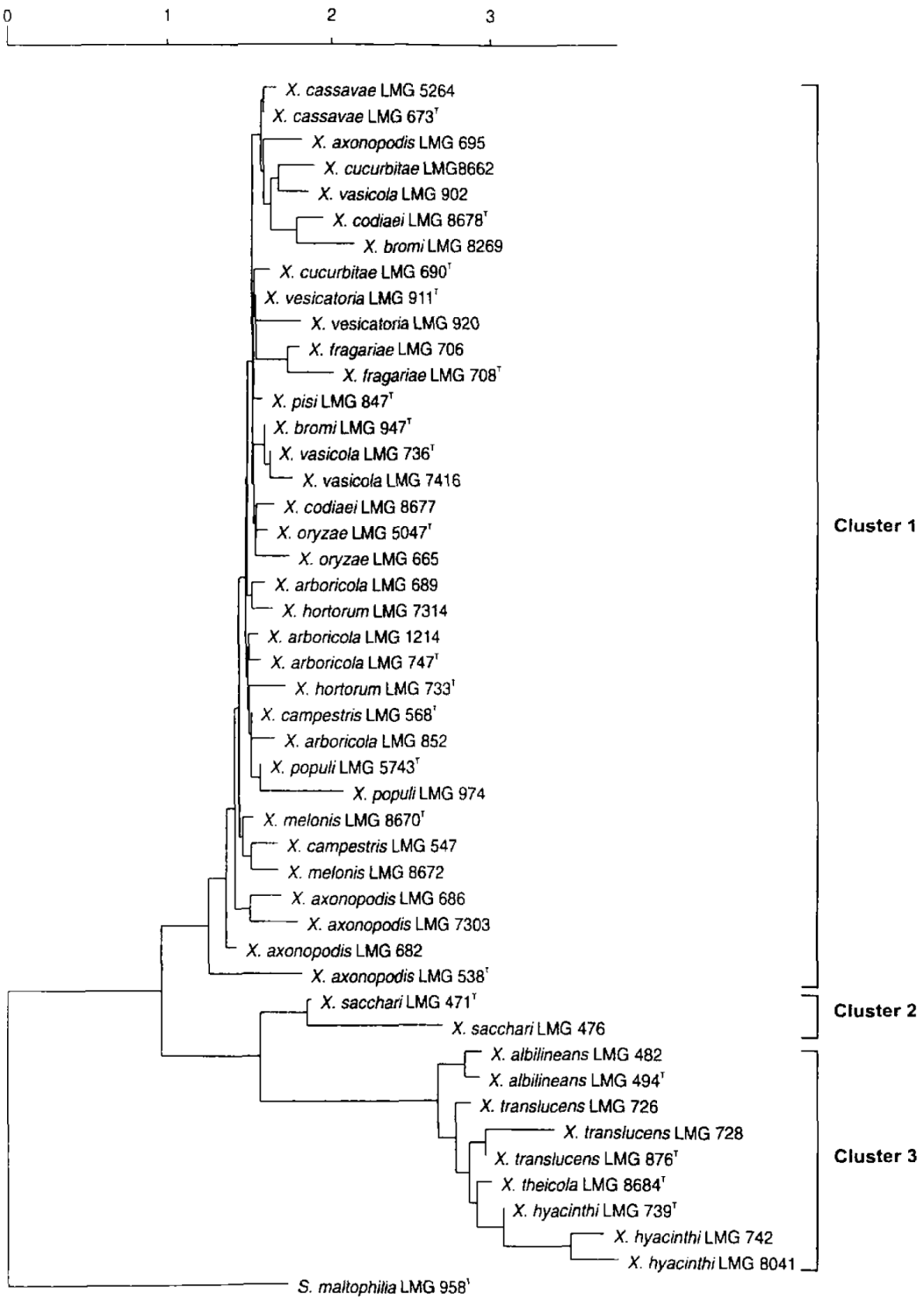

Figure 1.5. Phylogenetic relationship among *Xanthomonas* species based on partial 16S rRNA gene sequences. Original figure taken from Hauben *et al.*, 1997.

History of the genus. The first description of a plant disease known as "yellow disease of hyacinths" caused by a *Xanthomonas* strain was reported by Wakker in 1883. The disease was named *Bacterium hyacinthi*. Later, yellow-colonies were allocated to the genus *Pseudomonas* (Winslow *et al*., 1920) and afterwards to *Phytomonas*, a genus created to group all plant-pathogenic, yellow-pigmented bacteria

(Bergey *et al*., 1923). In 1930, Burkholder made a comparative study on a grand scale and he sorted out the major groups of bacteria that had previously been lumped into *Phytomonas*. Dowson (1939) classified plant-pathogenic bacteria mainly into three genera: (1) *Bacterium* Ehrenberg 1828, emend. Dowson 1939 for the Gram-negative bacteria with peritrichous flagella, (2) *Pseudomonas* Migula 1897, emend. Dowson 1939 for the green-fluorescent bacteria with polar flagella, and (3) a new genus, *Xanthomonas* Dowson 1939 for the Gram-negative, yellow pigmented bacteria with a single polar flagellum (Dowson, 1939; Garrett, 1981). Dowson also differentiated 19 species within this new genus (Van Den Mooter & Swings, 1990).

Initially, each variant of the genus *Xanthomonas* showing a different host range or producing different disease symptoms was classified as a separate species, reflected in the new host-new species concept (Starr, 1981). However, this led to an unreasonably large number of nomenspecies, which later resulted in a reclassification according to the classical nomenclature.

Burkholder and Starr discussed the impossibility of distinguishing the species within *Xanthomonas* by biochemical and physiological features alone without knowing their hosts, and they also criticized the new host – new species concept (Burkholder & Starr, 1948). Nevertheless, in *Bergey's Manual of Determinative Bacteriology*, 7th ed., Burkholder listed 60 species within the genus (Burkholder, 1957). The fact that the different "species" were almost indistinguishable later led to the reduction of the number of species to just 5: *X. albilineans*, *X. ampelina*, *X. axonopodis, X. campestris* and *X. fragariae* (Dye *et al*., 1974). The consequence was a large-scale partial merge into a single species, *Xanthomonas campestris* which was then subdivided into different pathovars (Dye *et al*., 1974). Within the genus some changes were made: the species *X. ampelina* was transferred to a new genus *Xylophilus*, as *Xylophilus ampelinus* (Willems *et al*., 1987) and new species were integrated in the genus *Xanthomonas*, as *X. populi* formerly classified as *Aplanobacter populi* (Ridé & Ridé, 1992). Later, another reclassification was proposed by Young *et al*. (1978) based on the former taxonomy system. The evolution number of *Xanthomonas* species is given in Table 1.1.

Table 1.1. Evolution of the number of *Xanthomonas* species

Reference	*Xanthomonas* species (no.)
Dowson (1939)	16
Burkholder (1957)	60
Dye *et al.* (1974)	5
Vauterin *et al.* (1995)	20
Parkinson *et al*. (2007)	27

Several studies attempted to delineate *Xanthomonas* species and clarify the relationships between the newly created pathovars using DNA Restriction Fragment Length Polymorphism (RFLP) profiling (Lazo

et al., 1987), fatty acids (Yang *et al*., 1993) and numerical taxonomy (Van Den Mooter & Swings, 1990). In 1995, based on DNA-DNA hybridization of 183 strains Vauterin *et al*. proposed reclassification of the genus *Xanthomonas* in 20 species (20 DNA-DNA homology groups) including four earlier defined species such as *X. albilineans, X. fragariae*, *X. populi* and *X. oryzae* (Vauterin *et al*., 1995). Although the new nomenclature was confirmed with the Bacteriological Code and supported by rep-PCR and Amplified Fragment Length Polymorphism (AFLP) (Rademaker *et al*., 2000; Rademaker *et al*., 2005), it has not ceased to be the object of controversy in terms of nomenclature (Schaad *et al*., 2000; Young *et al*., 2001). These heated debates led to the rejection of several names proposed by Vauterin *et al*. (1995) (Schaad *et al*., 2000). However the Taxonomy Committee of Plant Pathogenic Bacteria did not accept the names proposed by Schaad *et al*. because of misinterpretation of the Bacteriological Code (Young *et al*., 2001). In 2005, based on Rep-PCR results of 339 strains of the genus *Xanthomonas*, Rademaker *et al*. identified 20 groups, which correspond to the 20 DNA-DNA homology groups of Vauterin *et al*. (1995) (Rademaker *et al*., 2005). In the same study the species *X. axonopodis* was divided into six subgroups. Thereafter, new species were defined based on the polyphasic approach. These new species included *X. cynarae* (Trébaol *et al*., 2000), *X. euvesicatoria*, *X. perforans*, *X. gardneri* (Jones *et al*., 2004), *X. citri*, *X. fuscans*, *X. alfalfae* (Schaad *et al*., 2005; Schaad *et al*., 2006), *X. dyei* (Young *et al*., 2010). A last species, *X. maliensis*, has recently been described (Triplett *et al*., 2015).

Currently, diversity studies dealt with *Xanthomonas gyrase B* (*gyrB*) partial sequence analysis (Parkinson *et al*., 2007; Parkinson *et al*., 2009) and MLSA (Young *et al*., 2008). Although the classification of *Xanthomonas* is still changing up to this day, about 33 species are currently recognized (http://www.bacterio.net, last accessed on 25/01/2017 (Euzéby, 1997).

Although *gyrB* sequence analysis and MLSA offer a clear phylogenetic differentiation of the *Xanthomonas* species, the resolution is often insufficient to distinguish at the pathovar level. The recent genomic approaches to *Xanthomonas* classification seem promising (Rodriguez *et al*., 2012) and once more genomes will be sequenced this genomic based taxonomy will result in new changes in *Xanthomonas* nomenclature.

1.3 Bacterial blight of aroids

1.3.1 Introduction

Bacterial blight of aroids is caused by the bacterium which is up till recently named as *Xanthomonas axonopodis* pv. *dieffenbachiae* (Xad). The pathogen was first reported in the United States in 1939 (McCulloch & Pirone, 1939) and has since then spread globally. Several species within the family of Araceae have been indicated as host plants (Chase, 1987). Under favorable conditions the disease can cause significant damage to both plant stock and aroid production. The pathogen is a quarantine

organism in some major *Anthurium*-production countries and appears on the A2 list of the European and Mediterranean Plant Protection Organization (EPPO). That means that the pathogen is locally present in the EPPO region and is recommended for regulation as quarantine pest in order to limit its further spread (EPPO, 2009). This third part of the General Introduction focuses on biology, control and detection of the aroid pathogen Xad, and defines the economic and legislative implications of the bacterial blight disease.

1.3.2 Symptoms

The disease can occur either as a local or as a systemic infection (Fukui *et al*., 1998). Local infection appears on the leaves and spathe. Usually, the pathogen invades the leaf through the hydathodes (Sakai & Alvarez, 1990) and only occasionally through stomata. The first symptoms are small star-shaped water-soaked spots at the leaf margins, eventually with some yellowing, which appear necrotic under dry conditions. Leaf spots coalesce to large, V-shaped to irregular brown necrotic areas with a bright yellow margin. Systemic or vascular infection occurs when the bacterium spreads from the stem to other parts of the plant (Nishijima & Fujiyama, 1985). Older leaves and petioles are yellowing and easily break off to show dark brown vascular tissues at their base. Sometimes droplets of yellow bacterial ooze slime occur on infected petioles. Eventually the entire plant can be killed. Sometimes systemic infection also produces new water-soaked leaf spots, mainly near the main veins, when bacteria invade the leaf parenchyma from the infected vascular bundles. Some hosts only show leaf symptoms, while other hosts show both leaf and systemic symptoms. The symptom type may also depend on the infecting strain. Systemic infection may sometimes resemble foliar infection. This occurs when the bacterium moves upward from the vascular system in the stem into the leaf blade. In this case, water-soaked spots will occur near the main vein. The disease in *Anthurium* was originally described as a non-systemic leaf blight, but depending on the infecting strain, both leaf and systemic infections can occur (Figure 1.6.). Xad may occur in a latent form, also in tissue culture (Norman & Alvarez, 1994a; Fukui *et al*., 1996).

Figure 1.6. Typical symptoms of *X. axonopodis* pv. *dieffenbachiae* on *Anthurium*. Advanced foliar blight with necrotic areas surrounded by a bright yellow margin (a); early foliar blight symptoms, water-soaked spots at leaf margins (b); blacking of the spathe in the 'flower blight' stage (c); systemic infection resulting in death of potted plants (d). Photos by W. Nishijima and T. Vowell.

1.3.3 Causal Organism

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Xanthomonadales

Family: Xanthomonadaceae

Genus: *Xanthomonas*

Species: *Xanthomonas axonopodis*

Xad is a gram-negative, rod-shaped bacterium. Cells are about 0.3-0.4 µm diameter and 1.5 µm in length, with a single polar flagellum (McCulloch & Pirone 1939; Bradbury, 1986). On Wilbrink-N agar medium (Koike, 1965) yellow, mucoid, circular and convex colonies appear after 2 to 3 days. Separate colonies easily coalesce and form darker yellow, opaque areas. Xad is able to infect a broad range of aroids and the strains display a complex host specificity (Chase *et al*., 1992).

Taxonomically, strains of Xad are known to be heterogeneous and it has been suggested that they represent different taxa (Rademaker *et al*., 2005; Donahoo *et al*., 2013). Xad strains isolated from *Anthurium* and *Dieffenbachia* were shown to comprise two types on the basis of SDS-PAGE profiles (Vauterin *et al*., 1991) and rRNA gene restriction patterns (Berthier *et al*., 1993). Although both types belong to the same DNA homology group (group 9), their level of DNA homology was only 66% (Vauterin *et al*., 1995). In a follow-up study, DNA homology group 9 was divided into six subgroups, with strains originating from *Anthurium* placed in subgroup 9.4 and strains from *Dieffenbachia* in subgroup 9.6 suggesting that the different genetic groups identified in *X. axonopodis* probably represent different species (Rademaker *et al*., 2005). Similar results were reported by Hajri *et al*. (2009), who corroborated that *rpoD* sequence analysis grouped *X. axonopodis* pv. *dieffenbachiae* strains according to their host of isolation (*Anthurium*, *Dieffenbachia* and *Philodendron*). Furthermore, Xad from group 9.6 has been included in the emended species description of *X. citri* (Ah-You *et al*., 2009). The description referred to the strains not pathogenic to *Anthurium* spp..

1.3.4 Disease cycle and epidemiology

Xad seems primarily transmitted to aroid production fields through infected nursery stock (Norman & Alvarez, 1994a). Splashing water (rain or irrigation), aerosols, infested soil and possibly nematodes during planting are other sources of Xad-infection (Nishijima & Fujiyama, 1985; Alvarez *et al*., 1994). Although root damage by nematodes was associated with symptomless systemic infection in adult plants in the field, no experimental evidence was found that nematodes contribute to spreading of the bacterium (Fukui *et al*., 1998). Infected plant debris is another suspected primary source of Xad-infection. Studies have also shown that the pathogen can survive in plant debris for more than four months (Duffy, 2000) and its capacity to spread through aerosols was demonstrated using Andersen samplers and settling plates (Alvarez *et al*., 2006). Natural spread between glasshouse companies is very unlikely to occur, but all care should be given to the sanitary condition of plant material that is introduced in the cultures and greenhouse. Xad can also be spread by human assistance through contaminated tools, clothes, infected soil on footwear, vehicles and equipment (Nishijima & Fujiyama, 1985). Although the pathogen does not survive long in soil, this medium should not be overlooked as inoculum source (Duffy, 2000). Epiphytic survival and aroid leaf invasion by Xad, as well as disease development, are all favored by high temperature and humid conditions. Symptoms typically develop faster on young plants than on older, matured plants (Nishijima & Fujiyama, 1985). Disease severity was clearly higher in plants grown at 31°C than at 26°C or lower, independent of air humidity (Alvarez *et al*., 2006). Xad invades the leaf though hydathodes but in some cases under conditions that favor the opening of the stomata (light intensity and humidity) the bacterium enters the leaf though stomata. However, under low humidity, the bacteria never die, the lesions remain small and dry and

disease development is slower than under favorable conditions. Under moist conditions (high relative humidity), the bacteria multiply on the leaf surface and increase the probability of infection, the spots enlarge and merge to cover large areas. However, some infected plants can remain asymptomatic while the bacteria multiply and spread throughout the plant; such plants can exude guttation fluid containing bacteria (Norman *et al*., 1999). Studies have shown that the amino acids found in guttation fluid provide nutrients for invading bacteria (Sakai & Alvarez, 1990). These exudates can then be dispersed to new plants by dripping or splashing. Moreover, splash dispersal to plants at 5 meters distance occurred downwind of a sprinkler irrigated block of plants and behind a barrier installed to prevent bacterial movement by splash droplets. Therefore, appropriate precautions should be taken in case the propagation areas for tissue cultured plants are downwind of flower production areas (Alvarez *et al*., 1994).

1.3.5 Geographic distribution

The literature indicates that bacterial blight of aroids probably originated in South or Central America. The first scientific report dates back to 1939 (Table 1.2.) and described the disease as *Bacterium dieffenbachiae* (McCulloch & Pirone, 1939). Apparently, the disease first spread throughout the USA, probably through infected plant material (EPPO, 1997) and later the export of new plants spread the disease to other continents (Jouen *et al*., 2007). At present, the bacterium has spread globally, although several countries have taken measures to eradicate the pest (EPPO 2005, 2007).

Table 1.2. Distribution of *X. axonopodis* pv. *dieffenbachiae* around the world

Year of report	Geographical region	Reference
1939	New Jersey	(McCulloch & Pirone, 1939)
1952	Brazil	(Robbs, 1955)
1972	Hawaii	(Hayward, 1972)
1985	California	(Cooksey, 1985)
1985	Caribbean	(Prior *et al*., 1985)
1985	Florida	(Pohronezny *et al*., 1985)
1987	Venezuela	(Guevara & Debrot, 1987)
1990	Philippines	(Natural & Alvarez, 1990)
1990	French Polynesia	(Mu & Alvarez, 1990)
1992	Puerto Rico	(Cortes-Monllor, 1992)
1997	The Netherlands	(EPPO, 1997)
1998	Taiwan (China)	(Hseu & Lin, 1998)
2000	Italy	(Zoina *et al*., 2000)

Continued on next page

2000	Reunion Island	(Soustrade *et al*., 2000)
2002	China	(Ji *et al*., 2003)
2003	Turkey	(Aysan & Sahin, 2003)
2004	Romania	(Vlad *et al*., 2004)
2007	New Caledonia	(Jouen *et al*., 2007)
2008	Poland	(Pulawska *et al*., 2008)

1.3.6 Detection and identification

The basic detection method for Xad is visual inspection of aroid plants, isolation of the bacterium from symptomatic plants and identification by phenotypic and pathogenicity tests. However, visual inspections are not very effective because of latent infection, while identification by phenotypic and pathogenicity testing are time-consuming and have limited efficiency because of limitation of the sample size. Because the symptoms caused by Xad are easily confused with other pathogens as *Pseudomonas* and *Ralstonia solanacearum*, laboratory confirmation is required (Norman & Yuen, 1999). Since the main host of Xad in our region is *Anthurium*, the interest was directed to develop techniques for the detection of the pathogen from this host. Until recently, the detection of Xad was based on a reference method which consisted of isolation of the bacterium from suspect symptoms on semi-selective media (Norman & Alvarez, 1989), followed by identification of *Xanthomonas* colonies by immunofluorescence (IF) and/or enzyme-linked immunosorbent assays (ELISA) using specific monoclonal or polyclonal antibodies (EPPO, 2004). Although the enrichment of target bacteria on semi-selective media before ELISA improves the sensitivity, the method predisposes to false positive reactions due to immunological cross-reaction with other associated bacteria, for example epiphytic and saprophytic bacteria which often overgrow the pathogen even when semi-selective media are used (Norman & Alvarez, 1994b). Therefore, the final confirmation was still made by pathogenicity testing by inoculating host plants and scoring symptom development. With the introduction of PCR, a number of fingerprinting techniques for isolate identification were reported (Louws & Alvarez, 2000; Khoodoo & Jaufeerally-Fakim, 2004; Khoodoo *et al*., 2005; Robéne-Soustrade *et al*., 2006). A multiplex PCR for Xad based on three sets of primers, combined with a genus-specific monoclonal antibody was successfully applied in direct leaf tissue screening (Khoodoo *et al*., 2005). This PCR was developed to detect and differentiate all the different groups among Xad strains, resulting in very complex profiles. Subsequently a nested PCR with increased sensitivity claimed more reliable Xad detection in symptomless contaminated plants. The nested-PCR has been developed to specifically detect and identify *X. axonopodis* pv. *dieffenbachiae* strains pathogenic to *Anthurium* (Robéne-Soustrade *et al*., 2006). More recently, the nested-PCR has been compared to the reference

method (isolation and serological identification of bacterial colonies) and to other alternative serological methods in order to ensure the most appropriate detection scheme (Chabirand *et al*., 2014). The method has been included in the EPPO decision scheme as an alternative method to the biochemical and serological tests (EPPO 2009). Recent developments in *Xanthomonas* taxonomy, mainly the sequencing of several phylogenetically relevant genes (Young *et al*., 2008; Parkinson *et al*., 2009), have claimed rapid identification of *Xanthomonas* species and pathovars, including *X. axonopodis pv. dieffenbachiae*. The available reference sequence records can be used to confirm the identity of any suspected Xad-isolate.

1.3.7 Control

Once established, eradication of the bacterium is very difficult because of its systemic and latent nature. Prevention is the most important management practice. Sanitation and exclusion are the main cultural measures (Lipp *et al*., 1992). For a long time, the only available method to reduce bacterial blight was to remove suspect leaves showing symptoms and to eliminate systemically infected plants (Nishijima, 1988). Different products have been used for chemical control, such as streptomycin or oxytetracycline (Sato, 1983). Because of their high cost and the development of streptomycin-resistant strains these control methods were later abandoned (Nishijima, 1988). In Europa such antibiotic are not allowed, therefore cupric hydroxide and mancozeb, reported as having good effectiveness against Xad, have replaced the two antibiotic products (Knauss *et al*., 1972). In the absence of effective chemicals, long-term disease management measures include sanitation, cultural practices, biological control (Toves, 2008). Sanitation combined with resistant or tolerant cultivars is the most effective approach against the disease (Valencia *et al*., 2004). Disinfection of cutting tools is essential for preventing the spread of blight, since plant materials which show no symptoms have the potential for latent infection. Although the sanitation practices and disinfection of tools are useful, they are insufficient for stopping disease spread. Drip irrigation rather than overhead or sprinkler irrigation reduced the spread of the bacterium through aerosols and water splash in the cultures and significantly reduced the incidence of bacterial blight (Kamemoto & Kuehnle, 1989; Alvarez & Norman, 1993). Appropriate nutrition is important for the plant susceptibility to diseases and it was suggested that lower fertilizer rates for pot plants could result in lower susceptibility of leaves to Xad and greater flower production (Chase, 1988). Higher levels of ammonium fertilizer led to higher amounts of amino compounds in guttation fluid when compared to nitrate fertilizers (Sakai & Alvarez, 1990). Increased amount of amino compounds were associated with greater plant susceptibility to disease. The use of sufficient amounts of nitrate fertilizer for plant growth reduced the amount of amino compounds in guttation fluid, and was proposed to reduce blight incidence (Sakai, 1991; Sakai *et al*., 1992). Growing aroids under cool and shaded conditions slows the progression of the disease. Inoculated plants

exposed to temperature higher than 27°C were more susceptible to disease than inoculated plants exposed to lower temperature (Alvarez *et al*., 1990). Although greenhouse trials indicated a relationship between fertilizer treatments and blight susceptibility, field trials were indecisive (Higaki *et al*., 1992). It is generally agreed that the most cost-effective method to control blight would be through the development of resistant cultivars (Prior *et al*., 1985; Anaïs *et al*., 2000). Thus far, transgenic resistance to the blight pathogen using peptide biocides (Kuehnle *et al*., 2004) has not provided satisfactory levels of resistance. Although the treatments considerably reduced Xad numbers and symptom development, the disease could not be completely eradicated without significant plant mortality. At present, pathogen-free planting material, hygienic measures, controlled temperature and restricted use of overhead irrigation are the most effective control measures for bacterial blight.

1.3.8 Economic impact and legislative relevance

Bacterial blight, caused by Xad has been of major economic concern among the anthurium growers worldwide. Although the disease causes heavy losses on *Anthuriums* and other members of the family Araceae, limited information is available on effective damages caused by the pathogen (Kelaniyangoda & Wickramarathne, 2009). High disease incidence was reported in Hawaii where it destroyed the production of approximately 200 small farms in 1985 – 1989. The cut flower production dropped from approximately 30 to 15.6 million stems per year in 1990 (Alvarez *et al*., 2006) and the losses for 1987 exceeded 5 million dollars to the *Anthurium* cut flower industry in Hawaii. With the implementation of an integrated disease management program, losses were reduced to 5%. The pathogen produced also significant damage in Italy and Réunion Island where 80 to 100% of the plants showed symptoms (Soustrade *et al*., 2000; Zoina *et al*., 2000). Outbreak of Xad in Turkey was reported in *Anthurium* pot plants and disease incidences of 20 – 25% occurred (Aysan & Sahin, 2003). Application of active substances such as cupric hydroxide (Kocide 101) and mancozeb (manzate 200) already mentioned above may help decrease Xad mediated yield loss, even if they do not completely eradicate the disease. Economic losses can vary substantially among fields, greenhouses and production years. Direct field losses are an important part of the disease impact. The indirect cost associated with sanitary measures, disinfection actions as well as with tests in the certification of planting material are significant expenses spend every year to better control bacterial blight.

As discussed earlier, the most effective control measure for Xad is still the prevention of introduction with planting stock. Therefore, bacterial blight was first introduced in the list of quarantine diseases of the European and Mediterranean Plant Protection Organization (EPPO) in 1997 (EPPO 1997). Any nursery plant consignment, imported in or transported within the EU, should have an official declaration that the place of production has been free of Xad symptoms. Imported or transported planting stock batches where Xad is discovered have to be destroyed. Also nurseries in the US, wishing

to export plants of Araceae to any EU countries, must have plant passport inspections (art. 2.3 in Annex V part A of directive 2000/29/EC).

1.4 Virulence factors in *Xanthomonas*

The establishment of infection by phytopathogenic bacteria is mediated by virulence factors. Virulence factors can generally be defined as all bacterial products or strategies that contribute to the ability of the bacterium to cause disease. To successfully colonize host plants, plant pathogenic bacteria must be able to adhere to the plant surface invade the mesophyll or the vascular system to acquire nutrients and resist plant defense responses. The classical plant bacterial pathogens reside in the intercellular spaces and they deploy an arsenal of secreted virulence factors to modulate host cell processes (Figure 1.7). For *Xanthomonas* spp., the most extensively studied secretion system is the type III secretion system (T3SS). The T3SS is responsible for the secretion of various effectors into the host cell. Resistance mediated by *R*-genes of the host is predominantly based on the specific recognition of type III secreted effectors. The result of specific recognition is the hypersensitive response which inhibits pathogen proliferation. When not recognized, these effectors may suppress host defense mechanisms and promote virulence processes of the pathogen. Therefore, characterization of the genes encoding effector proteins may enable the identification of resistance mechanisms inside the host plants. In the following, the different secretion systems and other virulence factors present in *Xanthomonas* spp. and also potentially important for Xad infection will be described.

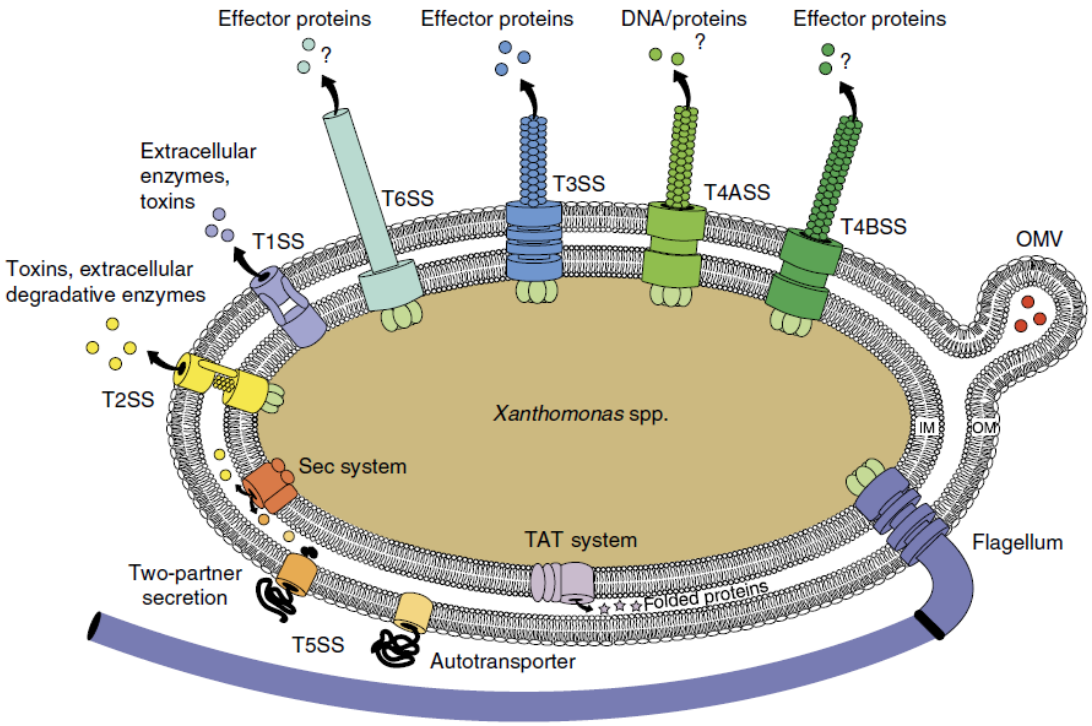

Figure 1.7. Schematic representation of protein secretion systems from *Xanthomonas* spp. Six types of protein secretion systems are encoded. T2S and T5S systems depend on the general secretory pathway (red: Sec) or the twin-arginine translocation (light purple: TAT) system for protein transport across the inner membrane. T3S, T4S and T6S systems are associated with extracellular pilus structures and presumably translocate proteins into the host cell. So far, protein translocation was experimentally proven for T3S systems. Only in a few cases does protein secretion depend on the formation of outer membrane vesicles (OMV). IM, inner membrane; OM, outer membrane; TAT, twin-arginine translocation. Original figure taken from Büttner & Bonas, 2010.

1.4.1 The type III secretion system (T3SS)

Type III secretion system (T3SS) is an important pathogenicity factor employed by most Gram-negative bacterial pathogens. It consists of more than 20 proteins which form a needle-like complex to deliver effector proteins directly from the bacterial cytoplasm into the host cells (Büttner & Bonas, 2002). In plant pathogens, the T3SS is encoded by *hrp* (hypersensitive response and pathogenicity) genes, essential for bacterial pathogenicity and hypersensitive response in host and non-host plants, respectively (Lindgren *et al*., 1986). At least nine *hrp* genes are conserved in plant and animal pathogenic bacteria and called *hrc* (hypersensitive response and conserved) genes (Bogdanove *et al*., 1996). In contrast to the conserved T3SS apparatus, the type III effectors vary considerably among different plant pathogens and even among different strains of the same species (Greenberg & Vinatzer 2003). This variation suggests that different strains have evolved different repertoires of virulence factors to infect and cause disease on specific host plants. To date, more than 100 different effector proteins that can be divided into 39 different so-called Xop (*Xanthomonas* outer protein) groups based on sequence relatedness (White *et al.*, 2009) are known that are secreted via the T3SS. According to experimental and bioinformatic analyses, approximately 20–30 effectors with overlapping activities are typically secreted by one single *Xanthomonas* strain (Büttner & Bonas, 2010). These effectors can fulfil multiple functions, such as interference with host immunity or they may facilitate nutritional and virulence processes of the pathogen (Büttner & He, 2009). At the same time, they represent essential determinants of pathogenicity on susceptible plants and are required for the induction of the hypersensitive response (HR) on resistant plants (White *et al*., 2009). The fact that plant pathogenic bacteria secrete many type III effectors could be an adaptive feature of plant pathogens and suggests that functional redundancy may exist among these effectors (Büttner & Bonas, 2003). Some effectors such as the transcription activator-like effectors (TALEs) are structurally and functionally well-characterized, and have been the subject of more than 20 years of ongoing research in the pepper and tomato pathogen *Xanthomonas campestris* pv. *vesicatoria* (Bogdanove *et al.*, 2010). TALEs (also called AvrBs3/PthA-family effectors) and closely related proteins have been found in several but not all phytopathogenic *Xanthomonas* species. TALEs consist of a common N-terminus required for type III secretion and a C-terminus containing a nuclear localization signal (NLS) and an acidic activation domain (AAD). Nearly all characterized and cloned *R*-genes that are effective against *Xanthomonas* spp. rely on detection of or interaction with TALEs.

1.4.2 The type II secretion system (T2SS)

A number of possible virulence factors are secreted via T2SS, including toxins, cell wall degrading enzymes (CWDEs), proteases, lipases and phosphatases. As extracellular pathogens, phytopathogenic bacteria encounter plant cell walls as barriers preventing access to the cytoplasmic contents of host cells. In order to infect the host plant effectively, many plant pathogens include a battery of cell wall degrading enzymes in their repertoire of virulence factors. These enzymes include pectinase, cellulases and proteases that work together to softer or break down plant cell walls, facilitating pathogen access and the release of nutrients for pathogen growth (Barras *et al*., 1994). *X. campestris* pv. *campestris* has an extensive collection of genes encoding putative cell wall degrading enzymes, including several pectic enzymes and cellulases (da Silva *et al*., 2002). Presumably, these enzymes contribute to the massive degeneration of plant tissue that occurs during development of black rot disease in plants infected with *X. campestris* pv. *campestris* (Agrios 2005). Xylanase is the enzyme that degrades xylan, a component of xylem vessels. *X. oryzae* pv. *oryzae* strains with a mutant *xynB*, the gene for xylanase secretion, have an abolished ability to accumulate xylanase in planta and attenuated virulence in rice (Rajeshwari *et al*., 2005).

Other secretion systems

Although poorly understood, *Xanthomonas* spp. may also make use of secretion systems other than T2SS and T3SS, that potentially also play important roles in the interaction with their host. In *Xanthomonas* spp. genes for all known protein transport systems of Gram-negative bacteria have been identified, i.e. type I, type IV, type V and type VI. For example, the type IV secretion system (T4SS) is known to contribute to virulence and it comprises 12 proteins, VirB1-VirB11 and VirD4 (Christie *et al*., 2005). The type IV secretion system (T4SS) is related to bacterial conjugation machines (Juhas *et al*., 2008) and is able to translocate proteins and/or protein-DNA complexes to the extracellular milieu or the host interior, in many cases contributing to the ability of the bacterial pathogen to colonize the host and evade its immune system (Backert & Meyer, 2006). In function, the T4SS very closely resembles the T3SS, which utilizes a flagellar export machine to inject effector molecules into the host cells (Macnab 1999). Both systems deliver substrates by a process requiring physical contact with target cells. Both systems require coupling or chaperone-like proteins for delivery of substrates to the respective transfer machines. Both systems are generally thought to export substrates in a one-step reaction via a trans-envelope channel. Finally, both systems elaborate extracellular pili or filaments that contribute in some way to substrate delivery. However, at least one fundamental difference exists between these two systems – the type IV systems can export long DNA polymers to recipient cells, whereas there is currently no evidence for transmission of nucleic acids via the type III machinery.

The type VI secretion machinery (T6SS) is a recently characterized secretion system that appears to constitute a phage-tail-spike-like injectisome that has the potential to introduce effector proteins directly into the cytoplasm of host cells (Filloux *et al*., 2008; Shrivastava & Mande, 2008), analogous to the T3SS and T4SS machineries. T6SS has diverse roles in virulence, symbiosis and interbacterial interactions (Records, 2011). Overall however, the identities and functions of T6SS effectors are still poorly understood.

1.4.3 Extracellular polysaccharides (EPS)

Almost all *Xanthomonas* spp. (except for *Xanthomonas albilineans*) produce a characteristic extracellular polysaccharide (EPS) called xanthan. Xanthan is a heteropolysaccharide consisting of repeating pentasaccharide units with a cellulose-like backbone and trisaccharide side chains of two mannose and one glucuronate residues. The production of xanthan in the bacteria is directed by several genes located on the *gum* gene cluster. The *gum* gene cluster typically consists of 12 genes which are highly conserved among *Xanthomonas* spp. (Katzen *et al.*, 1998). Due to the highly hydrated and anionic consistency of xanthan, it is expected that it protects bacteria from environmental stresses such as dehydration and toxic compounds. In addition to protection, for vascular pathogens, xanthan might be responsible for wilting of host plants by blocking the water traffic in xylem vessels (Chan & Goodwin, 1999). Although the xanthan production and its function related to pathogenicity exist, expression of the *gum* gene cluster most likely contributes to epiphytic survival and is not required for pathogenicity (Katzen *et al.*, 1998; Dunger *et al.*, 2007; Rigano *et al.*, 2007).

However, xanthan has also been suggested to suppress basal plant defense responses such as callose deposition in the plant cell wall, which presumably occurs by chelation of divalent calcium ions in the plant apoplast and is required for the activation of plant defense responses (Aslam *et al.*, 2008). Further, xanthan has been shown to be involved in the formation of bacterial biofilms in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* (Dow *et al.*, 2003; Rigano *et al.*, 2007; Torres *et al.*, 2007) in which bacteria attach to each other forming an extracellular polymeric matrix consisting of proteins, lipids and EPS (Sutherland, 2001). Biofilm formation might contribute to bacterial epiphytic survival before colonization of the plant intercellular space because it presumably provides protection against antibiotics and host defense responses prior to attachment of vascular bacteria to xylem vessels (Stoodley *et al.*, 2002).

1.4.4 Lipopolysaccharides (LPS)

In addition to EPS, lipopolysaccharides (LPS) represent another group of surface-associated virulence factors of *Xanthomona*s spp. Similar to EPS, LPS are essential components of the bacterial outer

membrane and may serves a dual role as physical barrier by protecting bacteria from antibacterial substances produced by plants and also as an inducer of plant defense-related genes (Newman *et al*., 2000). It comprises three covalently linked components: an outer membrane–bound moiety called lipid A, a core oligosaccharide and polysaccharide side chains (O-antigen) (Raetz & Whitfield, 2002). In *X. campestris* pv. *campestris*, the synthesis of LPS is directed by the *wxc* gene cluster, which comprises 15 different genes (Vorhölter *et al.*, 2001). Mutations in the *wxc* gene cluster causes higher sensitivity in unfavorable conditions for *Xanthomonas* spp. and might therefore lead to an attenuation of bacterial virulence as it has been shown for *X. campestris* pv. *campestris* and *X. campestris* pv. *citrumelo* (Kingsley *et al.*, 1993; Dow *et al.*, 1995; Newman *et al.*, 2001). Comparative sequence analysis of *wxc* gene clusters and whole genome sequences have revealed that LPS gene clusters of different *Xanthomonas* spp. are highly variable in number and identity of genes. Therefore, LPS genes presumably are subject to a strong diversifying selection in different species, pathovars or even strains (Lu *et al.*, 2008). Variations in LPS composition facilitate bacteria to avoid recognition of resistance mechanisms of the plant and presumably also affect bacterial resistance to phage adsorption and/or infection (Ojanen *et al*., 1993; Hung *et al*., 2002). It has been suggested that variation in the LPS gene cluster among *X. axonopodis* pv. *citrumelo*, *X. axonopodis* pv. *citri* and *X. campestris* pv. *vesicatoria* might contribute to their differences in virulence or symptom development in plant hosts rather than serving as a determinant of their differential host range (Jalan *et al*., 2011).

1.4.5 Gene cluster of regulation of pathogenicity factors (*rpf*)

It has been known that most *regulation of pathogenicity factor (rpf)* genes in xanthomonads regulates virulence in response to the diffusible signal factor, DSF. DSF is often regarded as a quorum-sensing (QS) molecule that allows bacteria to assess their population density. Gram-negative bacteria typically produce homoserine lactose derivates as main QS signals (de Kievit & Iglewski, 2000). When the extracellular concentration of a secreted QS signal rises above a specific threshold, the bacterium can recognize the signal, which in turn regulates the expression of genes involved in diverse group behaviors, such as swarming motility, biofilm formation, cell division, stress survival, and production of virulence factors (Fuqua *et al*., 2001). Investigation of the function of *rpf* genes, was showed that mutations in *rpfB*, *rpfC, rpfF*, and *rpfG* reduce the virulence and motility in *X. oryzae* pv. *oryzae* (*Xoo*) and decrease the expression of genes involved in the production of EPS, lipopolysaccharide (LPS), phytase, xylanase, and lipases (Jeong *et al*., 2008). In *X. campestris* pv. *campestris* (*Xcc*), the inactivation of *rpfI* gene reduced expression levels of proteases indicating that *rpfI* is responsible for tissue degeneration during *Xcc* infection (Dow *et al.*, 2000).

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Chapter 2:

Genetic characterization of strains named as *Xanthomonas axonopodis* pv. *dieffenbachiae* leads to a taxonomic revision of the *X. axonopodis* species complex

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Autors' contributions

MM, CB, DVP and CEC designed the experiment set-up. CEC did the practical work, performed the MLSA and drafted the manuscript. DVP and CI provided the necessary bacterial strains. VMC helped with the DNA extractions and sequencing. BS performed the whole-genome DNA sequencing and ANI calculations and CI helped with the biochemical analyses. CB supervised the work and assisted in writing. CB, CI and MM proofread the manuscript.

Abstract

Xanthomonas axonopodis pv. *dieffenbachiae* (Xad) is the causal agent of anthurium bacterial blight and listed as an A2 quarantine organism by EPPO. However, the name Xad covers a variety of strains. Here, 25 Xad strains and 88 phylogenetically related strains, including *Xanthomonas* type strains and representatives of other pathovars, were examined using a polyphasic taxonomic approach. Multilocus sequence analysis of seven genes showed that strains isolated from *Dieffenbachia*, *Philodendron*, and *Anthurium* cluster into three distinct phylogenetic groups (PG I, II and III), while the type strain of *X. axonopodis* clustered into a fourth group (PG IV). PG I included the type strains of *X. citri* subsp. *citri, X. citri* subsp. *malavacearum, X. fuscans* subsp. *fuscans* and *X. fuscans* subsp. *aurantifolii.* PG II included the type strains of *X. euvesicatoria, X. perforans*, *X. alfalfae* subsp. *alfalfae* and *X. alfalfae* subsp. *citrumelonis.* PG III included the type strains of *X. phaseoli*. Each PG was shown to represent a single species based on average nucleotide identity values, DNA-DNA hybridization data and phenotypic characteristics. Therefore, strains named as Xad belong to PG I, PG II and PG III, and not to *X. axonopodis* (PG IV). Taxonomic proposals are made: emendations of the descriptions of *X. citri, X. phaseoli* and *X. axonopodis*, to encompass the strains of PG I, PG III and PG IV, respectively; reclassification of *X. perforans* and *X. alfalfae* as *X. euvesicatoria* and emendation of the description of *X. euvesicatoria* to encompass all strains of PG II.

2.1 Introduction

Xanthomonas axonopodis pv. *dieffenbachiae* (Xad) is the causal agent of anthurium bacterial blight. It affects members of the Araceae (aroids) causing leaf spots, blight and in some cases plant decay. Ornamental aroids such as *Anthurium*, *Alocasia*, *Dieffenbachia* and *Philodendron* are widely grown for use as indoor foliage. Other aroids such as *Colocasia esculenta* (taro), *Alocasia* (elephant ear), *Cyrtosperma* (swamp taro) and *Xanthosoma* (tannia) are used for food, animal fodder or medicinal purposes in the tropics and subtropics. Difficulties in controlling the spread of the pathogen, as well as the severity of disease symptoms, especially on *Anthurium*, led to the classification of Xad as an A2 quarantine organism on the EPPO list (EPPO, 2009). The disease was first described in the United States (McCulloch & Pirone, 1939) and has since spread worldwide. The pathogen was originally named *Xanthomonas dieffenbachiae* according to the "new host-new species" concept by which a plant pathogenic xanthomonad isolated from a new host plant was classified as a new species. When the 'Approved Lists' (Skerman *et al*., 1980) were implemented, the species was classified in *X. campestris* and the original species name was preserved in a special-purpose pathovar nomenclature as *X. campestris* pv. *dieffenbachiae* (Dye *et al*., 1980). In 1995, pathovar *dieffenbachiae* was transferred, together with 33 other *X. campestris* pathovars to *X. axonopodis* based on DNA-DNA hybridization

(DDH) data (Vauterin *et al*., 1995). The complex species *X. axonopodis* represents a high heterogeneity, not only in terms of pathogenicity to a wide range of unrelated host plant genera, but also genetically, based on rep-PCR and AFLP data, housekeeping gene sequences and DDH data (Rademaker *et al*., 2000; Jones *et al*., 2004; Young *et al*., 2008; Parkinson *et al*., 2009).

Recent phylogenetic analyses of the genus *Xanthomonas* based on comparison of house-keeping gene sequences, including the *gyrase B* gene (*gyrB*) (Parkinson *et al*., 2009) and the RNA polymerase β subunit gene (*rpoB*) (Ferreira-Tonin *et al*., 2012), proved successful to reliably discriminate *Xanthomonas* species. In the study of Parkinson *et al*. (2009), four phylogenetic clades were discriminated among known *X. axonopodis* pathotype strains. A *gyrB* fragment was also selected as a suitable barcode for identification of quarantine *Xanthomonas* taxa in the Quarantine Barcoding of Life project and is currently implemented in Q-bank for use by phytodiagnostic laboratories (www.q-bank.eu/Bacteria). Also, it was reported that Xad strains cluster into separate groups according to their host of isolation (Rademaker *et al*., 2005; Hajri *et al*., 2009; Donahoo *et al*., 2013).

The objective of this study was to investigate 25 Xad strains isolated worldwide from four aroid hosts (*Anthurium*, *Dieffenbachia*, *Philodendron* and *Aglaonema*), together with 88 phylogenetically related *Xanthomonas* species and pathovars, using a polyphasic taxonomic approach that included multilocus sequence analysis (MLSA) using seven genes, DDH, calculation of whole-genome average nucleotide identity (ANI) values, and biochemical analyses.

2.2 Materials and methods

2.2.1 Bacterial strains

Twenty-five Xad strains and 88 phylogenetically related strains, including type strains and representatives of other *Xanthomonas* pathovars, were used in this study and are listed in Supplementary Table 2.1. Strains were obtained from CIRM-CFBP (International Centre for Microbial Resources-French Collection of Plant Associated Bacteria, France), NCPPB (National Collection of Plant Pathogenic Bacteria, York, UK), LMG (BCCM/LMG Bacteria Collection, Ghent University, Belgium) and from working collections of several research institutes. For long time storage, all strains were kept at -80°C on beads in cryovials (Microbank, Prolab Diagnostics). Supplementary Table 2.2 presents the pathotype strains for which *gyrB* sequences available in GenBank, were used in Supplementary Figure 2.1.

2.2.2 DNA extraction

All strains were grown on Wilbrink's medium with nitrate (Wilbrink-N) (Koike, 1965) and incubated for 48 h at 28°C. A single bacterial colony from Wilbrink-N was transferred in 3 mL trypticase soy broth (TSB; Becton, Dickinson and Company) and grown for 24 h in a shaking incubator (200 rpm) at 28°C. The pellet of 1 mL TSB culture was used for genomic DNA extraction by the DNeasy Blood & Tissue kit (QIAGEN). DNA concentration and purity (according to $A_{260/280}$ and $A_{260/230}$) were determined using a ND-1000 spectrophotometer (NanoDrop) and DNA aliquots were stored at -20°C for further use.

2.2.3 Multi-locus sequence analysis (MLSA)

MLSA using seven housekeeping genes (*atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, *lrp*, *rpoD*) was performed for 109 *Xanthomonas* strains. The seven loci were chosen based on previous sequence-based studies of *Xanthomonas* species (Cubero. & Graham, 2004; Fargier *et al*., 2011; Hamza *et al*., 2012). Primers and annealing temperatures for amplification of these housekeeping genes are given in Supplementary Table 2.3.

PCR reactions were done in a total volume of 25 μL consisting of 2 μL DNA template (25 ng μL^{-1}), 2.5 μL 10 x reaction buffer (Roche Applied Science) with 2 mM $MgCl_2$, 0.5 μL dNTPs (10 mM), 1 μL of each 10 mM primer, 0.2 µL FastStart Taq DNA polymerase (5 U μL^{-1}; Roche Applied Science) and 17.8 μL milli-Q water. PCR amplifications were performed in a C1000 thermal cycler (Bio-Rad) using the following program: 5 min at 95°C; 35 cycles of 0.5 min at 95°C; 1 min at 72°C; and a final extension for 10 min at 72°C. Products were visualized with the QIAxcel system and purified with NucleoSpin Gel and PCR clean-up (Macherey-Nägel). DNA concentration and quality were assessed in a ND-1000 spectrophotometer. Purified PCR products were sequenced directly and in both directions by Beckman Coulter Genomics (UK) using the same primers as for the PCR amplification.

Sequences were assembled with BIONUMERICS v. 7.0 software (Applied Maths), trimmed and deposited in Genbank with the accession numbers provided in Supplementary Table 2.4. Sequences were concatenated and aligned using CLUSTALW (Thompson *et al*., 1994) following the alphabetic order of the genes, ending in a sequence of 4815 bp (1–747 for *atpD*, 748–1683 for *dnaK*, 1684–2070 for *efp*, 2071–2955 for *glnA*, 2956–3483 for *gyrB*, 3484–3942 for *lrp*, 3943–4815 for *rpoD*). A similarity matrix of all sequences was calculated using the same software Wiley Online Library (Constantin *et al*., 2016). Evolutionary distances were computed using the maximum likelihood method with the Tamura-Nei model (Tamura & Nei, 1993). Phylogenetic trees for each independent gene alignment (only the *gyrB* tree is provided, Supplementary Figure 2.1) as well as for the concatenated sequence alignment (Figure 2.1) were generated, all using MEGA v.6 software (Tamura *et al*., 2013). Distance estimation was calculated using the *p*-distance substitution model with 1000 bootstrapping replications. The

nucleotide sequences of the seven genes were also translated into their corresponding amino acid sequences using the same software. The lengths of the peptide sequences for *atpD, dnaK, efp, glnA, gyrB, lrp* and *rpoD* were 249, 312, 129, 295, 176, 153 and 291 amino acids, respectively, resulting in a concatenated peptide length of 1605 amino acids. Individual and concatenated-peptide sequence trees were drawn in MEGA v.6 using the Tamura-Nei model and by estimating the gamma distribution with invariant sites parameters. *Xanthomonas vasicola* and *Xanthomonas oryzae* were used as the out-groups.

2.2.4 DNA-DNA hybridization

DNA was extracted at large scale using the method described by (Wilson, 1987) with minor modifications (Cleenwerck *et al*., 2002). DDH was performed under stringent conditions in a solution containing 50% of formamide (v/v) at 51°C, which corresponds to the T_{OR}+ 6°C, using a modified version of the microplate method described by Ezaki *et al*. (1989). The T_{OR} was determined using the following equation by Goris *et al*. (1998): 0.51×average %GC content + 47°C–36°C (correction for 50% formamide), with 66 mol% taken as the average %GC content of *X. axonopodis* (Vauterin *et al*., 1995). For every DNA pair, reciprocal reactions (e.g. A×B and B×A) were carried out. Per DNA pair, the presented DNA-DNA relatedness value (Supplementary Table 2.5) is the average of the mean value of A×B and that of B×A, while the value between parentheses is the difference between the mean value of A×B and that of B×A.

2.2.5 Whole-genome DNA sequencing, assembly and taxonomic analysis

Custom DNA library preparation and sequencing using multiplex Illumina TruSeq v. 3.0 technology was performed at Baseclear N.V., Leiden, The Netherlands. A paired-end (PE) DNA library with 2 x 50 bp reads was constructed for each strain to generate assemblies with ~ 30x coverage. Sequencing was performed on a Hiseq2500 instrument (Illumina Inc.). The FASTQ sequence reads were generated using the Illumina CASAVA pipeline v. 1.8.3. Initial quality assessment was based on data passing the Illumina Chastity filtering.

Subsequently, samples were demultiplexed and reads, containing adapters and/or PhiX control signal, were removed using an in-house filtering protocol from BASECLEAR. FASTQ files were delivered by FTP. First, the PE data set was quality trimmed at Q20 in a CLC BIOGENOMICS Genomics WORKBENCH v7.0 using a quality score of 0.05 and a maximum of two ambiguous nucleotides per read. Then, *de novo* assembly was performed with the trimmed PE dataset using automatic word and bubble size parameters, a minimum contig length of 200bp and autodetection of paired distances without scaffolding. Other genome quality statistics are shown in (Supplementary Table 2.6). The resulting contigs were exported in FASTA format and used for ANI calculations in JSpecies v1.2.1. (Richter &

Rosselló-Mora, 2009) under BIO-LINUX v. 7.0 (Field *et al*., 2006) with default settings for BLAST-based analysis (ANIb). Whole genome sequences downloaded from GenBank were included (Table 2.1). The similarity matrix was exported and used to construct a complete linkage tree in BIONUMERICS v. 7.5 (Figure 2.2). A scatter plot was constructed depicting the correlation between ANI values and MLSA similarities based on the concatenated gene sequences (Figure 2.3). The draft assemblies have been deposited in the GenBank WGS database with the accession numbers: JPYD00000000 (LMG 9322), JPYF00000000 (LMG 826), JPYC00000000 (LMG 27970), JPYG00000000 (LMG 495), JPYE00000000 (LMG 982), JPYB00000000 (LMG 695), JPYH00000000 (LMG 7399), JPUN00000000 (LMG 12749) and JPUO00000000 (LMG 9055).

2.2.6 Gas chromatographic analysis of FAMEs

Whole cell fatty acid methyl ester (FAME) compositions were determined using a 6890N gas chromatograph (Agilent Technologies). Cultivation of the strains, harvesting of the cells, fatty acid extraction and analysis of the FAME were performed according to the recommendations of the Sherlock Microbial Identification System (MIDI, Newark, Delaware, USA). Fatty acids were extracted from cultures grown on trypticase soy agar (BBL) for 24 h at 28° C under aerobic conditions. The peaks of the profiles were identified using the TSBA50 peak naming table and identification library v. 5.0. The profiles were also compared with profiles of selected type strains of *Xanthomonas* species generated from cells of the same age using the same method and present in the FAME-database of BCCM/LMG Supplementary Table 2.7).

2.2.7 Phenotypic analysis with the Biolog GEN III MicroPlate system

Xanthomonas strains for Biolog GEN III MicroPlate (Biolog Inc.) assays were grown for 24 h under aerobic conditions on BUG medium without blood at 33°C.

Inocula were prepared by picking up a 3 mm diameter area of cell growth from the surface of the agar plate using a sterile cotton stick, and by rubbing the stick against the bottom of a tube filled with inoculating fluid A. Each well of a Biolog GEN III microplate was filled with 100 µl of this fluid, and the plate was covered and incubated for 24 - 144 h at the same temperature as used to obtain the culture for inoculation. The microplates were read with a Biolog MicroStation microplate reader and analyzed using the Biolog Microbial Identification software, OmniLog Data Collection.

However, for some species and pathovars, such as *X. euvesicatoria*, [*X. campestris*] pv. *syngonii*, *X. axonopodis* pv. *axonopodis*, *X. fuscans* pv. *aurantifolii, X. campestris* pv. *campestris*, *X. bromi* and *X. oryzae* the color reactions were not sufficiently developed for accurate reading when they were grown

at 33°C. For these strains better results were obtained when the strains were grown at 28°C and the plates incubated at a lower temperature (28°C) up to 144 hours.

2.3 Results

2.3.1 Multilocus Sequence Analysis (MLSA)

MLSA of seven housekeeping genes (*atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, *lrp*, and *rpoD*) was performed for 109 *Xanthomonas* strains comprising 25 Xad strains and 84 strains of phylogenetically related pathovars. All individual gene trees revealed four large phylogenetic groups (PG I to IV) with the Xad strains distributed in three of these groups (PG I to III). The Xad strains in PG III, whether based on single genes (only the *gyrB* tree is shown in Supplementary Figure 2.1) or a concatenation of all seven genes (Figure 2.1), formed a homogeneous group clearly separated from all other known pathovars in this group. In contrast, the Xad strains in PG I and II were only differentiated from other closely related pathovars in the same group by the concatenated nucleotide sequences and by partial sequences of four genes (*atpD*, *dnaK*, *lrp*, and *rpoD)*, but not on sequences of *efp*, *glnA* and *gyrB* (Supplementary Figure 2.1). Most branch nodes were well supported by high bootstrap values, and quantitative support for the four PG's is also shown in the similarity matrix given in Wiley Online Library (Constantin *et al*., 2016). The analysis of peptide sequences was similar to that of the nucleotides (data not shown).

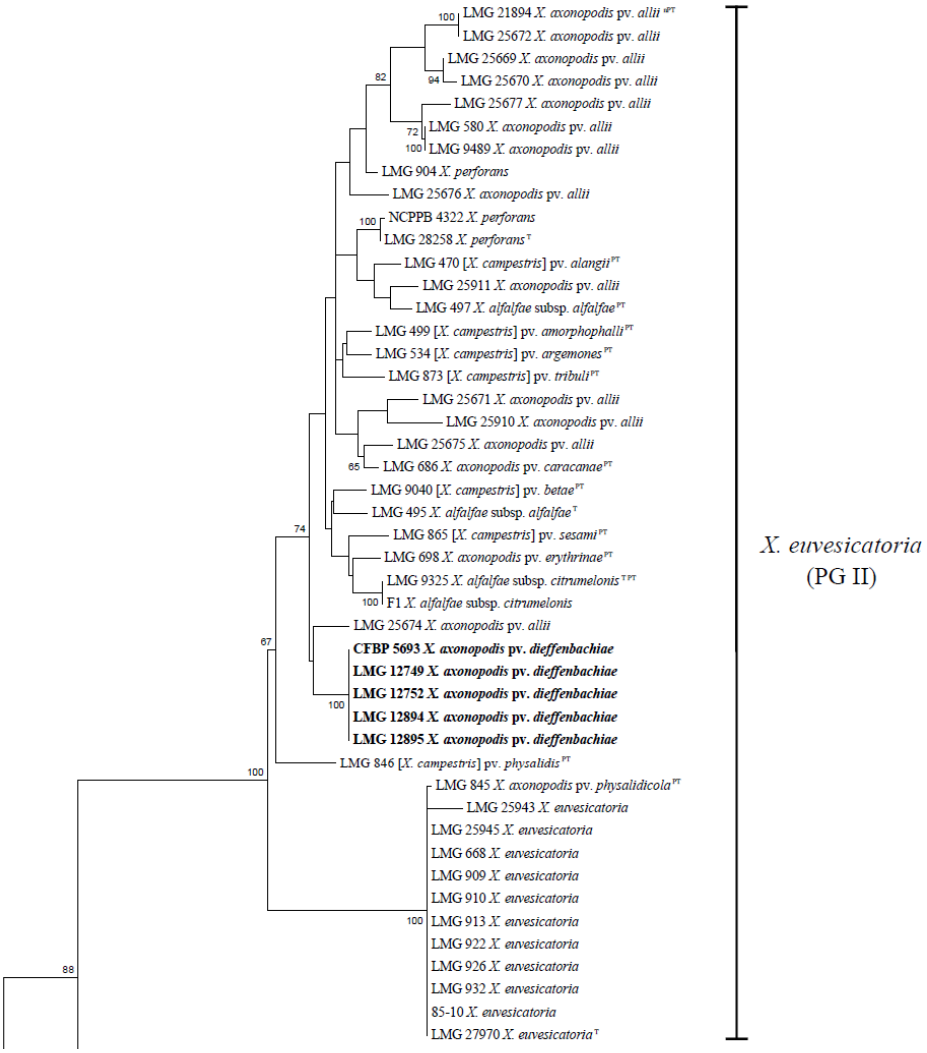
PG I comprises 35 strains including the Xad strains isolated from *Dieffenbachia.* This group is further divided in two subgroups, subgroup 1 (11 strains) and subgroup 2 (24 strains). Subgroup 1 included the type strains of *X. citri* subsp. *citri* and *X. citri* subsp. *malavacearum* and the pathotype strains of the *X. axonopodis* pathovars: *citri*, *malvacearum*, *glycines*, *punicae* and *mangiferaeindicae*. Subgroup 2 contains the Xad strains isolated from *Dieffenbachia* and the type strains of *X. fuscans* subsp. *fuscans* and *X. fuscans* subsp. *aurantifolii* as well as the pathotype strains of *X. axonopodis* pathovars: *anacardii*, *rhynchosiae*, *sesbaniae*, *vignaeradiatae*, *vignicola* together with [*X. campestris*] pv. *aracearum* and [*X. campestris*] pv. *thirumalacharii*.

PG II comprises 46 strains including the Xad strains isolated from *Philodendron* and the type strains of *X. euvesicatoria, X. perforans*, *X. alfalfae* subsp. *alfalfae* and *X. alfalfae* subsp. *citrumelonis*, as well as the pathotype strains of *X. axonopodis* pv. *alfalfae*, *X. axonopodis* pv. *allii*, *X. axonopodis* pv. *coracanae*, *X. axonopodis* pv. *erythrinae*, *X. axonopodis* pv. *physalidicola*, [*X. campestris*] pv. *alangii*, [*X. campestris*] pv. *amorphophalli*, [*X. campestris*] pv. *argemones*, [*X. campestris*] pv. *betae*, [*X. campestris*] pv. *physalidis*, [*X. campestris*] pv. *sesami* and [*X. campestris*] pv. *tribuli*.

PG III comprises 23 strains including the pathotype strain of Xad amongst other Xad strains isolated from *Anthurium* as well as the type strain of *X. phaseoli* and the pathotype strains of *X. axonopodis* pv. *manihotis*, *X. axonopodis* pv. *phaseoli* and [*X. campestris*] pv. *syngonii*.

PG IV comprises the type strain of *X. axonopodis* and two strains from the sugar-cane pathogen *X. axonopodis* pv. *vasculorum* including the pathotype strain.

Based on concatenated gene sequences, the similarity values within each PG were higher than 98.31% (PG I: ≥ 98.31%, PG II: ≥ 98.50%, PG III: ≥ 98.31% and PG IV: ≥ 98.93%), and the similarity values found between these groups ranged from 96.60 to 97.77% (Wiley Online Library, Constantin *et al*., 2016).



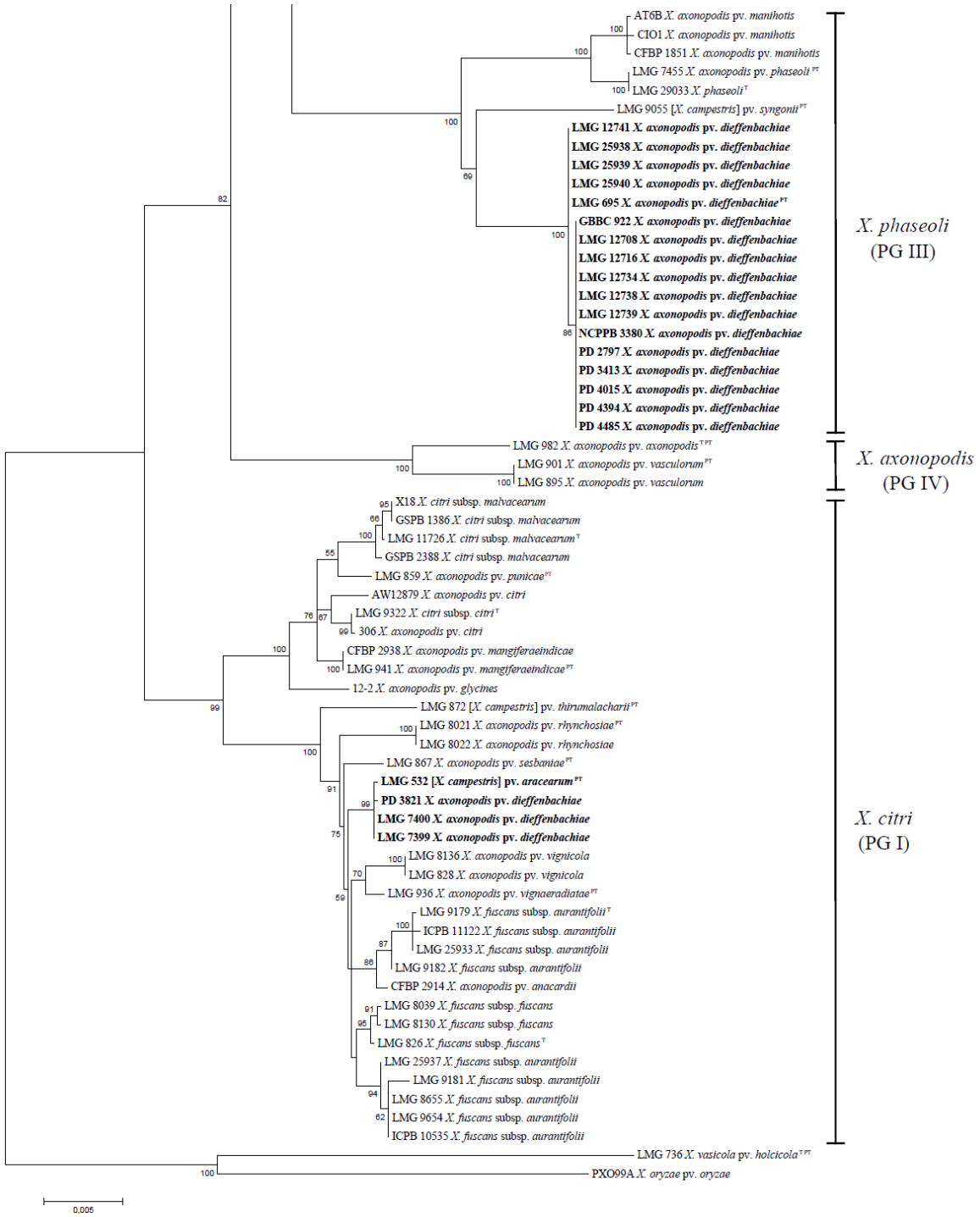


Figure 2.1. Maximum-likelihood phylogenetic tree of concatenated nucleotide sequences of partial *atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, *lrp* and *rpoD* genes. In bold are all strains pathogenic to aroids. Bootstrap values greater than 50% are shown for 1000 replicates. Horizontal scale bar (0.005) at the bottom represents number of nucleotide substitutions per site. Concatenated sequences of *X. vasicola* LMG 736^T and *X. oryzae* PXO99A were used as outgroups. T = type strain; PT = pathotype strain; nPT = neopathotype.

2.3.2 DNA-DNA hybridization

DNA-DNA hybridisations were made with a selection of strains from the four phylogenetic groups (PG I to IV) differentiated among *X. axonopodis* strains as indicated in Supplementary Table 2.5. Within PG I to PG III, DNA-DNA relatedness values of more than 70% were found, whereas relatedness values ranging from

57 to 62% were found with the type strain of *X. axonopodis* LMG 982^{T} (PG IV) (Supplementary Table 2.5). These results suggested that strains from PG I, PG II and PG III should not be classified as *X. axonopodis* as the values are clearly below 70% (Wayne *et al*., 1987). Among G I, PG II and PG III values up to 69% were obtained, complicating the decision if they warranted classification as distinct species.

2.3.3 Whole genome sequence analysis

Nine genomes representing three strains of Xad and six strains of phylogenetically related pathovars were sequenced. These genomes were compared with available whole genome sequences of 17 other *Xanthomonas* strains, and ANI values were calculated. The similarity matrix of ANI values is shown in Table 2.1. The ANI data confirmed that strains of PG I, PG II and PG III should not be classified as *X. axonopodis* and their ANI values with PG IV were clearly below the 95% value for species delineation cut-off (Goris *et al*., 2007; Richter & Rosselló-Mora, 2009). Within PG I, PG II and PG III ANI values above of more than 95.45% were found, with >98.37% for PG I subgroup1, >98.03% for PG I subgroup 2, >98.27% for PG II and >97.42% for PG III. The lowest ANI values (95.45-96.19%) were found within PG I between the subgroups 1 and 2. Further, the ANI values between PG I, PG II and PG III were not exceeding 94.14%, suggesting distinct species. A complete linkage tree based on the whole genome sequences was constructed and confirmed the topology found by MLSA (Figure 2.2). The ANI data matched the MLSA data as shown in Figure 2.3. In general, strains sharing an ANI value above 95% also showed at least ≥98.32% sequence similarity in MLSA.

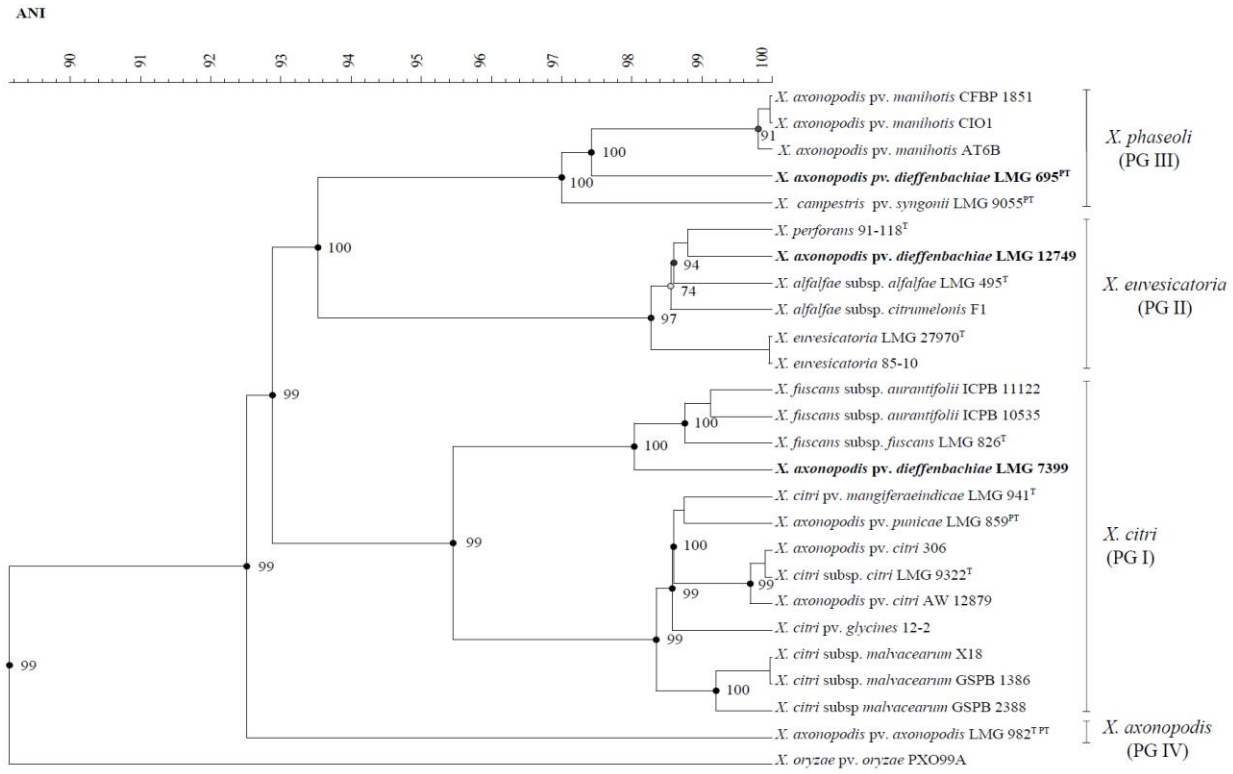


Figure 2.2. Gene content similarities among the genomes of *Xanthomonas* strains using complete linkage cluster analysis. *Xanthomonas oryzae* PXO99A was used as out-group. *Xanthomonas axonopodis* pv. *dieffenbachiae* strains are in bold. ANI = average nucleotide identity; PG = phylogenetic group; T = type strain; PT = pathotype strain. .

Table 2.1. Average nucleotide identity (ANI) pairwise comparison values among (draft) whole genome sequences of selected *Xanthomonas axonopodis* (*sensu* Vauterin *et al*., 1995) strains.

			1	2	3	4	5	6	7	8	9	10	11	12	13
X. euvesicatoria (PG II)	1	*X. perforans* 91-118^{T}	---												
	2[a]	*X. alfalfae* subsp. *alfalfae* LMG 495^{T}	98.66	---											
	3	*X. alfalfae* subsp. *citrumelonis* F1	98.72	98.55	---										
	4[a]	*X. axonopodis* pv. *dieffenbachiae* LMG 12749	98.79	98.6	98.55	---									
	5	*X. euvesicatoria* 85-10	98.55	98.44	98.47	98.41	---								
	6[a]	*X. euvesicatoria* LMG 27970^{T}	98.52	98.27	98.37	98.28	99.95	---							
X. phaseoli (PG III)	7	*X. axonopodis* pv. *manihotis* AT6B	93.72	93.63	93.58	93.66	93.67	93.52	---						
	8	*X. axonopodis* pv. *manihotis* CIO1	93.7	93.67	93.6	93.65	93.73	93.56	99.86	---					
	9	*X. axonopodis* pv. *manihotis* CFBP 1851	93.72	93.81	93.79	93.77	93.76	93.59	99.87	99.86	---				
	10[a]	*X. campestris* pv. *syngonii* LMG 9055^{PT}	93.85	93.82	93.74	93.73	93.77	93.85	97.03	97.09	97.06	---			
	11[a]	*X. axonopodis* pv. *dieffenbachiae* LMG 695^{PT}	93.73	93.72	93.68	93.67	93.59	93.62	97.42	97.64	97.7	97.21	---		
X. axonopodis (PG IV)	12[a]	*X. axonopodis* pv. *axonopodis* LMG 982$^{T, PT}$	92.62	92.83	92.78	92.76	92.66	92.63	92.89	92.89	92.91	92.91	93.02	---	
X. citri (PG I)	13	*X. citri* subsp. *malvacearum* X 18	94.09	94.11	94.06	94.14	94.08	94.0	93.19	93.15	93.18	93.27	93.2	92.66	---
	14	*X. citri* subsp. *malvacearum* GSPB 1386	94.07	94.01	93.91	94.02	94.13	94.02	93.14	93.14	93.15	93.0	93.16	92.62	99.97
subgroup 1	15	*X. citri* subsp. *malvacearum* GSPB 2388	93.72	93.68	93.62	93.71	93.78	93.67	93.12	93.1	93.12	92.88	93.06	92.58	99.24
	16	*X. citri* pv. *punicae* LMG 859^{PT}	93.87	93.81	93.82	93.82	93.83	93.75	93.18	93.15	93.13	92.98	93.14	92.59	98.55
	17	*X. axonopodis* pv. *citri* AW 12879	93.84	93.77	93.74	93.82	93.72	93.75	93.18	93.16	93.16	92.97	92.98	92.66	98.5
	18[a]	*X. citri* subsp. *citri* LMG 9322^{T}	93.84	93.86	93.84	93.87	93.72	93.71	93.11	93.1	93.13	92.96	93.15	92.65	98.5
	19	*X. axonopodis* pv. *citri* 306	93.85	93.87	93.86	93.89	93.72	93.7	93.1	93.06	93.13	92.99	93.11	92.66	98.5
	20	*X. citri* pv. *mangiferaeindicae* LMG 941^{PT}	93.85	93.89	93.87	93.86	93.77	93.81	93.16	93.16	93.17	93.08	93.22	92.59	98.46
	21	*X. citri* pv. *glycines* 12-2	93.88	93.83	93.92	93.84	93.8	93.75	93.15	93.13	93.22	92.98	93.18	92.64	98.48
	22[a]	*X. axonopodis* pv. *dieffenbachiae* LMG 7399	93.67	93.59	93.59	93.71	93.41	93.46	93.72	93.86	93.93	93.36	93.95	92.73	95.63
subgroup 2	23	*X. fuscans* subsp. *aurantifolii* ICPB 11122	93.69	93.67	93.67	93.7	93.66	93.7	93.18	93.19	93.21	93.06	93.16	92.64	95.86
	24[a]	*X. fuscans* subsp. *fuscans* LMG 826^{T}	93.7	93.67	93.63	93.65	93.6	93.56	93.2	93.29	93.34	92.97	93.11	92.71	96.04
	25	*X. fuscans* subsp. *aurantifolii* ICPB 10535	93.76	93.62	93.52	93.64	93.56	93.61	93.26	93.25	93.25	93.08	93.13	92.51	95.79
	26	*X. oryzae* pv. *oryzae* PXO99A	89.55	89.6	89.52	89.84	89.4	89.53	89.14	89.29	89.26	89.13	89.31	89.18	89.52

			14	15	16	17	18	19	20	21	22	23	24	25	26
X. euvesicatoria (PG II)	1	*X.perforans* 91-118^{T}													
	2[a]	*X. alfalfae* subsp. *alfalfae* LMG 495^{T}													
	3	*X. alfalfae* subsp. *citrumelonis* F1													
	4[a]	*X. axonopodis* pv. *dieffenbachiae* LMG 12749													
	5	*X. euvesicatoria* 85-10													
	6[a]	*X. euvesicatoria* LMG 27970^{T}													
X. phaseoli (PG III)	7	*X. axonopodis* pv. *manihotis* AT6B													
	8	*X. axonopodis* pv. *manihotis* CIO1													
	9	*X. axonopodis* pv. *manihotis* CFBP 1851													
	10[a]	*X. campestris* pv. *syngonii* LMG 9055^{PT}													
	11[a]	*X. axonopodis* pv. *dieffenbachiae* LMG 695^{PT}													
X. axonopodis (PG IV)	12[a]	*X. axonopodis* pv. *axonopodis* LMG 982$^{T, PT}$													
X. citri (PG I)	13	*X. citri* subsp. *malvacearum* X 18													
	14	*X. citri* subsp. *malvacearum* GSPB 1386	---												
subgroup 1	15	X. *citri* subsp. *malvacearum* GSPB 2388	99.2	---											
	16	*X. citri* pv. *punicae* LMG 859^{PT}	98.51	98.64	---										
	17	X. *axonopodis* pv. *citri* AW 12879	98.45	98.45	98.65	---									
	18[a]	X. *citri* subsp. *citri* LMG 9322^{T}	98.34	98.42	98.59	99.68	---								
	19	*X. axonopodis* pv. *citri* 306	98.4	98.49	98.68	99.71	99.9	---							
	20	*X. citri* pv. *mangiferaeindicae* LMG 941^{PT}	98.43	98.56	98.74	98.7	98.66	98.76	---						
	21	*X. citri* pv. *glycines* 12-2	98.37	98.47	98.57	98.66	98.6	98.58	98.66	---					
	22[a]	*X. axonopodis* pv. *dieffenbachiae* LMG 7399	95.6	95.49	95.71	95.45	95.54	95.61	95.56	95.58	---				
subgroup 2	23	*X. fuscans* subsp. *aurantifolii* ICPB 11122	95.81	95.94	95.91	96.0	95.92	95.99	96.11	96.06	98.03	---			
	24[a]	*X. fuscans* subsp. *fuscans* LMG 826^{T}	96.0	96.07	96.14	96.08	96.05	96.15	96.11	96.19	98.11	98.77	---		
	25	*X. fuscans* subsp. *aurantifolii* ICPB 10535	95.68	95.93	95.8	96.0	95.96	96.02	96.11	95.88	98.12	99.12	98.75	---	
	26	*X. oryzae* pv. *oryzae* PXO99A	89.37	89.64	89.25	89.54	89.55	89.65	89.38	89.36	89.44	89.47	89.66	89.41	---

Strains with ANI values above 95% are considered to belong to the same species (in grey shading). T = type strain; PT = pathotype (pathovar reference) strain.

[a]Whole genome sequences that were determined in this study (others were already available in GenBank with accession numbers provided in Supplementary Table 2.4).

X. perforans 91-118^{T} (Potnis *et al*., 2011) is the same strain as XV938, NCPPB 4321, LMG 28258.

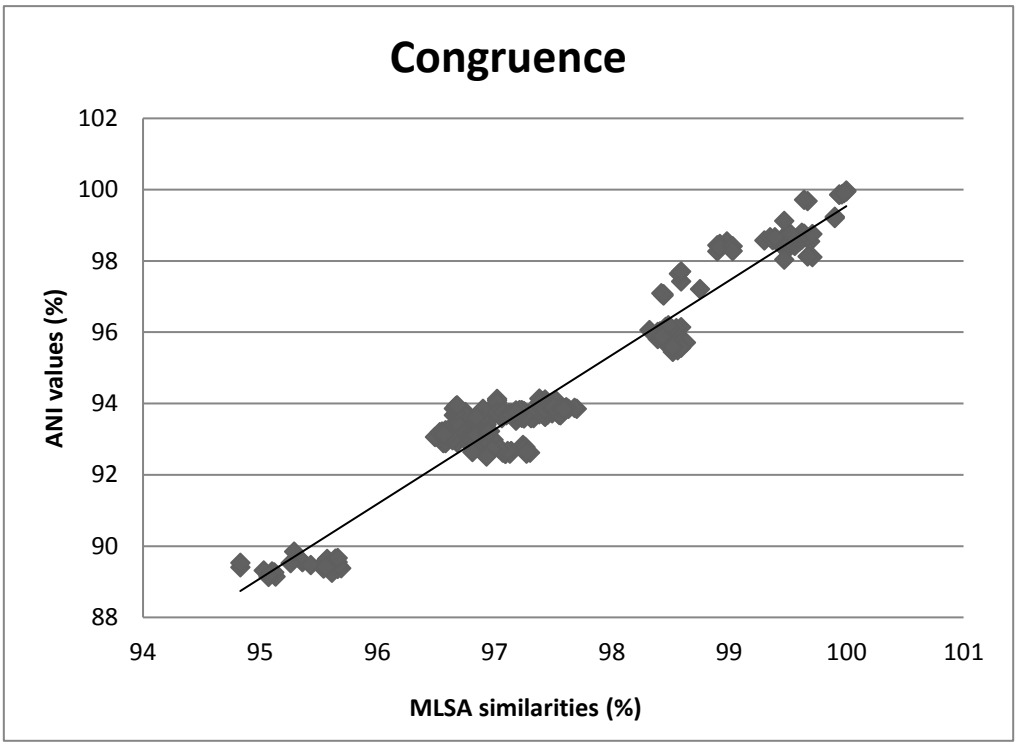


Figure 2.3. Scatter plot showing the congruence between average nucleotide identity (ANI) values and multilocus sequence analysis (MLSA) similarities based on concatenated partial sequences of seven genes (*atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, *lrp* and *rpoD*) from *Xanthomonas* strains. Each point represents the ANI similarity value from Table 2.1 with the corresponding MLSA similarity value among 26 strains.

2.3.4 Phenotypic analysis

The cellular fatty acid contents of 31 representative *X. axonopodis* strains and phylogenetically related strains are shown in Supplementary Table 2.7. All strains contained the three fatty acids characteristic for the genus *Xanthomonas* ($C_{11:0}$ iso, $C_{11:0}$ iso 3-OH, $C_{13:0}$ iso 3-OH) (Vauterin *et al*., 1995). Although the strains within the four phylogenetic groups (PG I to PG IV) had an overall similar fatty acids compositions, the amount of fatty acids, such as $C_{15:0}$ anteiso and $C_{17:0}$ iso, enabled the differentiation of PG IV (*X. axonopodis*) from the other three PGs.

The results of the Biolog GEN III metabolic fingerprinting tests for 23 representative strains are given in Supplementary Table 2.8. Strains from PG II were able to oxidize 31 substrates on average, followed by strains from PG III (mean number of 27 substrates). Strains from PG I and PG IV oxidized on average 20 and 6 substrates, respectively. For each phylogenetic group, a number of core substrates were identified that can be oxidized by all strains tested. Strains from PG I, PG II and PG III can oxidize core sets of 5, 19 and 13 substrates, respectively. Also, strains from PG IV were metabolically more versatile in the oxidation of substrates than strains from the other three groups, although they could only oxidase less than 20%. Based on GEN III data from this study, combinations of the phenotypic features

shown in Table 2.2 appear useful to differentiate the four groups PG I to PG IV from each other and from closely related *Xanthomonas* species.

Table 2.2. Distinguishing features for *X. citri* (PG I), *X. euvesicatoria* (PG II), *X. phaseoli* (PG III), *X. axonopodis* (PG IV) and related *Xanthomonas* species, based on Biolog GEN III data.

	Species[a]								
Substrate	1	2	3	4	5	6	7	8	9
D-Maltose	20 (/)	100	75 (+)	0	0	100	0	100	0
D-Melibiose	40 (-)	100	50 (-)	0	100	100	0	100	0
N-Acetyl-D-Glucosamine	100	100	100	66 (+)	100	100	100	0	100
α-D-Glucose	100	100	100	100	100	100	100	0	100
Glycerol	80 (+)	100	75 (+)	0	0	0	100	100	100
D-Glucose-6-PO4	40 (-)	50 (/)	75 (+)	33 (/)	0	100	100	100	0
D-Fructose-6-PO4	100	100	100	0	100	100	100	100	100
Gelatin	80 (+)	83 (+)	75 (+)	0	0	100	100	0	100
D-Saccharic acid	40 (-)	17 (/)	0	0	100	100	100	0	0
L-Lactic Acid	40 (-)	33 (/)	25 (/)	33 (+)	0	0	100	0	0
Antibiotic resistance assays									
Rifamycin SV	80 (+)	67 (+)	100	0	100	0	100	100	100
Nalidixic Acid	20 (-)	67 (+)	100	66 (+)	100	100	100	100	100
Aztreonam	40 (/)	67 (+)	100	33 (-)	100	100	100	100	100

Values are the percentage of intermediate and positive (i.e. / or +) strains. For species 1-4, the result of the type strain is given in parantheses. All data were generated in this study.
[a]Species: 1. *X. citri* (5 strains); 2. *X. euvesicatoria* (6 strains); 3. *X. phaseoli* (4 strains); 4. *X. axonopodis* (3 strains); 5. *X. campestris* LMG 568^T; 6. *X. melonis* LMG 8670^T; 7. *X. bromi* LMG 947^T; 8. *X. oryzae* LMG 5047^T; 9. *X. vasicola* LMG 736^T. The type strain of each species is included.

2.4 Discussion

For practical diagnosis, it is important that quarantine organisms can be unambiguously identified, preferably in a rapid manner. This study aimed to clarify the taxonomic position of strains of Xad, which are acknowledged to be heterogeneous, possibly representing different taxa (Rademaker *et al*., 2005; Donahoo *et al*., 2013). For this purpose, a well-chosen set of collection strains was examined by a polyphasic taxonomic approach that included MLSA, DDH, ANI calculation and biochemical analysis. The 16S rRNA gene was not investigated for the strains, as the taxonomic resolution of this gene is too low within the genus *Xanthomonas* (Hauben *et al*., 1997). Hence, additional techniques are required to resolve differentiation of *Xanthomonas* (sub) species (Moore *et al*., 1997).

DNA-DNA hybridization data are considered the 'gold standard' to delineate bacterial species (Wayne *et al*., 1987). However, DDHs have provided contradictory results within *Xanthomonas* (Vauterin *et al*., 1995; Schaad *et al*., 2005). The species *X. axonopodis* was emended, based mainly on DDH data, by

Vauterin *et al*. (1995) to include 34 pathovars previously classified in *X. campestris*. Strains within *X. axonopodis* shared 77 ± 15% DNA-DNA relatedness. Later, some of these pathovars were elevated to species rank such as *X. euvesicatoria*, *X. perforans*, *X. alfalfae*, *X. citri* and *X. fuscans* as they were claimed to show less than 48% DNA-DNA relatedness with the type strain of *X. axonopodis* (Jones *et al*., 2004; Schaad *et al*., 2005). Subsequently, Ah-You *et al*. (2009) proposed that *X. fuscans* should be considered a later heterotypic synonym of *X. citri* because of their close phylogenetic relationship and ΔT_m values below 5°C (Wayne *et al*., 1987). DDH data from this study revealed that strains of PG I, PG II and PG III did not belong to *X. axonopodis* but it was unclear if these groups formed distinct species. In the last decade, MLSA and ANI have been proposed as alternative methods for DDH experiments (Gevers *et al*., 2005; Goris *et al*., 2007; Young *et al*., 2008). The advantage of MLSA is that a large number of strains can be analyzed with a better portability than DDH data.

Multi-locus sequence analysis, based on several housekeeping gene sequences, has been used for species delineation within *Xanthomonas* (Young *et al*., 2008) and to obtain insight in *X. axonopodis sensu* Vauterin (1995) (Ah-You *et al*., 2009). To obtain reliable MLSA data, it is advised to use a minimum of five well-chosen housekeeping genes, universally present as single copies in the bacterial taxa studied (Stackebrandt *et al*., 2002). The MLSA data of the present study, based on seven loci, are congruent with those of previous studies using one or more of these loci (Ah-You *et al*., 2009; Parkinson *et al*., 2009; Almeida *et al*., 2010). The 109 strains included in this study, are found in four phylogenetic groups PG I to PG IV, which respectively correspond to the clades previously described by Parkinson *et al*. (2009) as *X. fuscans* clade, *X. euvesicatoria* species complex, *X. euvesicatoria* species complex sister clade and *X. axonopodis* clade. In addition these groups relate to the *X. axonopodis* subgroups previously identified by Rademaker *et al*. (2005), i.e. PG I to subgroups 9.5 and 9.6; PG II to subgroup 9.2, PG III to subgroup 9.4 and PG IV to subgroup 9.3.

The type strain of *X. axonopodis* belongs to PG IV. Strains of pathovar *dieffenbachiae* are distributed over three phylogenetic groups (PG I to III), with PG I containing strains mostly isolated from *Dieffenbachia*, PG II containing strains isolated from *Philodendron*, and PG III containing strains isolated from *Anthurium* except one that was isolated from *Dieffenbachia*. Similar results were reported by Hajri *et al*. (2009) who corroborated that *rpoD* sequence analysis grouped pathovar *dieffenbachiae* strains according to their host of isolation (*Anthurium*, *Dieffenbachia* and *Philodendron*). Also in the study of Rademaker *et al*. (2005), Xad strains isolated from *Anthurium* and those isolated from *Dieffenbachia* belonged to different subgroups (9.4 and 9.6, respectively).

Previous studies have suggested that the different genetic groups identified in *X. axonopodis* probably represent different species (Rademaker *et al*., 2005; Young *et al*., 2008). This is confirmed here by ANI values, DDH data and in a lesser extent by phenotypic features. More specifically for the Xad strains, the present data indicate that they belong to three different species. Based on this data, and in

compliance with the rules of the International Code of Nomenclature of Prokaryotes (Parker *et al*., 2015) and the International Standards for Naming Pathovars (Dye *et al*., 1980; Young *et al*., 1991), a number of taxonomic proposals are made and specified further in this paper.

In this study, the MLSA data was congruent with the ANI data; MLSA is, therefore, proven suitable for accurate identification of strains of *X*. *axonopodis sensu* Vauterin *et al*. (1995) at the species level. Based on the similarity matrix Wiley Online Library (Constantin *et al*., 2016), similarities above 98.3% suggest species level classification. Values between species were not exceeding 97.7%. Similar results for *X*. *axonopodis sensu* Vauterin *et al*. (1995) were reported by Young *et al*. (2008).

On the basis of this and other studies, it is clear that the formal bacterial classification and nomenclature based on the Bacterial Code (1990 Revision) is often conflicting with the pathovar infrasubspecific classification applied to the phytopathogenic diversity within *Xanthomonas* (Parker *et al*., 2015, Young *et al*., 1992). The use of pathovars is well established for the genus *Xanthomonas* and clearly indicates differences in pathogenic ability within a species. However, for some pathogenic *Xanthomonas* subspecies have been created instead of pathovars (*X. fuscans* subsp. *fuscans*, *X. fuscans* subsp. *aurantifolii*, *X. citri* subsp. *citri* and *X. citri* subsp. *malvacearum* in PG I), (*X. alfalfae* subsp. *alfalfae* and *X. alfalfae* subsp. *citrumelonis* in PG II) (Schaad *et al*., 2006). In the present autors' opinion, these subspecies should be lowered in rank to the pathovar level. Even though the two subgroups distinguished within PG I (*X. citri*) could merit the creation of subspecies, it seems unappropriate to create subspecies for groups of strains that are meant to be distinguished on the basis of their pathogenicity on a certain host. This approach was, for example, already implemented by Ah-You *et al*. (2009); those authors regarded *X. fuscans* as a later heterotypic synonym of *X. citri* and, for consistency within *Xanthomonas*, designated the two subspecies of *X. fuscans* as pathovars, rather than subspecies, of *X. citri*.

Here, a first taxonomic framework for the *X. axonopodis* species complex is proposed, removing subspecies and describing pathovars instead, allowing more extended studies that tackle the host-pathogen relation more in depth. The proposed classification system is a combination of a rational taxonomy and a convenient pathovar classification system for phytosanitary management. In conclusion, the combination of genotypic and phenotypic data of a considerable set of strains of *X. axonopodis sensu* Vauterin *et al*. (1995), allows for several taxonomic proposals: emendation of the descriptions of *X. citri*, *X. phaseoli* and *X. axonopodis*, to encompass the strains of PG I, PG III and PG IV, respectively; reclassification of *X. perforans*, *X. alfalfae* as *X. euvesicatoria* and emendation of the description of *X. euvesicatoria* to encompass all strains of PG II. In addition, it is proposed to lower the investigated subspecies in rank to the pathovar level and reclassify the investigated pathovars now included in *X. axonopodis* according the species classification proposed here. However, it is clear that

still more pathovars of *X. axonopodis* are misclassified at the species level and require further revision, such as *X. axonopodis* pv. *begoniae* (Rademaker subgroup 9.1).

In conclusion, strains now considered as Xad have been shown to belong to three phylogenetic groups representing separate species. Only Xad strains within PG III, which include the pathotype strain, can maintain the pathovar epithet 'dieffenbachiae' in a new combination as *X. phaseoli* pv. *dieffenbachiae* comb. nov. The Xad strains comprised within PG I and PG II should be named as novel pathovars respectively within *X. citri* and *X. euvesicatoria,* respectively, if this is supported by pathogenicity tests and characterization of pathogenicity genes in further studies. It is clear that a revision of the phytosanitary status of these xanthomonads pathogenic to Araceae is required. This new classification has consequences not only for these aroid pathogens on the EPPO A2 quarantine list but also for other EU regulated *Xanthomonas* plant pathogens (Council Directive 2000/29/EC).

Emended description of *Xanthomonas citri* (*ex* Hasse 1915) Gabriel *et al.* 1989 emend. Ah-You *et al*. 2009

The characteristics are as described for the genus (Vauterin *et al*., 2009) extended with data from this study. Using the Biolog GEN III MicroPlate system dextrin, N-acetyl-D-glucosamine, α-D-glucose, D-galactose, D-Fructose-6-PO4, α-keto-glutaric acid, pH 6 are oxidized, but the following substrates are not: D-turanose, stachyose, α-D-lactose, β-methyl-D-glucoside, N-acetyl-β-D-galactosamine, N-acetyl-neuraminic acid, D-fucose, L-rhamnose, D-mannitol, D-serine, p-hydroxy-phenylacetic acid, D-malic acid, γ-amino-butyric acid, β-hydroxy-D,L-butyric acid, fomnic acid, 4% NaCl, 8% NaCl, fusidic acid, D-serine, troleando-mycin, guanidine HCl, lithium chloride. The oxidization of the following substrates is strain-dependent: D-maltose (20% of the strains), D-melibiose (40% of the strains), glycerol (80% of the strains), D-glucose-6-PO4 (40% of the strains), gelatin (80% of the strains), D-saccharic acid (40% of the strains), L-lactic acid (40% of the strains), rifamicin SV (80% of the strains), nalidixic acid (20% of the strains) and aztreonam (40% of the strains). The fatty acids $C_{15:0}$ iso and summed feature 3 ($C_{16:1}$ ω7c / $C_{15:0}$ iso 2-OH) are present in significant amounts in cells grown on TSA (BBL 11768) for 24h under aerobic conditions. *X. citri* can be differentiated from the phylogenetic close *Xanthomonas* species by MLSA (Ah-You *et al*., 2009; this study).

The type strain is LMG 9322^T = ICPB 10518^T.

X. citri pv. *citri* (Hasse 1915) comb. nov.

= *X. citri* subsp. *citri* (Hasse 1915) Schaad *et al*. 2007.

Description of Schaad *et al*. (2006) extended with the description of the species (this study).

Pathotype strain: LMG 682; NCPPB 409.

The pathotype strain of *X. citri* pv. *citri* (LMG 682) and the type strain of *X. citri* subsp. *citri* (LMG 9322) are members of the same taxon (this study). Future pathogenicity studies should clarify if the type strain of *X. citri* subsp. *citri* can be classified in this pathovar.

X. citri pv. *malvacearum* (Smith 1901) comb. nov.
= *X. citri* subsp. *malvacearum* (Smith, 1901) Schaad *et al*. 2007.
Description of Schaad *et al.* (2006) extended with the description of the species (this study)
Pathotype strain: LMG 761; NCPPB 633.
X. citri pv. *malvacearum* has the same pathogenicity as *X. citri* subsp. *malvacearum* (Schaad *et al*., 2006)

X. citri pv. *glycines* (Nakano 1919) comb. nov.
= *X. axonopodis* pv. *glycines* (Nakano 1919) Vauterin *et al.* 1995.
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 712; NCPPB 554.

X. citri pv. *punicae* (Hingorani and Singh 1959) comb. nov.
= *X. axonopodis* pv. *punicae* (Hingorani and Singh 1959) Vauterin *et al*. 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 859; NCPPB 466.

X. citri pv. *mangiferaindicae* (Patel *et al*. 1948) comb. nov.
= *X. axonopodis* pv. *mangiferaeindicae* (Patel *et al*. 1948) Ah-You *et al*. 2007.
Description of Ah-You *et al.* (2007) extended with the description of the species (this study).
Pathotype strain: LMG 941; NCPPB 490.

X. citri pv. *fuscans* (Schaad *et al*. 2007) comb. nov.
= *X. fuscans* subsp. *fuscans* Schaad *et al.* 2007
Description of Schaad *et al.* (2006) extended with the description of the species (this study).
The pathotype strain is the type strain of *X. fuscans* subsp. *fuscans*: LMG 826; NCPPB 381.
The pathovar contains both the non-fuscous strains (*X. axonopodis* pv. *phaseoli* GL2 & GL3) and the fuscous strains (*X. fuscans* subsp. *fuscans*).

X. citri pv. *aurantifolii* (Schaad *et al*. 2007) comb. nov.
= *X. fuscans* subsp. *aurantifolii* Schaad *et al.* 2007

Description of Schaad *et al.* (2006) extended with the description of the species (this study).
The pathotype strain is the type strain of *X. fuscans* subsp. *aurantifolii*: LMG 9179; NCPPB 3236.

X. citri pv. *anacardii* (Ah-You *et al*. 2007) comb. nov.
= *X. axonopodis* pv. *anacardii* Ah-You *et al*. 2007
Description of Ah-You *et al.* (2007) extended with the description of the species (this study).
Pathotype strains: CFBP 2913; ICMP 4088.

X. citri pv. *rhynchosiae* (Sabet *et al*. 1969) comb. nov.
= *X. axonopodis* pv. *rhynchosiae* (Sabet *et al*. 1969) Vauterin *et al*. 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strains: LMG 8021; NCPPB 1827.

X. citri pv. *sesbaniae* (Patel *et al*. 1952a) comb. nov.
= *X. axonopodis* pv. *sesbaniae* (Patel *et al*. 1952a) Vauterin *et al*. 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 867; NCPPB 582.

X. citri pv. *vignaeradiatae* (Sabet *et al*. 1969) comb. nov.
= *X. axonopodis* pv. *vignaeradiatae* (Sabet *et al*. 1969) Vauterin *et al.* 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 936; NCPPB 2058.

X. citri pv. *vignicola* (Burkholder 1944) comb. nov.
= *X. axonopodis* pv. *vignicola* (Burkholder 1944) Vauterin *et al*. 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 8752; NCPPB 1838.

X. citri pv. *aracearum* (Berniac 1974) comb. nov.
= *X. campestris* pv. *aracearum* (Berniac 1974) Dye *et al*. 1978.
Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 532; NCPPB 2832.

X. citri pv. *thirumalacharii* (Padhya & Patel 1964) comb. nov.
= *X. campestris* pv. *thirumalacharii* (Padhya & Patel 1964) Dye *et al*. 1978.

Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 872; NCPPB 1452.

Emended description of *Xanthomonas euvesicatoria* (Jones *et al*. 2006)

The characteristics are as described for the genus (Vauterin *et al.*, 2009) extended with data from this study. Using the Biolog GEN III MicroPlate system dextrin, D-maltose, D-cellobiose, gentiobiose, sucrose, D-melibiose, N-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, glycerol, D-fructose-6-PO4, L-glutamic acid, citric acid, α-keto-glutaric acid, L-malic acid, acetic acid, pH 6, 1% NaCl, 1% sodium lactate, lincomycin are oxidized, but the following substrates are not: stachyose, N-acetyl-neuraminic acid, D-sorbitol, D-mannitol, D-serine, p-hydroxy-phenylacetic acid, γ-amino-butyric acid, β-hydroxy-D,L-butyric acid, 8% NaCl, potassium tellurite. The oxidization of the following substrates is strain-dependent: D-glucose-6-PO4 (50% of the strains), gelatin (80% of the strains), D-saccharic acid (20% of the strains), L-lactic acid (30% of the strains), rifamicin SV (65% of the strains), nalidixic acid (65% of the strains) and aztreonam (65% of the strains). The fatty acids $C_{15:0}$ iso and summed feature 3 ($C_{16:1}$ ω7c / $C_{15:0}$ iso 2-OH) are present in significant amounts in cells grown on TSA (BBL 11768) for 24h under aerobic conditions. *X. euvesicatoria* can be differentiated from the phylogenetic close *Xanthomonas* species by MLSA (Ah-You *et al*., 2009; this study).

The type strain is LMG 27970^T = NCPPB 2968^T.

X. euvesicatoria pv. *euvesicatoria* (Jones *el al*. 2006) comb. nov.
= *X. euvesicatoria* Jones *el al*. 2006
Description of Jones *et al.* (2004) extended with the description of the species (this study).
The pathotype strain is the type strain of *X. euvesicatoria*: LMG 27970; NCPPB 2968.

X. euvesicatoria pv. *perforans* (Jones *el al*. 2006) comb. nov.
= *X. perforans* Jones *el al*. 2006
Description of Jones *et al.* (2004) extended with the description of the species (this study).
The pathotype strain is the type strain of *X. perforans*: LMG 28258; NCPPB 4321.

X. euvesicatoria pv. *alfalfae* (*ex* Riker *et al*. 1935) comb. nov.
= *X. alfalfae* subsp. *alfalfae* (*ex* Riker *et al*. 1935) Schaad *et al*. 2007
Description of Schaad *et al.* (2006) extended with the description of the species (this study).
Pathotype strain: LMG 497; NCPPB 2062.

The pathotype strain of *X. axonopodis* pv. *alfalfae* (LMG 497) and the type strain of *X. alfalfae* subsp. *alfalfae* (LMG 495) are members of the same taxon (this study). Future pathogenicity studies should clarify if the type strain of *X. alfalfae* subsp. *alfalfae* can be classified in this pathovar.

X. euvesicatoria pv. *citrumelonis* (Schaad *et al*., 2007) comb. nov.
= *X. alfalfae* subsp. *citrumelonis* Schaad *et al*., 2007
Description of Schaad *et al.* (2006) extended with the description of the species (this study).
The pathotype strain is the type strain of *X. alfalfae* subsp. *citrumelonis*: LMG 9325; NCPPB 4376.

X. euvesicatoria pv. *allii* (Kadota *et al*. 2000) comb. nov.
= *X. axonopodis* pv. *allii* (Kadota *et al*. 2000) Roumagnac *et al*. 2004
Description of Roumagnac *et al.* (2004) extended with the description of the species (this study).
Pathotype strain: LMG 21894; NCPPB 4355.

X. euvesicatoria pv. *coracanae* (Desai *et al*. 1965) comb. nov.
= *X. axonopodis* pv. *coracanae* (Desai *et al*. 1965) Vauterin *et al*. 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 686; NCPPB 1786.

X. euvesicatoria pv. *erythrinae* (Patel *et al*. 1952b) comb. nov.
= *X. axonopodis* pv. *erythrinae* (Patel *et al*. 1952b) Vauterin *et al*. 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 698; NCPPB 578.

X. euvesicatoria pv. *physalidicola* (Goto & Okabe, 1958) comb. nov.
= *X. axonopodis* pv. *physalidicola* (Goto & Okabe, 1958) Vauterin *et al.* 1995
Description of Vauterin *et al.* (1995) extended with the description of the species (this study).
Pathotype strain: LMG 845, NCPPB 761.

X. euvesicatoria pv. *alangii* (Padhya & Patel 1962) comb. nov.
= *X. campestris* pv. *alangii* (Padhya & Patel 1962) Dye *et al*. 1978
Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 470; NCPPB 1336.

X. euvesicatoria pv. *amorphophalli* (Jindal *et al*. 1972) comb. nov.
= *X. campestris* pv. *amorphophalli* (Jindal *et al*. 1972) Dye *et al*. 1978
Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 499; NCPPB 2371.

X. euvesicatoria pv. *argemones* (Srinivasan *et al*. 1961) comb. nov.
= *X. campestris* pv. *argemones* (Srinivasan *et al*. 1961) Dye *et al*. 1978
Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 534; NCPPB 1593.

X. euvesicatoria pv. *betae* (Robbs *et al*. 1981) comb. nov.
= *X. campestris* pv. *betae* Robbs *et al*. 1981
Description of Robbs *et al*. (1981) extended with the description of the species (this study).
Pathotype strain: LMG 9040; NCPPB 2592.

X. euvesicatoria pv. *physalidis* (Srinivasan *et al*. 1962) comb. nov.
= *X. campestris* pv. *physalidis* (Srinivasan *et al*. 1962) Dye *et al*. 1978
Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 846; NCPPB 1756.

X. euvesicatoria pv. *sesami* (Sabet & Dowson, 1960) comb. nov.
= *X. campestris* pv. *sesami* (Sabet & Dowson, 1960) Dye *et al*. 1978
Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 865; NCPPB 631.

X. euvesicatoria pv. *tribuli* (Srinivasan & Patel, 1956) comb. nov.
= *X. campestris* pv. *tribuli* (Srinivasan & Patel, 1956) Dye *et al*. 1978
Description of Dye *et al.* (1978) extended with the description of the species (this study).
Pathotype strain: LMG 873; NCPPB 1454.

Emended description of *Xanthomonas axonopodis* Starr and Garcés 1950 emend. Vauterin *et al*. 1995

The characteristics are as described for the genus (Vauterin *et al.*, 2009) extended with data from this study. Using the Biolog GEN III MicroPlate system α-D-glucose, α-keto-glutaric acid (weakly), pH 6, 1%

NaCl are oxidized, but the following substrates are not*:* D-maltose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-β-D-mannosamine, N-acetyl-β-D-galactosamine, N-acetyl-neuraminic acid, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycerol, D-fructose-6-PO4, D-serine, gelatin, L-arginine, L-aspartic acid, L-histidine, L-pyroglutamic acid, L-serine, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, quinic acid, D-saccharic acid, p-hydroxy-phenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, D-malic acid, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, propionic acid, fomnic acid, pH 5, 4% NaCl, 8% NaCl, fusidic acid, D-serine, troleando-mycin, rifamycin SV, minocycline, guanidine HCl, niaproof 4, vancomycin, lithium chloride, potassium tellurite, sodium butyrate, sodium bromate. The oxidization of the following substrates is strain-dependent: N-acetyl-D-glucosamine (65% of the strains), D-glucose-6-PO4 (30% of the strains), L-lactic acid (30% of the strains), nalidixic acid (65% of the strains) and aztreonam (30% of the strains). The fatty acids $C_{15:0}$ iso, summed feature 3 ($C_{16:1}$ ω7c / $C_{15:0}$ iso 2-OH) and $C_{17:0}$ iso are present in significant amounts in cells grown on TSA (BBL 11768) for 24h under aerobic conditions. *X. axonopodis* can be differentiated from the phylogenetic close *Xanthomonas* species by MLSA (Ah-You *et al*., 2009; this study).

The type strain is LMG 982^T = NCPPB 457^T.

Emended description of *X. phaseoli* (ex Smith 1897) Gabriel *et al*. 1989

The characteristics are as described for the genus (Vauterin *et al.*, 2009) extended with data from this study. Using the Biolog GEN III MicroPlate system dextrin, D-trehalose, D-cellobiose, gentiobiose, sucrose, N-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fructose-6-PO4, L-glutamic acid, citric acid, acetoacetic acid, pH 6, lincomycin, tetrazolium blue, rifamicin SV, nalidixic acid, aztreonam are oxidized, but the following substrates are not: stachyose, D-raffinose, α-D-lactose, β-methyl-D-glucoside, N-acetyl-neuraminic acid, D-sorbitol, D-mannitol, L-histidine, L-pyroglutamic acid, D-gluconic acid, quinic acid, D-saccharic acid, γ-amino butyric acid, α-hydoxy-butyric acid, β-hydoxy-D,L-butyric acid, pH 5, 4% NaCl, 8% NaCl, minocycline. The oxidization of the following substrates is strain-dependent: D-maltose (75% of the strains), D-melobiose (50% of the strains), glycerol (75% of the strains), D-glucose-6-PO4 (75% of the strains), gelatin (75% of the strains), L-lactic acid (25% of the strains). The fatty acids $C_{15:0}$ iso and summed feature 3 ($C_{16:1}$ ω7c / $C_{15:0}$ iso 2-OH) are present in significant amounts in cells grown on TSA (BBL 11768) for 24h under aerobic conditions. *X. phaseoli* can be differentiated from the phylogenetic close *Xanthomonas* species by MLSA (Ah-You *et al*., 2009; this study).

The type strain is LMG 29033^T = ATCC 49119^T.

X. phaseoli pv. *dieffenbachiae* (McCulloch & Pirone 1939) comb. nov.

= *X. axonopodis* pv. *dieffenbachiae* (McCulloch & Pirone 1939) Vauterin *et al*. 1995

Description of Vauterin *et al.* (1995) extended with the description of the species (this study).

Pathotype strain: LMG 695; NCPPB 1833.

X. phaseoli pv. *manihotis* (Bondar 1915) comb. nov.

= *X. axonopodis* pv. *manihotis* (Bondar 1915) Vauterin *et al*. 1995

Description of Vauterin *et al.* (1995) extended with the description of the species (this study).

Pathotype strain: LMG 784; NCPPB 1834.

X. phaseoli pv. *phaseoli* (Smith 1897) comb. nov.

= *X. axonopodis* pv. *phaseoli* (Smith 1897) Vauterin *et al*. 1995

Description of Vauterin *et al.* (1995) extended with the description of the species (this study).

Pathotype strain: LMG 7455; NCPPB 3035.

The pathotype strain of *X. axonopodis* pv. *phaseoli* (LMG 7455) and the type strain of *X. phaseoli* (ATCC 49119) are members of the same taxon (this study). Future pathogenicity studies should clarify if the type strain of *X. phaseoli* can be classified in this pathovar.

This pathovar includes only the strains pathogenic to bean classified in *X. axonopodis* (subgroup 9.4 of Rademaker *et al*., 2005).

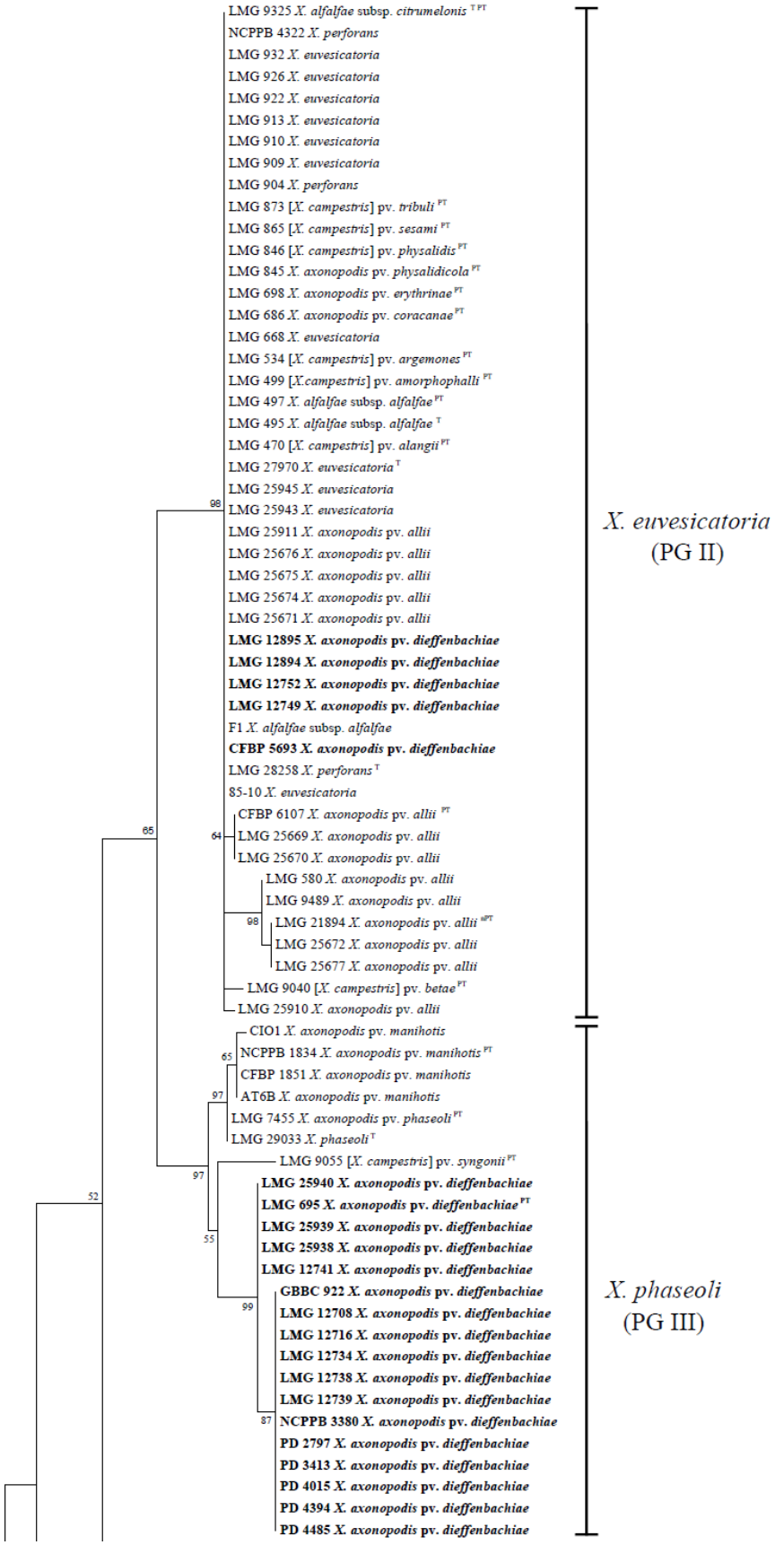
X. phaseoli pv. *syngonii* (Dickey & Zumoff, 1987) comb. nov.

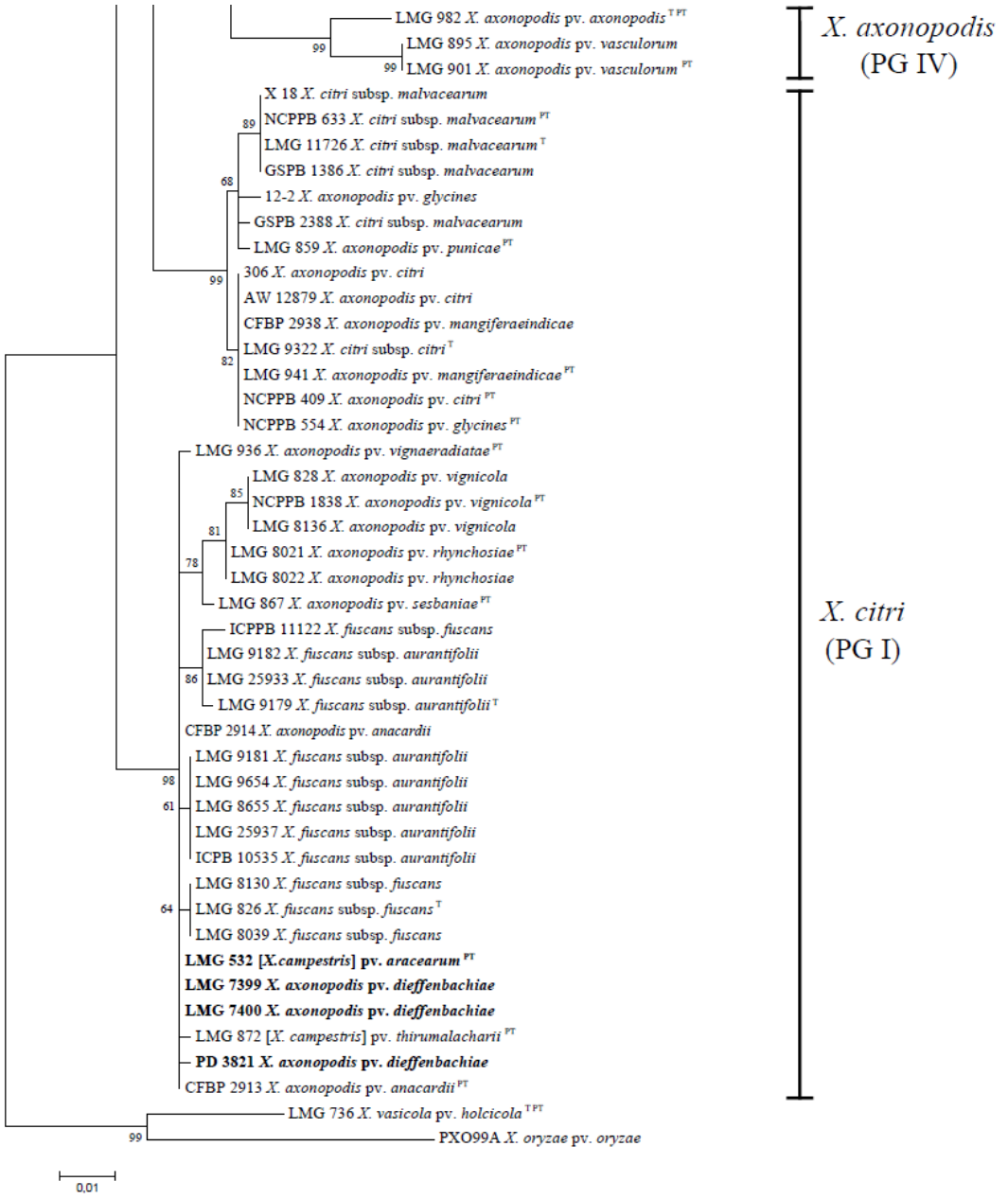
= *X. campestris* pv. *syngonii* Dickey & Zumoff, 1987

Description of Dickey & Zumoff, (1987) extended with the description of the species (this study).

Pathotype strain: LMG 9055; NCPPB 3586

Supplementary material





Supplementary Figure 2.1. Maximum-likelihood phylogenetic tree of partial *gyrB* nucleotide sequences. Xad strains are in bold. Bootstrap values greater than 50% are shown for 1000 replicates. Horizontal scale bar (0.01) at the bottom represents number of nucleotide substitutions per site. Partial *gyrB* sequences of *X. vasicola* LMG 736^{T} and *X. oryzae* PXO99A were used as outgroups. T = type strain. PT = pathotype strain. nPT = neopathotype.

Supplementary Table 2.1 Strains used in this study. Taxa are named according to the proposals made in this study with former names also provided in the table.

Proposed species classification	Strain no.	Former (sub)species classification (List of Prokaryotic Names with Standing in Nomenclature)[a]	Former name (below species level) according to Bull *et al*. (2010 & 2012)[b]	Host	Geographic origin	Year
X. citri (PG I, *RG 9.5* & *9.6*)[c]	**LMG 9322^{T}** [1 * †]	*X. citri*	*X. citri* subsp. *citri* [2]	*Citrus aurantifolia*	USA	1989
n = 35	306	*X. citri*	*X. citri* / *X. axonopodis* pv. *citri* [1, 2]	*Citrus* sp.	—	—
	AW 12879	*X. citri*	*X. citri* / *X. axonopodis* pv. *citri* [1, 2]	*Citrus aurantifolia*	USA	—
	GSPB 1386	*X. citri*	*X. citri* subsp. *malvacearum* [2]	*Gossypium herbaceum*	Nicaragua	1994
subgroup 1	GSPB 2388	*X. citri*	*X. citri* subsp. *malvacearum* [2]	*Gossypium herbaceum*	Nicaragua	1986
n = 11	**LMG 11726** [§1 †]	*X. citri*	*X. citri* subsp. *malvacearum* [2]	*Gossypium* sp.	USA	1944
	X 18	*X. citri*	*X. citri* subsp. *malvacearum* [2]	*Gossypium herbaceum*	Burkina Faso	—
	12-2	*X. citri*	*X. axonopodis* pv. *glycines* [1, 2]	*Glycine max*	—	—
	LMG 859^{PT} [1]	*X. axonopodis*	*X. axonopodis* pv. *punicae*	*Punica granatum*	India	1957
	CFBP 2938	*X. citri*	*X. axonopodis* pv. *mangiferaeindicae* [2]	*Schinus molle*	La Réunion	1986
	LMG 941^{PT} [1]	*X. citri*	*X. axonopodis* pv. *mangiferaeindicae* [2]	*Mangifera indica*	India	1957
	LMG 826[‡ 3 * †]	*X. citri*	*X. fuscans* subsp. *fuscans* [2]	*Phaseolus vulgaris*	Canada	1957
	LMG 8039	*X. citri*	*X. fuscans* subsp. *fuscans* [2]	*Phaseolus vulgaris*	Africa	1982
	LMG 8130	*X. citri*	*X. fuscans* subsp. *fuscans* [2]	*Phaseolus vulgaris*	Australia	1972
	ICPB 10535	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus* sp.	—	—
	ICPB 11122	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus* sp.	—	—
	LMG 8655	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus aurantifolia*	Brazil	1981
	LMG 9179[¶ 3 †]	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus limon*	Argentina	1989
subgroup 2	LMG 9181	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus aurantifolia*	Brazil	1989
n = 24	LMG 9182	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus aurantifolia*	Mexico	1989
	LMG 9654	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus aurantifolia*	Brazil	1989
	LMG 25933	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus limon*	Argentina	1981
	LMG 25937	*X. citri*	*X. fuscans* subsp. *aurantifolii* [2]	*Citrus aurantifolia*	Brazil	2000
	LMG 7399 [* †]	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Dieffenbachia sp.*	USA	1950
	LMG 7400	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Dieffenbachia sp.*	USA	1950
	PD 3821	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Aglaonema* cv. silver	USA	2000
	CFBP 2914	*X. citri*	*X. axonopodis* pv. *anacardii* [2]	*Mangifera indica*	Brazil	—

Proposed species classification	Strain no.	Former (sub)species classification (List of Prokaryotic Names with Standing in Nomenclature)[a]	Former name (below species level) according to Bull *et al*. (2010 & 2012)[b]	Host	Geographic origin	Year
	LMG 8021^{PT} [3]	*X. citri*	*X. axonopodis* pv. *rhynchosiae* [2]	*Rhynchosia memnonia*	Sudan	1965
	LMG 8022	*X. citri*	*X. axonopodis* pv. *rhynchosiae* [2]	*Rhynchosia memnonia*	Sudan	1966
	LMG 867^{PT} [3]	*X. citri*	*X. axonopodis* pv. *sesbaniae* [2]	*Sesbania sesban*	India	1958
	LMG 936^{PT} [3]	*X. citri*	*X. axonopodis* pv. *vignaeradiatae* [2]	*Vigna radiata*	Sudan	1966
	LMG 828	*X. citri*	*X. axonopodis* pv. *vignicola* [2, 3]	*Vigna unguiculata*	Sudan	1956
	LMG 8136	*X. citri*	*X. axonopodis* pv. *vignicola* [2, 3]	—	India	—
	LMG 532^{PT} [3]	[*X. campestris*]	[*X. campestris*] pv. *aracearum*	*Xanthosoma sagittifolium*	Guadeloupe	—
	LMG 872^{PT} [3]	[*X. campestris*]	[*X. campestris*] pv. *thirumalacharii*	*Triumfetta pilosa*	India	1961
	LMG 495^{T} [4 * †]	*X. alfalfae* subsp. *alfalfae*	*X. alfalfae* subsp. *alfalfae*	*Medicago sativa*	India	1954
	LMG 497^{PT} [4]	*X. axonopodis* pv. *alfalfae*	*X. alfalfae* subsp. *alfalfae*	*Medicago sativa*	India	1954
	F1 [5]	*X. alfalfae* subsp. *citrumelonis*	*X. alfalfae* subsp. *citrumelonis*	*Citrus* sp.	USA	1984
	LMG 9325$^{T, PT}$ [4 †]	*X. alfalfae* subsp. *citrumelonis*	*X. alfalfae* subsp. *citrumelonis*	*Citrus* sp.	USA	1989
	85-10 [6]	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum annuum*	—	—
	LMG 668	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum annuum* cv. VR2	Cook Islands	1978
	LMG 909	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum* sp.	Côte D'ivoire	1979
	LMG 910 †	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum* sp.	Morocco	1979
	LMG 913	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum* sp.	Senegal	1979
	LMG 922	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum frutescens*	USA	1939
	LMG 926	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum frutescens*	Hungary	1957
	LMG 932	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum frutescens*	Brazil	1971
	LMG 25943	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum annuum*	Brazil	1981
	LMG 25945	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum annuum*	Brazil	1983
X. euvesicatoria (PG II, *RG 9.2*)	**LMG 27970^{T}** [4 * †]	*X. euvesicatoria*	*X. euvesicatoria*	*Capsicum frutescens*	USA	1977
n = 46	**LMG 28258^{T}** [4,6 †]	*X. perforans*	*X. perforans*	*Lycopersicon esculentum*	USA	1991
	LMG 904	*X. perforans*	*X. perforans*	—	—	—
	NCPPB 4322	*X. perforans*	*X. perforans*	*Lycopersicon esculentum*	USA	1993
	LMG 580	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	USA	1976
	LMG 9489	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	USA	1989
	LMG 21894^{nPT} [7]	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	France	—

Proposed species classification	Strain no.	Former (sub)species classification (List of Prokaryotic Names with Standing in Nomenclature)[a]	Former name (below species level) according to Bull *et al*. (2010 & 2012)[b]	Host	Geographic origin	Year
	LMG 25669	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium fistulosum*	Japan	1998
	LMG 25670	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium sativum*	Cuba	1986
	LMG 25671	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	Barbados	—
	LMG 25672	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	Mauritius	1997
	LMG 25674	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	USA	1997
	LMG 25675	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	Brazil	1998
	LMG 25676	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	South Africa	—
	LMG 25677	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	Venezuela	2001
	LMG 25910	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	Brazil	1993
	LMG 25911	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium cepa*	Brazil	1998
	LMG 686^{PT} [4]	*X. axonopodis*	*X. axonopodis* pv. *coracanae*	*Eleusine coracana*	India	1965
	CFBP 5693	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Philodendron scandens*	USA	—
	LMG 12749 [†]	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Philodendron* sp.	USA	1992
	LMG 12752	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Philodendron* sp.	USA	1992
	LMG 12894	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Philodendron* sp.	USA	1992
	LMG 12895	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae* [2]	*Philodendron* sp.	USA	1992
	LMG 698^{PT} [4]	*X. axonopodis*	*X. axonopodis* pv. *erythrinae*	*Erythrina variegata*	India	1953
	LMG 845^{PT} [4]	*X. axonopodis*	*X. axonopodis* pv. *physalidicola*	*Physalis alkekengi*	Japan	1960
	LMG 470^{PT} [4]	[*X. campestris*]	[*X. campestris*] pv. *alangii*	*Alangium salviifolium*	India	1960
	LMG 499^{PT} [4]	[*X. campestris*]	[*X. campestris*] pv. *amorphophalli*	*Amorphophallus campanulatus*	India	1968
	LMG 534^{PT} [4]	[*X. campestris*]	[*X. campestris*] pv. *argemones*	*Argemone mexicana*	India	1961
	LMG 9040^{PT} [4]	[*X. campestris*]	[*X. campestris*] pv. *betae*	*Beta vulgaris* var. Hortensis	Brazil	1973
	LMG 846^{PT} [4]	[*X. campestris*]	[*X. campestris*] pv. *physalidis*	*Physalis minima*	India	1961
	LMG 865^{PT} [4]	[*X. campestris*]	[*X. campestris*] pv. *sesami*	*Sesamum indicum*	Sudan	1958
	LMG 873^{PT} [4]	[*X. campestris*]	[*X. campestris*] pv. *tribuli*	*Tribulus terrestris*	India	1956
	GBBC 922	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	Anthurium sp.	The Netherlands	2002
X. phaseoli (PG III, *RG 9.4*)	**LMG 695^{PT}** [8 * †]	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Brazil	1965
n = 22	LMG 12708	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Guadeloupe	1992

Proposed species classification	Strain no.	Former (sub)species classification (List of Prokaryotic Names with Standing in Nomenclature)[a]	Former name (below species level) according to Bull *et al*. (2010 & 2012)[b]	Host	Geographic origin	Year
	LMG 12716	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Guadeloupe	1992
	LMG 12734	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Martinique	1992
	LMG 12738	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Venezuela	1992
	LMG 12739	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Hawaii	1992
	LMG 12741	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Puerto Rico	1992
	LMG 25938	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Brazil	1994
	LMG 25939	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Dieffenbachia* hib., var. Camilia	Brazil	1995
	LMG 25940	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Brazil	1998
	NCPPB 3380	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	Jamaica	—
	PD 2797	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	The Netherlands	1995
	PD 3413	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium* sp.	The Netherlands	1998
	PD 4015	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium andreanum*	The Netherlands	2000
	PD 4394	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium andreanum*	The Netherlands	2002
	PD 4485	*X. axonopodis*	*X. axonopodis* pv. *dieffenbachiae*	*Anthurium 'Leni'*	The Netherlands	2002
	AT6B[9]	*X. axonopodis*	*X. axonopodis* pv. *manihotis*[8]	*Manihot esculenta*	—	—
	CFBP 1851[9]	*X. axonopodis*	*X. axonopodis* pv. *manihotis*[8]	*Manihot esculenta*	USA	—
	CIO1[9]	*X. axonopodis*	*X. axonopodis* pv. *manihotis*[8]	*Manihot esculenta*	—	—
	LMG 29033^{T}[10†]	*X. phaseoli*	*X. axonopodis* pv. *phaseoli*	*Phaseolus vulgaris*	USA	—
	LMG 7455^{PT}[8 †]	*X. axonopodis*	*X. axonopodis* pv. *phaseoli*	*Phaseolus vulgaris*	USA	1978
	LMG 9055^{PT}[8 * †]	[*X. campestris*]	[*X. campestris*] pv. *syngonii*	*Syngonium podophyllum*	USA	1984
X. axonopodis (PG IV, *RG 9.3*)	**LMG 982$^{T, PT}$**[11 * †]	*X. axonopodis*	*X. axonopodis* pv. *axonopodis*	*Axonopus scoparius*	Columbia	1949
n = 3	LMG 895 [†]	*X. axonopodis*	*X. axonopodis* pv. *vasculorum*	*Saccharum officinarum*	Australia	1946
	LMG 901^{PT}[11 †]	*X. axonopodis*	*X. axonopodis* pv. *vasculorum*	*Saccharum officinarum*	Mauritius	1979
	LMG 568$^{T, PT}$ †	*X. campestris*	*X. campestris* pv. *campestris*	*Brassica oleracea*	UK	1957
	LMG 8670$^{T, PT}$ †	*X. melonis*	*X. campestris* pv. *melonis*	*Cucumis melo*	Brazil	1974
	LMG 947^{T} †	*X. bromi*	*X. bromi*	*Bromus carinatus*	France	1980
	LMG 5047$^{T, PT}$ †	*X. oryzae*	*X. oryzae* pv. *oryzae*	*Oryza sativa*	India	1965
	PXO99A	*X. oryzae*	X. *oryzae* pv. *oryzae*	*Oryza sativa*	Los Banos	—

Proposed species classification	Strain no.	Former (sub)species classification (List of Prokaryotic Names with Standing in Nomenclature)[a]	Former name (below species level) according to Bull *et al*. (2010 & 2012)[b]	Host	Geographic origin	Year
	LMG 736$^{T, PT}$	*X. vasicola*	X. *vasicola* pv. *holcicola*	*Sorghum vulgare*	New Zealand	1969

[a] Conform to the rules of the International Code of Nomenclature of Prokaryotes; [b] Conform to the rules of the International Standards for Naming Pathovars of Plant Pathogenic Bacteria; [c] PG: phylogenetic groups determined in this study; *RG:* refers to the subgroups identified by Rademaker *et al*. (2005).
T = type strain; nPT = neopathotype strain; PT = pathotype (pathovar reference) strain; **§** LMG 11726 is the type strain of *X. citri* subsp. *malvacearum*; ‡ LMG 826 is the type strain of *X. fuscans* subsp. *fuscans*; ¶ LMG 9179 is the type strain of *X. fuscans* subsp. *aurantifolii* (all are in bold). LMG = BCCM/LMG Bacteria Collection, Ghent University, Belgium; CFBP = French Collection of Plant Associated Bacteria; NCPPB = National Collection of Plant Pathogenic Bacteria, York, UK; GBBC = ILVO Plant Crop Protection, Merelbeke, Belgium.
* strains investigated by DNA-DNA hybridization; † strains investigated by FAME and Biolog GEN III. All *X. campestris pathovars* not examined by Vauterin *et al.* (1995) and that are shown here not to belong to *X. campestris* are placed in parentheses [ ].
[1] classified as *X. citri* according to Parkinson *et al*. (2009); [2] classified as *X. citri* according to Ah-you *et al.* (2009), with the comment that for *X. axonopodis* pv. *dieffenbachiae*, only strains non-pathogenic to *Anthurium* were re-classified as *X. citri*; [3] classified as *X. fuscans* according to Parkinson *et al*. (2009); [4] classified in the '*X. euvesicatoria* species complex' according to Parkinson *et al*. (2009); [5] Schaad *et al*. (2006); [6] Potnis *et al*. (2011); [7] neopathotype (nPT) strain proposed by Roumagnac *et al*. (2004); [8] classified in the '*X. euvesicatoria* species complex sister clade' according to Parkinson *et al*. (2009); [9] see Bart *et al*. (2012); [10] classified as *X. phaseoli* according to Gabriel *et al*. (1989); [11] classified as *X. axonopodis* according to Parkinson *et al.* (2009)

Supplementary Table 2.2. *X. citri*, *X. euvesicatoria* and *X. phaseoli* strains for which *gyrB* sequences, available in GenBank, were used in Supplementary Figure 2.1.

Proposed species classification	**Strain no.**	**Former (sub)species classification**	**Former name (below species level) according to Bull *et al*. (2010 & 2012)**	**Host**	**Geographic origin**	**Year**	***gyrB* accession number**
X. citri (PG I)	**NCPPB 409**[PT 1]	*X. citri*	*X. axonopodis* pv. *citri*	*Citrus limon*	New Zealand	1956	EU285147
subgroup 1	**NCPPB 633**[PT 1]	*X. citri*	*X. axonopodis* pv. *malvacearum*	*Gossypium sp.*	Sudan	1958	EU285125
	NCPPB 554[PT 1]	*X. citri*	*X. axonopodis* pv. *glycines*	*Glycine max*	Sudan	1956	EU285151
subgroup 2	**CFBP 2913**[PT 2]	*X. citri*	*X. axonopodis* pv. *anacardii*	*Mangifera indica*	Brazil	—	EU015357
	NCPPB 1838[PT 3]	*X. citri*	*X. axonopodis* pv. *vignicola*	*Vigna sinensis*	USA	1942	EU285134
X. euvesicatoria (PG II)	**CFBP 6107**[PT 4]	*X. axonopodis*	*X. axonopodis* pv. *allii*	*Allium fistulosum*	Japan	1998	EU015310
X. phaseoli (PG III)	**NCPPB 1834**[PT 5]	*X. axonopodis*	*X. axonopodis* pv. *manihotis*	*Manihot esculenta*	Brazil	1965	EU285133

CFBP = French Collection of Plant Associated Bacteria; NCPPB = National Collection of Plant Pathogenic Bacteria, York, UK.
PT = pathotype (pathovar reference) strain.
[1] classified as *X. citri* according to Parkinson *et al*. (2009); [2] classified as *X. citri* according to Ah-you *et al.* (2009); [3] classified as *X. fuscans* according to Parkinson *et al*. (2009); [4] classified in '*X. axonopodis* subgroup 9.2 of Rademaker *et al*. (2005)', containing the type strain of *X. euvesicatoria*, according to Ah-you *et al*. (2009); [5] classified in the '*X. euvesicatoria* species complex sister clade' according to Parkinson *et al*. (2009).

Supplementary Table 2.3. Primers and annealing temperatures used in this study for PCR amplification and sequencing of seven housekeeping genes.

Locus	Function	Forward primer	Reverse primer	Annealing temperature (°C)	Fragment length (bp)	Use	Reference
atpD	ATP synthase beta chain	GGGCAAGATCGTTCAGAT	GCTCTTGGTCGAGGTGAT	64	747	PCR & sequencing	Bui Thi Ngoc *et al*., (2010)
dnaK	Heat shock protein 70	GGTGGAAGACCTGGTCAAGA	TCCTTGACYTCGGTGAACTC	54	940	PCR & sequencing	Young *et al*., (2008)
efp	Elongation factor P	TCATCACCGAGACCGAATA	TCCTGGTTGACGAACAGC	63	389	PCR & sequencing	Bui Thi Ngoc *et al*., (2010)
glnA	Glutamine synthetase	ATCAAGGACAACAAGGTCG	GCGGTGAAGGTCAGGTAG	60	887	PCR & sequencing	Hajri *et al*., (2012)
gyrB	Gyrase subunit beta	AAGCAGGGCAAGAGCGAGCTGTA	CAAGGTGCTGAAGATCTGGTC	50	530	PCR & sequencing	Parkinson *et al*., (2007)
lrp	Leucine responsive-regulatory protein	GCGACGGCTGGAGCGCGACG	GCGGTAGGACGCCATCTCGC	55	460	PCR & sequencing	Cubero *et al*., (2004)
rpoD	RNA polymerase sigma 70-factor	TGGAACAGGGCTATCTGACC	CATTCYAGGTTGGTCTGRTT	54	873	PCR & sequencing	Young *et al*., (2008)

Supplementary Table 2.4. Accession numbers of sequences for seven genes used in this study for MLSA.

Strain no.	*atpD*	*dnaK*	*efp*	*glnA*	*gyrB*	*lrp*	*rpoD*
12-2*	Genbank: NZ_AJJO00000000						
306*	Genbank: NC_003919						
85-10*	Genbank: NC_007508						
91-118*	Genbank: NZ_AEQW00000000						
AT6B*	Genbank: NZ_AKCX00000000						
AW 12879*	Genbank: NC_020815						
CFBP 1851*	Genbank: NZ_AKCY00000000						
CFBP 2914	KJ491108	KJ491198	KJ491288	KJ491378	KJ491468	KJ491558	KJ491648
CFBP 2938	KJ491109	KJ491199	KJ491289	KJ491379	KJ491469	KJ491559	KJ491649
CFBP 5693	KJ491110	KJ491200	KJ491290	KJ491380	KJ491470	KJ491560	KJ491650
CIO1*	Genbank:NZ_AKCZ00000000						
F1*	Genbank:NC_016010						
GBBC 922	KJ491111	KJ491201	KJ491291	KJ491381	KJ491471	KJ491561	KJ491651
GSPB 1386*	Genbank: NZ_AHIB00000000						
GSPB 2388*	Genbank: NZ_AHIC00000000						
ICPB 10535*	Genbank: NZ_ACPY00000000						
ICPB 11122*	Genbank: NZ_ACPX00000000						
LMG 11726	KJ491112	KJ491202	KJ491292	KJ491382	KJ491472	KJ491562	KJ491652
LMG 12708	KJ491113	KJ491203	KJ491293	KJ491383	KJ491473	KJ491563	KJ491653
LMG 12716	KJ491114	KJ491204	KJ491294	KJ491384	KJ491474	KJ491564	KJ491654
LMG 12734	KJ491115	KJ491205	KJ491295	KJ491385	KJ491475	KJ491565	KJ491655
LMG 12738	KJ491116	KJ491206	KJ491296	KJ491386	KJ491476	KJ491566	KJ491656
LMG 12739	KJ491117	KJ491207	KJ491297	KJ491387	KJ491477	KJ491567	KJ491657
LMG 12741	KJ491118	KJ491208	KJ491298	KJ491388	KJ491478	KJ491568	KJ491658
LMG 12749	KJ491119	KJ491209	KJ491299	KJ491389	KJ491479	KJ491569	KJ491659
LMG 12752	KJ491120	KJ491210	KJ491300	KJ491390	KJ491480	KJ491570	KJ491660
LMG 12894	KJ491121	KJ491211	KJ491301	KJ491391	KJ491481	KJ491571	KJ491661
LMG 12895	KJ491122	KJ491212	KJ491302	KJ491392	KJ491482	KJ491572	KJ491662
LMG 21894	KJ491123	KJ491213	KJ491303	KJ491393	KJ491483	KJ491573	KJ491663
LMG 25669	KJ491124	KJ491214	KJ491304	KJ491394	KJ491484	KJ491574	KJ491664
LMG 25670	KJ491125	KJ491215	KJ491305	KJ491395	KJ491485	KJ491575	KJ491665
LMG 25671	KJ491126	KJ491216	KJ491306	KJ491396	KJ491486	KJ491576	KJ491666
LMG 25672	KJ491127	KJ491217	KJ491307	KJ491397	KJ491487	KJ491577	KJ491667
LMG 25674	KJ491128	KJ491218	KJ491308	KJ491398	KJ491488	KJ491578	KJ491668
LMG 25675	KJ491129	KJ491219	KJ491309	KJ491399	KJ491489	KJ491579	KJ491669
LMG 25676	KJ491130	KJ491220	KJ491310	KJ491400	KJ491490	KJ491580	KJ491670
LMG 25677	KJ491131	KJ491221	KJ491311	KJ491401	KJ491491	KJ491581	KJ491671
LMG 25910	KJ491132	KJ491222	KJ491312	KJ491402	KJ491492	KJ491582	KJ491672
LMG 25911	KJ491133	KJ491223	KJ491313	KJ491403	KJ491493	KJ491583	KJ491673
LMG 25933	KJ491134	KJ491224	KJ491314	KJ491404	KJ491494	KJ491584	KJ491674
LMG 25937	KJ491135	KJ491225	KJ491315	KJ491405	KJ491495	KJ491585	KJ491675
LMG 25938	KJ491136	KJ491226	KJ491316	KJ491406	KJ491496	KJ491586	KJ491676

LMG 25939	KJ491137	KJ491227	KJ491317	KJ491407	KJ491497	KJ491587	KJ491677
LMG 25940	KJ491138	KJ491228	KJ491318	KJ491408	KJ491498	KJ491588	KJ491678
LMG 25943	KJ491139	KJ491229	KJ491319	KJ491409	KJ491499	KJ491589	KJ491679
LMG 25945	KJ491140	KJ491230	KJ491320	KJ491410	KJ491500	KJ491590	KJ491680
LMG 27970*	Genbank: JPYC00000000						
LMG 470	KJ491141	KJ491231	KJ491321	KJ491411	KJ491501	KJ491591	KJ491681
LMG 495*	Genbank: JPYG00000000						
LMG 497	KJ491142	KJ491232	KJ491322	KJ491412	KJ491502	KJ491592	KJ491682
LMG 499	KJ491143	KJ491233	KJ491323	KJ491413	KJ491503	KJ491593	KJ491683
LMG 532	KJ491144	KJ491234	KJ491324	KJ491414	KJ491504	KJ491594	KJ491684
LMG 534	KJ491145	KJ491235	KJ491325	KJ491415	KJ491505	KJ491595	KJ491685
LMG 580	KJ491148	KJ491238	KJ491328	KJ491418	KJ491508	KJ491598	KJ491688
LMG 668	KJ491149	KJ491239	KJ491329	KJ491419	KJ491509	KJ491599	KJ491689
LMG 686	KJ491150	KJ491240	KJ491330	KJ491420	KJ491510	KJ491600	KJ491690
LMG 695	KJ491151	KJ491241	KJ491331	KJ491421	KJ491511	KJ491601	KJ491691
LMG 698	KJ491152	KJ491242	KJ491332	KJ491422	KJ491512	KJ491602	KJ491692
LMG 736	KJ491154	KJ491244	KJ491334	KJ491424	KJ491514	KJ491604	KJ491694
LMG 7399	KJ491155	KJ491245	KJ491335	KJ491425	KJ491515	KJ491605	KJ491695
LMG 7400	KJ491156	KJ491246	KJ491336	KJ491426	KJ491516	KJ491606	KJ491696
LMG 7455	KJ491157	KJ491247	KJ491337	KJ491427	KJ491517	KJ491607	KJ491697
LMG 8021	KJ491158	KJ491248	KJ491338	KJ491428	KJ491518	KJ491608	KJ491698
LMG 8022	KJ491159	KJ491249	KJ491339	KJ491429	KJ491519	KJ491609	KJ491699
LMG 8039	KJ491160	KJ491250	KJ491340	KJ491430	KJ491520	KJ491610	KJ491700
LMG 8130	KJ491161	KJ491251	KJ491341	KJ491431	KJ491521	KJ491611	KJ491701
LMG 8136	KJ491162	KJ491252	KJ491342	KJ491432	KJ491522	KJ491612	KJ491702
LMG 826	KJ491163	KJ491253	KJ491343	KJ491433	KJ491523	KJ491613	KJ491703
LMG 828	KJ491164	KJ491254	KJ491344	KJ491434	KJ491524	KJ491614	KJ491704
LMG 845	KJ491165	KJ491255	KJ491345	KJ491435	KJ491525	KJ491615	KJ491705
LMG 846	KJ491166	KJ491256	KJ491346	KJ491436	KJ491526	KJ491616	KJ491706
LMG 859*	Genbank: NZ_CAGJ00000000						
LMG 865	KJ491167	KJ491257	KJ491347	KJ491437	KJ491527	KJ491617	KJ491707
LMG 8655	KJ491168	KJ491258	KJ491348	KJ491438	KJ491528	KJ491618	KJ491708
LMG 867	KJ491169	KJ491259	KJ491349	KJ491439	KJ491529	KJ491619	KJ491709
LMG 872	KJ491170	KJ491260	KJ491350	KJ491440	KJ491530	KJ491620	KJ491710
LMG 895†	EU015167	EU498763	FJ376344	HQ591131	HQ591262	KM668207	EU499088
LMG 901†	EU015169	EU498798	FJ376346	HQ591132	HQ591263	KM668206	EU499130
LMG 873	KJ491171	KJ491261	KJ491351	KJ491441	KJ491531	KJ491621	KJ491711
LMG 904	KJ491172	KJ491262	KJ491352	KJ491442	KJ491532	KJ491622	KJ491712
LMG 9040	KJ491173	KJ491263	KJ491353	KJ491443	KJ491533	KJ491623	KJ491713
LMG 9055	KJ491174	KJ491264	KJ491354	KJ491444	KJ491534	KJ491624	KJ491714
LMG 909	KJ491175	KJ491265	KJ491355	KJ491445	KJ491535	KJ491625	KJ491715
LMG 910	KJ491176	KJ491266	KJ491356	KJ491446	KJ491536	KJ491626	KJ491716
LMG 913	KJ491177	KJ491267	KJ491357	KJ491447	KJ491537	KJ491627	KJ491717
LMG 9179	KJ491178	KJ491268	KJ491358	KJ491448	KJ491538	KJ491628	KJ491718
LMG 9181	KJ491179	KJ491269	KJ491359	KJ491449	KJ491539	KJ491629	KJ491719

LMG 9182	KJ491180	KJ491270	KJ491360	KJ491450	KJ491540	KJ491630	KJ491720
LMG 922	KJ491181	KJ491271	KJ491361	KJ491451	KJ491541	KJ491631	KJ491721
LMG 926	KJ491182	KJ491272	KJ491362	KJ491452	KJ491542	KJ491632	KJ491722
LMG 932	KJ491183	KJ491273	KJ491363	KJ491453	KJ491543	KJ491633	KJ491723
LMG 9322	KJ491184	KJ491274	KJ491364	KJ491454	KJ491544	KJ491634	KJ491724
LMG 9325	KJ491185	KJ491275	KJ491365	KJ491455	KJ491545	KJ491635	KJ491725
LMG 936	KJ491186	KJ491276	KJ491366	KJ491456	KJ491546	KJ491636	KJ491726
LMG 941*	Genbank: NZ_CAHO00000000						
LMG 9489	KJ491187	KJ491277	KJ491367	KJ491457	KJ491547	KJ491637	KJ491727
LMG 9654	KJ491188	KJ491278	KJ491368	KJ491458	KJ491548	KJ491638	KJ491728
LMG 982	KJ491189	KJ491279	KJ491369	KJ491459	KJ491549	KJ491639	KJ491729
NCPPB 3380	KJ491190	KJ491280	KJ491370	KJ491460	KJ491550	KJ491640	KJ491730
NCPPB 4322	KJ491191	KJ491281	KJ491371	KJ491461	KJ491551	KJ491641	KJ491731
PD 2797	KJ491192	KJ491282	KJ491372	KJ491462	KJ491552	KJ491642	KJ491732
PD 3413	KJ491193	KJ491283	KJ491373	KJ491463	KJ491553	KJ491643	KJ491733
PD 3821	KJ491194	KJ491284	KJ491374	KJ491464	KJ491554	KJ491644	KJ491734
PD 4015	KJ491195	KJ491285	KJ491375	KJ491465	KJ491555	KJ491645	KJ491735
PD 4394	KJ491196	KJ491286	KJ491376	KJ491466	KJ491556	KJ491646	KJ491736
PD 4485	KJ491197	KJ491287	KJ491377	KJ491467	KJ491557	KJ491647	KJ491737
PXO99A*	Genbank: NC_010717						
X 18*	Genbank: CM002136						

* Whole genome sequences downloaded from GenBank.

† Only lrp gene sequences generated in this study, sequences of the other genes downloaded from GenBank.

Supplementary Table 2.5. DNA-DNA relatedness among selected strains of *Xanthomonas axonopodis* (*sensu* Vauterin *et al.*, 1995).

	Strain	1	2	3	4	5	6	7	8
X. euvesicatoria (PG II)	1. *X. alfalfae* subsp. *alfalfae* LMG 495^{T}	100							
	2. *X. euvesicatoria* LMG 27970^{T}	78 (0)	100						
X. phaseoli (PG III)	3. *X. axonopodis* pv. *syngonii* LMG 9055^{PT}		63 (20)	100					
	4. *X. axonopodis* pv. *dieffenbachiae* LMG 695^{PT}	69 (7)		84 (45)	100				
X. axonopodis (PG IV)	5. *X. axonopodis* LMG 982^{T}	57 (4)	58 (14)		62 (27)	100			
X. citri (PG I)	6. *X. citri* subsp. *citri* LMG 9322^{T}	64 (26)	57 (13)	64 (20)	65 (15)	61 (14)	100		
	7. *X. axonopodis* pv. *dieffenbachiae* LMG 7399						82 (6)	100	
	8. *X. fuscans* subsp. *fuscans* LMG 826^{T}						84 (3)	88 (14)	100

For every DNA pair reciprocal reactions (e.g. A×B and B×A) were carried out. The presented DNA-DNA relatedness values are the average of the mean value of A×B and that of B×A, while the values between parentheses are the difference between the mean value of A×B and that of B×A. T = type strain; PT = pathotype (pathovar reference) strain.

Supplementary Table 2.6. Main characteristics of assembled draft genomes of *Xanthomonas* strains generated in this study.

WGS	*X. citri* (PG I)			*X. euvesicatoria* (PG II)			*X. phaseoli* (PG III)		*X. axonopodis* (PG IV)
	LMG 826^{T}	LMG 7399	LMG 9322^{T}	LMG 495^{T}	LMG 12749	LMG 27970^{T}	LMG 9055^{PT}	LMG 695^{PT}	LMG 982$^{T, PT}$
Total clean reads	7565129	10473504	2933630	6709111	3048059	7148448	7972771	8525313	5517280
Total bp reads used	381570338	528737707	148761579	339005127	154610187	361162989	392805460	429912446	278609524
Sequence coverage	78,45499456	103,8527276	29,04023756	67,7688623	31,80348367	71,60594553	79,58030885	86,01531264	63,02963649
N50	17723	31929	23058	29661	51301	13901	26041	61344	15863
Number of contigs	760	495	661	488	296	869	575	228	700
Maximum contig length	94705	139980	153966	110335	155040	51661	110431	316089	52118
Total contig length	4863557	5091226	5122602	5002373	4861423	5043757	4935963	4998092	4420294
GC %	64,87	64,52	64,73	64,77	65,09	65,65	64,85	64,87	64,52
GenBank accesion numbers	JPYF00000000	JPYH00000000	JPYD00000000	JPYG00000000	JPUN00000000	JPYC00000000	JPUO00000000	JPYB00000000	JPYE00000000

N50= minimum number of contigs needed to cover 50% of the assembly

Supplementary Table 2.7. Cellular fatty acid contents (%) of *Xanthomonas axonopodis* (*sensu* Vauterin *et al*., 1995) strains and a selection of type strains of *Xanthomonas* species, representing the diversity of the genus.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
$C_{10:0}$	-	2.4	2.1	2.6	-	-	-	-	2.7	-	-	-	-	2.4	2.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{11:0}$ iso	6.4	6.0	7.4	3.4	4.6	9.5	4.3	5.5	9.1	5.0	3.8	5.8	4.9	4.5	6.1	5.7	9.9	12.4	8.4	6.2	5.5	3.6	3.9	4.3	5.2	4.8	6.7	4.6	7.7	8.0	4.5
$C_{11:0}$ iso 3-OH	3.8	3.7	2.5	2.6	3.3	4.2	2.8	3.1	6.1	2.3	2.6	2.4	2.3	3.6	3.3	3.0	4.4	5.9	6.3	3.8	4.8	2.3	3.0	2.7	3.3	3.0	5.2	2.4	5.0	2.6	2.8
$C_{12:0}$ 3-OH	4.3	9.3	5.7	9.7	2.6	6.0	4.7	5.2	10.5	4.5	5.6	3.8	5.6	9.9	9.4	5.0	4.6	5.1	3.7	2.3	4.1	3.7	3.8	4.8	3.8	2.2	4.2	3.8	2.4	5.3	-
$C_{12:0}$ iso 3-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.6	-
$C_{13:0}$ 2-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{13:0}$ iso 3-OH	8.2	6.1	6.0	5.0	7.5	9.7	5.1	6.9	11.3	5.0	5.4	5.5	5.2	5.0	5.4	6.6	12.1	12.7	7.9	4.8	5.6	3.7	3.9	3.7	4.4	4.6	7.9	5.5	3.0	2.4	2.3
$C_{14:0}$	-	2.6	5.5	6.2	-	-	-	-	-	-	-	-	2.0	3.1	3.2	-	-	-	-	-	2.2	-	-	-	-	-	-	-	-	-	-
$C_{14:0}$ iso	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.4	-
$C_{15:0}$ anteiso	11.2	8.0	12.5	5.8	22.7	10.9	21.0	6.1	6.4	9.6	11.0	11.5	10.3	14.4	7.3	-	2.6	-	12.1	5.5	3.1	-	2.5	11.9	16.2	7.2	10.5	4.9	5.0	6.4	-
$C_{15:0}$ iso	29.0	22.7	17.8	19.4	19.4	16.9	15.4	26.9	14.8	22.0	20.6	24.6	21.7	17.9	17.8	21.3	16.6	17.8	22.8	30.1	16.2	4.4	13.8	23.1	24.6	30.7	27.5	37.3	23.8	20.9	-
$C_{16:0}$	2.8	4.8	6.1	5.0	-	-	3.4	5.1	4.0	4.3	5.3	2.7	4.6	5.2	4.9	8.5	-	-	2.4	4.1	6.1	17.5	12.5	4.8	2.7	2.2	-	3.0	7.4	5.6	15.8
$C_{16:0}$ iso	2.1	-	2.0	-	3.0	-	2.3	-	-	2.6	2.1	3.2	2.3	-	-	-	-	-	-	-	-	-	-	-	3.1	-	-	-	-	7.8	2.8
$C_{16:1}$ ω9c	-	-	2.2	-	-	2.0	2.0	2.5	-	-	-	-	2.1	-	-	-	-	-	-	-	2.9	3.3	3.9	-	-	-	-	-	2.7	-	-
$C_{17:0}$ anteiso	-	-	-	-	3.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.6
$C_{17:0}$ iso	2.7	2.9	-	-	6.1	5.1	5.0	5.2	4.3	7.0	6.0	3.8	3.4	2.9	3.8	11.7	10.3	9.4	6.0	10.3	8.0	18.1	6.6	7.6	4.4	8.1	6.2	5.7	5.2	2.5	18.5
$C_{17:1}$ ω8c	-	-	-	-	-	-	-	-	-	-	-	-	2.0	-	-	2.1	-	-	-	-	-	-	-	-	2.5	-	-	-	-	-	-
$C_{18:1}$ ω7c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.0
$C_{18:1}$ ω9c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
iso $C_{17:1}$ ω9c	4.1	3.2	2.2	-	10.4	5.7	5.6	3.8	3.3	5.8	4.4	4.3	3.1	2.9	2.8	6.4	16.3	15.0	8.1	10.1	20.2	9.1	12.1	6.1	3.9	10.7	10.9	8.4	6.5	2.0	26.0
SF 3	16.7	21.8	21.6	28.6	8.0	15.9	18.1	18.0	15.1	17.9	19.1	14.6	19.4	23.6	21.1	20.4	13.4	12.5	9.8	10.0	14.0	23.2	21.0	18.9	13.2	18.8	10.9	11.9	22.8	15.4	12.5
unknown 11.799	3.0	2.7	-	2.3	2.6	3.9	2.4	2.7	4.9	2.3	2.5	2.4	2.4	3.4	3.1	3.5	3.1	3.2	2.7	-	2.8	-	2.5	-	2.1	-	2.8	2.1	-	-	-

SF 3, summed feature 3 ($C_{16:1}$ *ω7c* / $C_{15:0}$ iso 2-OH).

1-5. *X. citri* (PG I): 1. *X. citri* subsp. *citri* LMG 9322^{T} (this study); 2. *X. citri* subsp. *malvacearum* LMG 11726^{T} (this study); 3. *X. fuscans* subsp. *fuscans* LMG 826^{T} (this study); 4. *X. fuscans* subsp. *aurantifolii* LMG 9179^{T} (this study); 5. *X. axonopodis* pv. *dieffenbachiae* LMG 7399 (this study); **6-11. *X. euvesicatoria* (PG II):** 6. *X. euvesicatoria* LMG 27970^{T}; 7. *X. euvesicatoria* LMG 910 (this study); 8. *X. alfalfae* subsp. *alfalfae* LMG 495^{T} (this study); 9. *X. alfalfae* subsp. *citrumelonis* LMG 9325^{T} (this study); 10. *X. perforans* LMG 28258^{T}; 11. *X. axonopodis* pv. *dieffenbachiae* LMG 12749 (this study); **12-15. *X. phaseoli* (PG III):** 12. *X. phaseoli* LMG 29033^{T} (this study);13. *X. axonopodis* pv. *phaseoli* LMG 7455^{PT} (this study); 14. [*X. campestris*] pv. *syngonii* LMG 9055^{PT} (this study); 15. *X. axonopodis* pv. *dieffenbachiae* LMG 695^{PT} (this study);**16-18. *X. axonopodis* (PG IV):** 16. *X. axonopodis* pv. *axonopodis* LMG 982$^{T, PT}$ (this study); 17. *X. axonopodis* pv. *vasculorum* LMG 901^{PT} (this study); 18. *X. axonopodis* pv. *vasculorum* LMG 895 (this study); 19. *X. campestris* LMG 568^{T}; 20. *X. melonis* LMG 8670^{T} (this study); 21. *X. bromi* LMG 947^{T} (this study); 22. *X. oryzae* LMG 5047^{T}; 23. *X. vasicola* LMG 736^{T} (this study); 24. *X. vesicatoria* LMG 911^{T}; 25. *X. arboricola* LMG 747^{T}; 26. *X. gardneri* LMG 962^{T}, 27. *X. cassavae* LMG 673^{T}; 28. *X. maliensis* LMG 27592^{T}; 29. *X. translucens* LMG 876^{T}; 30. *X. sacchari* LMG 471^{T}; 31. *X. albilineans* LMG 494^{T}. -, not detectable or trace amount (< 2 %). Cultivation conditions prior to fatty acid extraction and fatty acid analyses were identical for all strains. Values are mean percentages. T = type strain; PT = pathotype (pathovar reference) strain.

Supplementary Table 2.8. Phenotypic features of *X. citri* (PG I), *X. euvesicatoria* (PG II), *X. phaseoli* (PG III), *X. axonopodis* (PG IV) and the type strains of the phylogenetically closest *Xanthomonas* species, obtained through Biolog GEN III MicroPlate assays.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
	33°C 24h	33°C 24h	33°C 24h	28°C 144h	33°C 24h	28°C 24h	33°C 24h	33°C 24h	33°C 24h	33°C 24h	33°C 24h	33°C 24h	33°C 24h	28°C 72h	33°C 24h	28°C 72h	33°C 24h	33°C 24h	28°C 24h	33°C 24h	28°C 24h	28°C 144h	33°C 24h
Carbon source oxidation assays																							
Dextrin	+	+	+	+	/	+	+	+	+	+	+	+	+	/	+	/	/	/	+	+	+	/	+
D-Maltose	/	-	-	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	-	+	-
D-Trehalose	/	/	+	+	/	+	+	+	+	+	/	+	+	+	+	+	-	-	+	+	/	/	/
D-Cellobiose	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	/	-	-	+	+	+	+	+
Gentiobiose	/	/	+	+	/	+	+	+	+	+	+	+	+	+	+	/	-	-	/	+	-	/	-
Sucrose	/	-	+	+	/	+	+	+	+	+	+	+	+	+	+	+	-	/	+	+	+	-	/
D-Turanose	-	-	-	-	-	-	-	-	-	-	/	/	/	-	-	-	-	-	-	-	-	-	-
Stachyose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	/	-	/	-	-	-	-	/	-	-	-	-	-	-	-	-	/	-	-	-
α-D-Lactose	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-	-	-	-	-	+	-	/	-
D-Melibiose	-	-	/	/	-	+	+	+	/	+	+	-	+	-	/	-	-	-	+	+	-	/	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-	-	/	-	-	-	-	-	-	-	-	-	-	/	-	/	-
D-Salicin	-	-	-	-	-	/	-	-	/	/	/	-	/	-	-	-	-	-	-	+	-	/	-
N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	/	+	+	+	-	+	+	+	+	-	+
N-Acetyl-β-D-Mannosamine	-	-	-	/	-	-	-	-	/	-	/	/	-	-	-	-	-	-	-	-	-	-	-
N-Acetyl-β-D-Galactosamine	-	-	-	-	-	-	-	-	/	-	-	-	/	-	-	-	-	-	-	-	-	/	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	+	+	+	-	+
D-Mannose	+	+	+	+	/	+	+	+	+	+	+	+	+	/	+	/	-	+	+	+	+	-	+
D-Fructose	+	+	+	+	/	+	+	+	+	+	+	+	+	+	+	/	-	/	+	+	+	-	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	/	+	+	+	+	+
3-Methyl Glucose	-	/	/	/	-	/	/	-	/	/	/	-	/	-	/	/	-	-	-	/	-	+	-
D-Fucose	-	-	-	-	-	+	-	-	/	/	/	-	/	-	/	/	-	-	/	/	/	+	-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15	16	17	18	19	20	21	22
	33°C	33°C	33°C	28°C	33°C	28°C	33°C	33°C	33°C	33°C	33°C	33°C	33°C	28°C	33°C	28°C	33°C	33°C	28°C	33°C	28°C	28°C	33°C
	24h	24h	24h	144h	24h	24h	24h	24h	24h	24h	24h	24h	24h	72h	24h	72h	24h	24h	24h	24h	24h	144h	24h
L-Fucose	+	+	+	+	/	+	+	+	+	+	+	+	+	-	/	+	-	-	+	+	+	+	+
L-Rhamnose	-	-	-	-	-	/	/	-	/	/	/	-	/	-	/	-	-	-	-	-	-	/	-
Inosine	-	-	-	/	-	-	-	-	/	/	-	/	/	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-
D-Arabitol	/	-	-	/	-	-	-	-	/	-	/	/	/	-	-	-	-	-	-	-	/	/	-
myo-Inositol	-	-	/	-	-	-	-	-	/	-	/	-	/	-	-	-	-	-	-	-	-	/	-
Glycerol	+	/	/	+	-	/	+	/	+	+	/	+	/	-	+	-	-	-	-	-	/	/	+
D-Glucose-6-PO4	-	-	+	+	-	/	-	-	+	-	+	+	/	-	+	/	-	-	-	/	/	+	-
D-Fructose-6-PO4	/	/	/	+	/	+	/	/	+	/	+	/	+	/	/	-	-	-	/	/	/	+	/
D-Aspartic Acid	-	-	-	/	-	/	-	-	+	-	/	-	/	-	/	/	-	-	-	-	/	/	-
D-Serine	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	/	/	/	-	-
Gelatin	+	/	/	/	-	+	+	-	+	+	/	+	/	-	+	-	-	-	-	+	/	-	/
Glycyl-L-Proline	/	/	-	+	/	+	/	+	/	+	/	+	/	-	/	+	/	-	/	+	+	+	-
L-Alanine	/	/	/	+	/	+	/	/	/	+	/	+	+		/	/	-	-	-	+	+	-	-
L-Arginine	-	-	-	/	-	/	-	-	/	-	/	-	/	-	-	-	-	-	-	-	/	-	-
L-Aspartic Acid	-	-	/	/	-	/	-	/	+	+	/	/	/	/	/	-	-	-	-	-	/	-	/
L-Glutamic Acid	+	/	+	+	/	+	+	+	+	+	+	+	+	+	+	/	-	/	/	+	+	/	/
L-Histidine	-	-	/	-	-	/	-	-	/	-	/	-	-	-	-	-	/	-	-	-	/	+	-
L-Pyroglutamic Acid	-	-	-	/	-	/	-	-	/	-	-	-	-	-	-	-	-	-	-	-	-	/	-
L-Serine	-	-	-	-	/	+	/	-	/	+	/	+	+	+	/	-	-	-	/	/	/	-	-
Pectin	+	+	+	+	/	+	+	/	+	+	+	+	+	/	/	/	-	/	+	+	+	-	+
D-Galacturonic Acid	/	/	/	/	-	/	/	/	/	/	/	/	/	-	-	-	-	-	/	/	/	/	/
L-Galactonic Acid Lactone	-	-	/	/	-	-	-	-	-	-	/	/	/	-	-	-	-	-	-	-	-	-	-
D-Gluconic Acid	-	-	-	/	-	/	-	-	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucuronic Acid	-	-	/	/	-	/	-	-	/	/	/	-	/	-	-	-	-	-	-	-	/	/	/
Glucuronamide	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	/
Mucic acid	-	-	/	-	-	/	-	-	-	-	-	-	-	/	-	/	-	-	+	+	/	-	-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15	16	17	18	19	20	21	22
	33°C	33°C	33°C	28°C	33°C	28°C	33°C	33°C	33°C	33°C	33°C	33°C	33°C	28°C	33°C	28°C	33°C	33°C	28°C	33°C	28°C	28°C	33°C
	24h	24h	24h	144h	24h	24h	24h	24h	24h	24h	24h	24h	24h	72h	24h	72h	24h	24h	24h	24h	24h	144h	24h
Quinic Acid	-	-	-	+	-	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Saccharic acid	-	-	-	+	+	/	-	-	-	-	-	-	-	-	-	-	-	-	+	+	/	-	-
p-Hydroxy-Phenylacetic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-	-	-	-	-	-
Methyl Pyruvate	/	-	-	-	/	/	/	/	-	/	-	-	/	+	/	-	-	-	/	/	/	-	/
D-Lactic Acid Methyl Ester	-	-	/	/	-	/	-	-	/	-	-	/	/	-	-	-	-	-	-	-	-	-	-
L-Lactic Acid	-	-	/	/	-	/	-	/	-	-	-	/	-	-	-	+	-	-	-	-	/	-	-
Citric Acid	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+
α-Keto-Glutaric Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	/	/	/	+	/	/	+	+
D-Malic Acid	-	-	-	-	-	/	-	-	-	-	-	/	-	-	-	-	-	-	/	-	/	-	-
L-Malic Acid	+	/	+	+	/	+	+	+	+	+	+	+	/	-	+	+	-	/	+	+	+	/	+
Bromo-Succinic Acid	/	-	+	+	-	+	/	/	/	/	+	+	-	-	/	/	-	-	+	/	+	+	+
Tween 40	-	-	/	+	/	+	+	+	-	/	/	+	+	/	+	/	-	-	/	/	/	-	-
γ-Amino-Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	/	-	/	-	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-
α-Keto-Butyric Acid	-	-	/	+	/	/	/	/	/	/	/	/	/	/	/	-	/	/	/	-	-	-	-
Acetoacetic Acid	+	/	-	-	+	+	+	+	/	+	+	+	+	+	+	/	/	/	/	/	+	-	+
Propionic Acid	-	-	+	-	/	+	/	+	/	+	/	+	/	+	/	-	-	-	/	/	/	-	/
Acetic Acid	+	/	+	-	+	+	+	+	+	+	+	+	+	+	/	+	-	/	+	+	+	+	+
Fomnic Acid	-	-	-	-	-	-	-	-	-	/	-	+	/	-	-	-	-	-	-	-	-	-	-
Chemical sensitivity assays																							
pH 6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	+	+	+	+	+	+
pH 5	-	-	-	-	/	-	-	/	-	/	-	-	-	-	-	-	-	-	/	/	/	-	/
1% NaCl	+	+	+	-	+	+	+	+	+	+	+	+	+	/	+	+	/	+	+	+	+	+	+
4% NaCl	-	-	-	-	-	-	/	-	-	/	-	-	-	-	-	-	-	-	+	-	/	-	-
8% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1% Sodium Lactate	+	+	+	-	+	+	+	+	+	+	+	/	+	-	+	+	-	/	+	+	+	+	+

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15	16	17	18	19	20	21	22
	33°C	33°C	33°C	28°C	33°C	28°C	33°C	33°C	33°C	33°C	33°C	33°C	33°C	28°C	33°C	28°C	33°C	33°C	28°C	33°C	28°C	28°C	33°C
	24h	24h	24h	144h	24h	24h	24h	24h	24h	24h	24h	24h	24h	72h	24h	72h	24h	24h	24h	24h	24h	144h	24h
Fusidic Acid	-	-	-	-	-	/	-	-	-	-	-	-	/	/	-	-	-	-	/	-	/	/	-
D-Serine	-	-	-	-	-	/	/	-	-	-	-	-	/	-	-	-	-	-	/	-	+	/	-
Troleando-mycin	-	-	-	-	-	/	-	-	-	-	-	/	/	-	-	-	-	-	/	-	/	/	-
Rifamycin SV	+	/	/	-	+	+	+	/	-	+	-	+	+	+	/	-	-	-	+	-	+	+	+
Minocycline	/	-	-	-	-	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/
Lincomycin	+	+	+	-	/	+	+	+	+	+	+	+	+	+	+	+	-	/	+	+	+	+	+
Guanidine HCl	-	-	-	-	-	/	/	-	/	/	/	/	/	-	/	-	-	-	+	/	-	-	-
Niaproof 4	/	-	-	-	-	/	-	-	-	/	-	-	-	-	/	-	-	-	/	-	-	/	/
Vancomycin	/	-	-	-	-	/	-	+	-	/	/	-	/	/	/	-	-	-	/	-	/	/	-
Tetrazolium Violet	/	/	/	/	+	+	/	/	/	+	+	/	+	/	+	/	/	/	+	+	/	/	/
Tetrazolium Blue	-	+	+	-	/	+	+	/	+	+	+	+	+	+	+	/	/	/	+	/	+	/	+
Nalidixic Acid	-	-	-	/	-	+	-	/	-	+	/	/	+	/	+	+	-	/	+	/	+	/	/
Lithium Chloride	-	-	-	-	-	-	-	-	-	/	/	-	/	-	/	-	-	-	/	+	-	-	-
Potassium Tellurite	/	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-	/	-	-	/	-
Aztreonam	/	-	-	-	+	+	/	+	-	+	-	+	+	/	/	-	-	/	/	+	/	/	/
Sodium Butyrate	-	-	-	-	/	/	-	/	/	/	-	/	/	-	-	-	-	-	/	-	/	/	-
Sodium Bromate	/	-	-	-	-	-	-	/	-	-	-	-	/	-	-	-	-	-	-	/	/	-	/

1-5. *X. citri* (PG I): 1. *X. citri* subsp. *citri* LMG 9322^{T} (this study); 2. *X. citri* subsp. *malvacearum* LMG 11726^{T} (this study); 3. *X. fuscans* subsp. *fuscans* LMG 826^{T} (this study); 4. *X. fuscans* subsp. *aurantifolii* LMG 9179^{T} (this study); 5. *X. axonopodis* pv. *dieffenbachiae* LMG 7399 (this study); **6-11. *X. euvesicatoria* (PG II)**: 6. *X. euvesicatoria* LMG 27970^{T}; 7. *X. euvesicatoria* LMG 910 (this study); 8. *X. alfalfae* subsp. *alfalfae* LMG 495^{T} (this study); 9. *X. alfalfae* subsp. *citrumelonis* LMG 9325^{T} (this study); 10. *X. perforans* LMG 28258^{T}; 11. *X. axonopodis* pv. *dieffenbachiae* LMG 12749 (this study); **12-15. *X. phaseoli* (PG III)**: 12. *X. phaseoli* LMG 29033^{T} (this study); 13. *X. axonopodis* pv. *phaseoli* LMG 7455^{PT} (this study); 14. [*X. campestris*] pv. *syngonii* LMG 9055^{PT} (this study); 15. *X. axonopodis* pv. *dieffenbachiae* LMG 695^{PT} (this study); **16-18. *X. axonopodis* (PG IV)**: 16. *X. axonopodis* pv. *axonopodis* LMG 982^{T}, ^{PT} (this study); 17. *X. axonopodis* pv. *vasculorum* LMG 901^{PT} (this study); 18. *X. axonopodis* pv. *vasculorum* LMG 895 (this study); 19. X. *campestris* LMG 568^{T}; 20. *X. melonis* LMG 8670^{T} (this study); 21. *X. bromi* LMG 947^{T} (this study); 22. *X. oryzae* LMG 5047^{T}; 23. *X. vasicola* LMG 736^{T} (this study). +, positive ; /, intermediate positive; -, negative. Prior to inoculation of the Biolog GEN III microplates, all strains were grown for 24 h under aerobic conditions on BUG medium without blood, at 28 or 33 °C, depending on the strain. The Biolog GEN III microplates were read with a Biolog MicroStation microplate reader after incubation for 24 to 144 h at the same temperature as used to obtain the culture for inoculation.

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Chapter 3:

Pathogenicity and virulence gene content of *Xanthomonas* strains infecting Araceae, formerly known as *Xanthomonas axonopodis* pv. *dieffenbachiae*

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Autors' contributions

MM, CB, VVJ and CEC designed the experiment set-up. CEC did the practical work and drafted the manuscript. VVJ provided the necessary plants. VMC, VVJ and CEC performed pathogenicity tests. BS performed the whole-genome DNA sequencing and genome depositions. HA helped with the genome analysis. CB supervised the work and assisted in writing. CB, HA, VVJ and MM proofread the manuscript.

Abstract

Bacterial leaf blight of aroids is caused by a heterogeneous group of xanthomonads listed as *X. axonopodis* pv. *dieffenbachiae* (Xad) on the EPPO A2 quarantine list. Recently, Xad strains were shown not to belong to *X. axonopodis* but to the species *X. citri*, *X. phaseoli* and *X. euvesicatoria*. Here, to verify the pathovar designation, eleven representative strains were tested for pathogenicity on six aroid genera. They had overlapping host ranges, only the strain isolated from *Syngonium* showed host specificity. The *X. citri* strains, isolated from various hosts, showed dissimilarity in virulence to the tested aroid genera. The *X. phaseoli* strains, isolated from *Anthurium* and *Syngonium*, were generally more virulent and, additionally, induced systemic infections. The *X. euvesicatoria* strains, isolated from *Philodendron*, were scored as not pathogenic on the tested aroids. Four representative strains were genome sequenced and showed a variable virulence-associated gene content. Pathogenicity to aroids was correlated with the presence of three specific T3 effector genes and with a T6SS gene sequence. Together, the phylogenetic and pathogenic differentiation among Xad strains justifies the installation of three pathovar epithets for the pathogens on aroids: *X. phaseoli* pv. *dieffenbachiae* comb. nov. for the strains isolated from *Anthurium*; *X. phaseoli* pv. *syngonii* comb. nov. for the strain isolated from *Syngonium*; and *X. citri* pv. *aracearum* comb. nov. for the strains isolated from *Aglaonema*, *Xanthosoma* and *Dieffenbachia*. It is proposed that phytosanitary regulations for xanthomonads on aroids are restricted to these three pathovars.

3.1 Introduction

Bacterial leaf blight of aroids is caused by xanthomonads named as *Xanthomonas axonopodis* pv. *dieffenbachiae* (Xad) and was reported for the first time on *Dieffenbachia maculata* in New Jersey, USA (McCulloch & Pirone, 1939). Although the first known host of Xad was *Dieffenbachia*, a strain isolated from *Anthurium* has been designated as pathovar reference strain of Xad (LMG 695^{PT}). The disease primarily affects foliage aroids, i.e. *Aglaonema*, *Anthurium*, *Caladium*, *Dieffenbachia*, *Epipremnum*, *Philodendron*, *Rhaphidophora*, *Scindapsus*, *Spathiphyllum* and *Syngonium* (Chase *et al*., 1992), but also edible aroid species, i.e. *Colocasia esculenta* (taro), *Alocasia* (elephant ear), *Cyrtosperma* (swamp taro) and *Xanthosoma* (tannia). Some of these hosts have a high economic importance in the EU where they are propagated and grown as ornamentals in commercial greenhouses. The Netherlands is the leading producer of *Anthurium* cut flowers and pot plants. Because of its economic importance for the EU, Xad is considered an organism recommended for regulation as quarantine pest (EPPO, 2009).

Strains isolated from *Syngonium* have been named as pathovar *syngonii* based on host range, symptomatology and some physiological differences from the other Xad strains (Dickey & Zumoff, 1987; Lipp *et al*., 1992), although this separation has also been questioned (Chase *et al*., 1992).

Bacterial leaf blight of aroids is known under different symptomatologies, with local infections on leaves and spathe, and systemic infections (Fukui *et al*., 1998). Early symptoms are small star-shaped spots eventually with some yellowing and water soaking and become necrotic under dry conditions. Leaf spots can coalesce to large, V-shaped or irregular brown necrotic areas with a bright yellow margin. Systemic infections occur when the pathogen invades the vascular system further spreading to other parts of the plant. Eventually the entire plant can be killed. In *Anthurium* the disease can display both leaf and systemic infections (Fukui *et al*., 1998).

Xad seems primarily transmitted to aroid production fields through infected nursery stock (Norman & Alvarez, 1994). Splashing water (rain or irrigation), aerosols, infested soil and possibly nematodes during planting are other sources of Xad-infection. Infected plant debris is another suspected primary source of Xad-infection. Studies have also shown that the pathogen can survive in plant debris for more than four months (Duffy, 2000). Epiphytic survival and aroid leaf invasion by Xad, as well as disease development, are all favored by high temperature and humid conditions. Symptoms typically develop faster on young plants than on older, matured plants. Xad invades the leaf through hydathodes or wounds but in some cases, under conditions that favor the opening of the stomata (light intensity and humidity) the bacterium enters the leaf through stomata. Under moist conditions (high relative humidity), the bacteria multiply on the leaf surface and increase the probability of infection, and once inside the plant tissue, the spots enlarge and merge to cover large areas. However, some infected plants can remain asymptomatic while the bacteria multiply and spread throughout the plant; such plants can exude guttation fluid containing bacteria (Norman *et al*., 1999). Studies have shown that the amino acids found in guttation fluid provide nutrients for invading bacteria. These exudates can then be dispersed to new plants by dripping or splashing.

Several earlier studies reported on the heterogeneity of Xad strains and suggested that they may represent different pathovars or species, but it was only recently that a taxonomic revision was made (Constantin *et al*., 2016). The study by Constantin *et al*., (2016) reclassified Xad strains to three species, with the strains isolated from *Anthurium and Syngonium* to *X. phaseoli*, strains isolated from *Philodendron* to *X. euvesicatoria,* and strains isolated from *Dieffenbachia*, *Xanthosoma* and *Aglaonema* to *X. citri*. The *X. phaseoli* pv. *dieffenbachiae* strains are predominantly isolated from *Anthurium* and *X. citri* pv. *aracearum* have been isolated from various aroid hosts such as: *Aglaonema*, *Alocasia*, *Caladium*, *Colocasia*, *Dieffenbachia*, *Epipremnum*, *Rhaphidophora Spathiphyllum* and *Xanthosoma*, (Lipp *et at*., 1992). Until now, all Xad strains infecting aroids are considered EU regulated, with even more ambiguity on the position of the pathogen occurring on *Syngonium*, known as *X. campestris* pv. *syngonii*. For the implementation of sanitary regulation it is of utmost importance to rely on a correct identification of the target pathogen and its pathogenic impact.

Many candidate pathogenicity factors have been identified in *Xanthomonas*. The most important are the protein secretion systems and their effectors (Buttner & Bonas, 2010). Of special interest is the type III secretion system (T3SS) encoded by the *hrp* (Hypersensitive Response and Pathogenicity) gene cluster (Ryan *et al*., 2011) and its type III secretion effector (T3E) repertoire, which play an important role in plant-pathogen interactions and in defining host range (Hajri *et al*., 2009; White *et al*., 2009). Also, other important elements, such as extracellular polysaccharides (EPS) and cell wall degrading enzymes (CWDE) (Buttner & Bonas, 2010), are known to be involved in pathogenicity and virulence of xanthomonads. Knowledge on the virulence elements of Xad is limited: so far, only one study looked at the T3E repertoire of Xad strains isolated from three different aroid hosts and concluded that they were almost identical (Hajri *et al.*, 2009).

The objective of this study was to evaluate the pathogenicity of strains previously named Xad in order to identify these strains as groups of Araceae pathogens within their respective species, incorporating the phylogenomic and pathogenic diversity, and possible plant health regulatory implications. Therefore, pathogenicity tests were made on six aroid genera with strains representative for the three species differentiated among Xad strains, and whole genome sequences were explored for sets of pathogenicity genes present.

3.2 Materials and methods

3.2.1 Bacterial strains and inoculum preparation

Ten Xad strains and one strain of *Xanthomonas campestris* pv. *syngonii* were used in this study (Table 3.1). The strains were received from LMG (BCCM/LMG Belgian Coordinated Collections, Bacteria Collection), CIRM-CFBP (International Centre for Microbial Resources-French Collection of Plant Associated Bacteria), and PD (Culture Collection of Plant Pathogenic Bacteria, Plant Protection Service, the Netherlands) and were grown on Wilbrink-N at 28° C for 48h. Strain allocation to the respective species group was based on MLSA of seven housekeeping genes (Constantin *et al*., 2016). Inoculum for the pathogenicity tests was prepared from cultures grown for 24 hours. Bacterial cells were suspended in 10 mM phosphate buffer (PB) and the suspensions were adjusted to A_{600} = 0.1 OD, corresponding to approximately 1 x 10^8 CFU/ml, and then further diluted to approximately 1 x 10^6 CFU/ml, which was verified by dilution plating. Within one hour after preparation, aliquots of 100 µL (about 10^5 cells) were used for inoculation of plants.

Table 3.1. Pathogenic *Xanthomonas* strains used in this study, isolated from different aroid plants and different geographical origins.

Strain[a]	Received as	New species allocation[b]	Proposed pathovar name[c]	Plant origin	Geographical origin	Year of isolation
LMG 695[PT]	*X. axonopodis* pv. *dieffenbachiae*	*X. phaseoli*	*dieffenbachiae*	*Anthurium* sp.	Brazil	1965
LMG 25940	***X. axonopodis* pv. *dieffenbachiae***	***X. phaseoli***	***dieffenbachiae***	*Anthurium* sp.	Brazil	1998
PD 4485	*X. axonopodis* pv. *dieffenbachiae*	*X. phaseoli*	*dieffenbachiae*	*Anthurium 'Leni'*	The Netherlands	2002
PD 4015	*X. axonopodis* pv. *dieffenbachiae*	*X. phaseoli*	*dieffenbachiae*	*Anthurium andreanum*	The Netherlands	2000
LMG 9055[PT]	***X. campestris* pv. *syngonii***	***X. phaseoli***	***syngonii***	*Syngonium podophyllum*	USA	1984
PD 3821	*X. axonopodis* pv. *dieffenbachiae*	*X. citri*	*aracearum*	*Aglaonema* 'Silver'	USA	2000
LMG 532[PT]	*X. campestris* pv. *aracearum*	*X. citri*	*aracearum*	*Xanthosoma sagittifolium*	Guadeloupe	1972
LMG 7399	***X. axonopodis* pv. *dieffenbachiae***	***X. citri***	***aracearum***	*Dieffenbachia* sp.	USA	1950
LMG 12894	*X. axonopodis* pv. *dieffenbachiae*	*X. euvesicatoria*		*Philodendron* sp.	USA	1992
LMG 12749	***X. axonopodis* pv. *dieffenbachiae***	***X. euvesicatoria***		*Philodendron* sp.	USA	1992
CFBP 5693	*X. axonopodis* pv. *dieffenbachiae*	*X. euvesicatoria*		*Philodendron scandens*	USA	NA

PT: pathotype strain. Whole genome sequences were produced for the strains in bold. NA: not available.
[a]LMG: BCCM/LMG Bacteria Collection, Belgium, CFBP: French Collection of Plant Associated Bacteria, PD: Culture Collection of Plant Pathogenic Bacteria, Plant Protection Service, The Netherlands.
[b] According to Constantin *et al*., 2016.
[C] This study.

3.2.2 Pathogenicity tests on aroids

The eleven strains listed in Table 3.1 were inoculated on six aroid genera: *Anthurium andraeanum* 'Hearts Desire'*, Aglaonema commutatum* 'Maria', a complex interspecific hybrid *Alocasia watsoniana* x *Alocasia sanderiana* 'Polly', *Dieffenbachia maculata* 'Camille', *Syngonium podophyllum* 'White Butterfly' and *Philodendron scandens* subsp. *oxycardium*. The selected aroid genera are commonly used for pathogenicity tests with Araceae pathogens and they are the host plant for many of the tested strains (Chase *et al*., 1992; Robéne-Soustrade *et al*., 2006). The eleven tested strains are representative of the three phylogenetic groups. Two inoculation methods were used to evaluate the capability of the strains to cause either local and/or systemic infection.

In a first evaluation for pathogenicity, the eleven strains were infiltrated in the leaf mesophyll, each strain on five plants per aroid genera. This is the method commonly used for pathogenicity testing of Xad strains on aroids (Lipp *et al*., 1992; Berthier *et al*.; 1993; Robène-Soustrade *et al.*, 2006). On each plant, two young fully developed leaves were inoculated at two different points per leaf (four inoculations per plant). The bacterial suspension was infiltrated with a syringe into the mesophyll and the visible infiltrated area was marked with a pencil. The plants were placed in covered, large transparent polypropylene boxes to obtain maximum humidity conditions. On the same day of inoculation, the plants were transferred to a greenhouse under conditions of 100% humidity and day/night temperatures of 28±1/22±1°C, which are optimal for disease development. Symptom development was monitored over a two-month period. Symptoms were visually examined and rated using the scale illustrated in Figure 3.1. Each leaf inoculation point was rated resulting in 20 ratings for each strain per aroid genera.

In a second evaluation for pathogenicity, the eleven strains were tested for their capability to cause disease symptoms when introduced in the vascular system. A 10 µl droplet of bacterial suspension was placed on a petiole and the petiole was then pierced through the droplet with a 25GA 5/8 inoculation needle. Each strain was tested on two plants per aroid genera, and on each plant the petioles of two young fully developed leaves were inoculated. The point of inoculation was wrapped with parafilm to seal the wound.

The plants were observed for two months for symptom development, and inoculation responses were then further verified by re-isolation from the affected plant tissue. A sample, 1 cm^2, was taken from the leaf tissue, cut into small pieces and transferred to sterile distilled water with shaking for a few seconds at 800 rpm. The extracts were left for 10-15 minutes to allow diffusion of bacteria out of the tissue, and then plated on Wilbrink-N medium. The identity of re-isolated bacteria was checked by partial *gyr*B gene sequencing (Parkinson *et al*., 2009). The differences between the strain from *Dieffenbachia* and the strains from *Anthurium* or *Syngonium* are 29 anf 28 SNPs, respectively. The difference between the strain from *Anthurium* and the strain from *Syngonium* is 8 SNPs. The

differences between the strain from *Philodendron* and those from *Anthurium, Syngonium* and *Dieffenbachia* are 14, 15 and 24 SNPs, respectively.

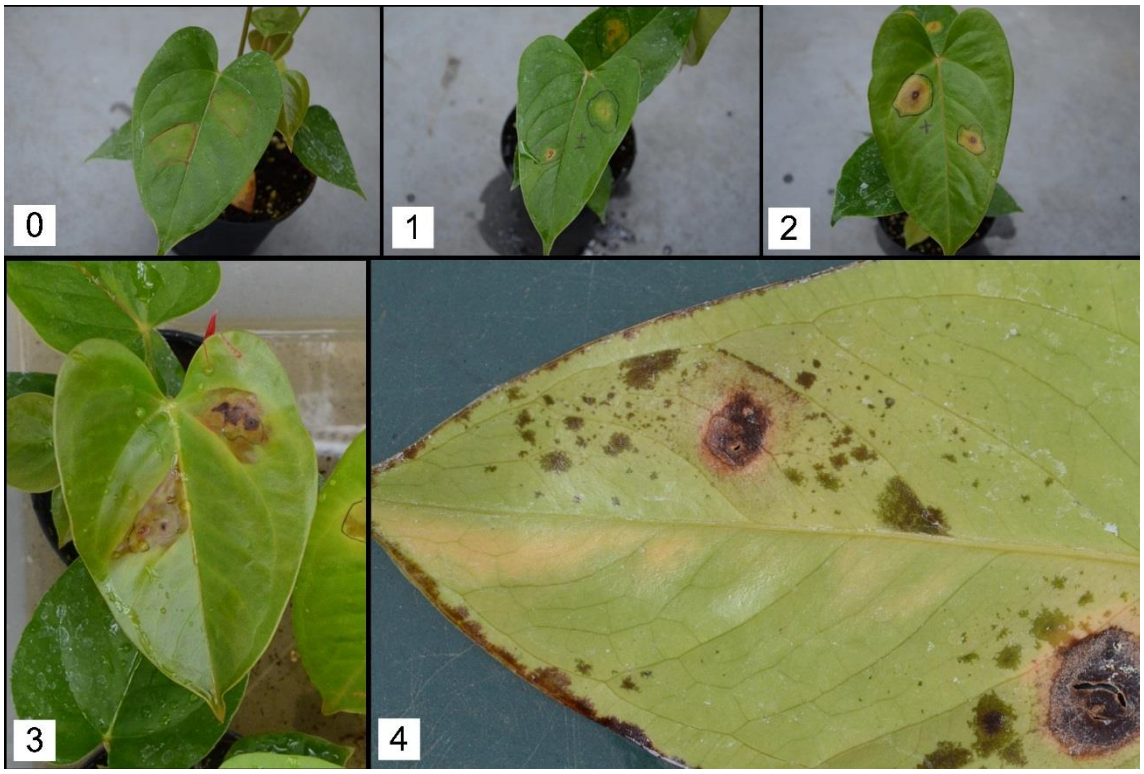

Figure 3.1. Symptoms produced after infiltration of bacterial suspensions of *Xanthomonas axonopodis* pv. *dieffenbachiae* strains into the leaf mesophyll of *Anthurium*. Disease scores: 0 = no symptoms; 1 = discoloration or necrosis smaller than the infiltrated leaf area; 2 = water-soaked or discolored area not expanding beyond the infiltrated leaf area; 3 = water-soaked or discolored area expanding beyond the infiltrated leaf area; 4 = oily spots and/or yellowish leaf parts, some associated with wilting of the leaf.

3.2.3 Whole-genome sequencing, assembly and gene annotations

Four strains were used for whole genome analysis: LMG7399, LMG9055, LMG12749 and LMG25940 (Table 3.1). A 350 bp insert whole genome shotgun library was constructed previously for LMG 7399, LMG 9055 and LMG 12749 to generate rough assemblies with +/- 30X coverage for average nucleotide identity (ANI) analyses (Constantin *et al*., 2016). These 30X assemblies were insufficient for a complete genome comparison; therefore a second sequencing run was initiated in this study to obtain a better assembly for in-depth genome analysis. A 500 bp insert library using multiplex Illumina TruSeq v3.0 technology was constructed for each strain to generate assemblies with +/- 100X coverage. Paired-end (PE) sequencing (2x91bp) was performed on an Illumina Hiseq2000 instrument at BGI, Hong Kong. Subsequently, demultiplexed samples were trimmed (based on a threshold of *Q* = 20) with an extra adapter and duplicate removal step using CLC GENOMICS WORKBENCH v. 7.5.

De novo assembly was performed with the trimmed PE datasets using DNASTAR SEQMAN NGEN v. 12.1.0 build 137, MIRA v. 4.0.2, SOAPDENOVO2 v. 2.04, CLC GENOMICS WORKBENCH v. 7.5 or SPADES v. 3.1.0, with or without scaffolding and with a minimum contig size of 200 bp. Assembly QC metrics were calculated

for all resulting assemblies using QUAST v. 2.3. The best assemblies were chosen based on N50 values, number of contigs and % of mapped reads. Annotation was performed using RAST (Overbeek *et al*., 2014).

3.2.4 Comparative genome analysis

For comparative genomic analysis, orthologous groups were determined by a reciprocal BLAST approach. The predicted proteins of the four genomes were downloaded from the RAST server. Pairwise comparisons between the protein sets of each genome were done by performing BLASTP searches. Sequences were considered orthologous when they were each other's top hit in the BLAST searches, with a minimum of 60% identity and a minimum query length coverage of 75%. The resulting orthologous groups were manually checked. Genome assemblies were also submitted to PATRIC (Wattam *et al*., 2013) for additional comparative analyses using the PROTEIN FAMILY SORTER tool to confirm the results from the reciprocal BLAST searches. To identify putative type III secretion effectors, BLASTN and TBLASTN searches were carried out for all known T3E genes listed on the Xanthomonas.org website (White *et al*., 2009) against the four genome assemblies (LMG 25940, LMG 7399, LMG 12749, LMG 9055). The resulting BLAST hits (E < 1e-10) were manually checked for frameshifts or stop-codons. A similar strategy was used for plant cell wall degrading enzymes (CWDE), LPS cluster, type II, type IV and type VI secretion systems, where known gene and/or protein sequences of different *Xanthomonas* species were used as query in BLAST searches. In addition, the RAST annotations were manually screened for possible additional homologs. To identify type VI secretion system (T6SS) effectors a T6SS-related protein class (COG3519) was used as bait (Vandroemme *et al.*, 2013). Sequences which showed similarity to the T6SS-related COG3519 family proteins retrieved from other *Xanthomonas* genomes were collected and compared in BIONUMERICS v. 7.0 software (Applied Maths). A tree was generated with MEGA v.6 software using Maximum Likelihood algorithm and Tamura-Nei model with 1000 bootstrap replicates.

3.3 Results

3.3.1 Pathogenicity tests on aroids

The pathogenicity tests by leaf infiltration produced different degrees of symptoms depending on the strain and the aroid used (Table 3.2). No symptoms were observed in the control plants infiltrated with sterile buffer. In general, the cultivars of *Aglaonema* and *Philodendron* used in these tests were the most and the least susceptible aroids, as expressed in a mean pathogenicity score of 2.6 and 1.6, respectively (Table 3.2). *Aglaonema commutatum* 'Maria' was susceptible to nearly all tested strains, but was the least susceptible to the strains originally isolated from *Philodendron*. On the other hand,

Philodendron scandens was the least susceptible aroid and was also not susceptible to the strains originally isolated from *Philodendron*; sometimes no symptoms at all could be observed on the inoculated plants during the 2-month observation period.

The pathogenicity tests by pricking the leaf petiole differentiated two groups among the Xad strains, those able to cause systemic infection and those that did not. Systemic infection was only observed for the strains that belong to the species *X. phaseoli*, which are the ones originally isolated from *Anthurium* and *Syngonium*. The strains originally isolated from *Aglaonema*, *Xanthosoma*, *Dieffenbachia* and *Philodendron* that belong to the species *X. citri* and *X. euvesicatoria*, did not cause systemic infection by the pin-prick inoculation of leaf petioles.

Table 3.2. Pathogenicity scores for the *Xanthomonas axonopodis* pv. *dieffenbachiae* strains two months after inoculation of the leaves or in the petiole vascular tissue of six aroid plant species.

				Virulence index[c]					
Strain	Species name[a]	Pathovar name[b]	Original host	*Aglaonema*	*Alocasia*	*Anthurium*	*Dieffenbachia*	*Philodendron*	*Syngonium*
LMG 695[PT]	*X. phaseoli*	pv. *dieffenbachiae*	*Anthurium* sp.	2.2 ± 0.5	1.7 ± 0.8	2 ± 0.0/S	2.7 ± 0.9	1.6 ± 0.6	2 ± 0.3
PD 4485	*X. phaseoli*	pv. *dieffenbachiae*	*Anthurium 'Leni'*	2.8 ± 0.8	2.4 ± 0.9	3.9 ± 0.3/S	2.1 ± 0.3/S	1.6 ± 0.6	1.8 ± 0.4
PD 4015	*X. phaseoli*	pv. *dieffenbachiae*	*Anthurium andreanum*	2.6 ± 0.8	2.3 ± 0.5	4 ± 0.0/S	2.3 ± 0.5	1.4 ± 0.5	2 ± 0.0
LMG 25940	*X. phaseoli*	pv. *dieffenbachiae*	*Anthurium* sp.	2.1 ± 0.3	4 ± 0.0	2.5 ± 0.9	2.6 ± 0.7/S	1.8 ± 0.4	2 ± 0.5
LMG 9055[PT]	*X. phaseoli*	pv. *syngonii*	*Syngonium podophyllum*	2.9 ± 0.6	0 ± 0.0	1 ± 0.6	0.5 ± 0.8	0.9 ± 0.9	4 ± 0.0/S
PD 3821	*X. citri*	pv. *aracearum*	*Aglaonema* 'Silver'	3.8 ± 0.4	3 ± 0.6	0.7 ± 0.7	0.9 ± 0.8	2 ± 0.4	2.8 ± 1.0
LMG 532[PT]	*X. citri*	pv. *aracearum*	*Xanthosoma sagittifolium*	2.7 ± 0.9	0.7 ± 0.8	1.3 ± 0.6	1.4 ± 0.6	1.4 ± 0.6	2.3 ± 0.7
LMG 7399	*X. citri*	pv. *aracearum*	*Dieffenbachia* sp.	2.9 ± 0.9	3 ± 0.5	2 ± 0.2	2.3 ± 0.7	1.8 ± 0.4	2.3 ± 0.5
LMG 12894	*X. euvesicatoria*		*Philodendron* sp.	2 ± 0.0	0.6 ± 0.8	1.3 ± 0.6	1.9 ± 0.4	1.8 ± 0.7	1.3 ± 0.5
LMG 12749	*X. euvesicatoria*		*Philodendron* sp.	2 ± 0.0	1.5 ± 0.7	1.6 ± 0.5	2 ± 0.0	1.2 ± 0.6	1.1 ± 0.5
CFBP 5693	*X. euvesicatoria*		*Philodendron scandens*	2.2 ± 0.6	1.6 ± 0.6	1.4 ± 0.5	1.8 ± 0.4	1.6 ± 0.5	1.4 ± 0.5
	Mean[¶]			2.6	1.9	2	1.9	1.6	2.1

PT = pathotype strain.

[a] According to Constantin *et al*., 2016.

[b] Proposed in this study.

[c] Given values are the mean (± standard deviation) of 20 leaf inoculations (four inoculations per plant, five plants per strain), each leaf inoculation point was rated using the following scale: 0 = no symptoms, 1 = discoloration or necrosis smaller than the infiltrated leaf area, 2 = water-soaked or discoloration not expanding beyond the infiltrated leaf area, 3 = water-soaked area expanding beyond the infiltrated leaf area, 4 = oily spots and/or yellowish leaf parts, some associated with wilting of the leaf; S= systemic infection observed in at least one of the four inoculated leaf petioles upon pricking of the vascular tissue.

The *X. phaseoli* strains (strains originally isolated from *Anthurium* and *Syngonium*)

The strains isolated from *Anthurium* (Table 3.2) infected all studied aroid plants with leaf pathogenicity scores ≥ 2 (except *Philodendron*). Disease severity varied with the strain/aroid combination but, within two weeks, leaf infiltration resulted in a light green to yellowish discoloration of the inoculated leaf area. In some cases, the symptoms spread over the leaf surface, with oily spots and yellowish patchy areas. Four to five weeks after inoculation, the lesions were necrotic and often extended beyond the infiltrated area. After two months, about 50% of the inoculated leaves were wilted and dead.

The strains isolated from *Anthurium* also caused a systemic infection by pin-pricking of the leaf petiole in *Anthurium* and/or *Dieffenbachia* (Figure 3.2a,b). In *Anthurium*, this resulted in water-soaked and necrotic spots along the veins, spreading towards the leaf margins, and finally covering the entire leaf. After 1-2 weeks the whole leaf turned yellow, the petiole began to rot at the base and the leaf dropped. In *Dieffenbachia*, the systemic infection appeared as brown zones in the midrib together with water-soaked spots in the surrounding mesophyll (Figure 3.2b). The strain LMG 9055 isolated from *Syngonium* displayed strong host specialization by causing severe leaf symptoms and systemic infection only in its original host *Syngonium* (Figure 3.3). The strain from *Syngonium* also caused leaf spots on *Aglaonema* but did not cause any symptoms on the other tested aroids. In *Syngonium*, symptoms spread over the whole leaf and to other uninoculated leaves. About 2 weeks after leaf infiltration, the first symptoms appeared as oily spots that, after 4 weeks, became necrotic and surrounded by a bright yellow margin. The lesions developed a papery appearance. In the final stage, diseased leaves turned yellow and completely withered.

Figure 3.2. Systemic infection caused by *X. phaseoli* strains, originally isolated from *Anthurium*, after leaf petiole pin-prick inoculation of LMG 695 into *Anthurium* (a) and LMG 25940 into *Dieffenbachia* (b).

Figure 3.3. *X. phaseoli* strain LMG 9055, originally isolated from *Syngonium,* caused typical blight symptoms on *Syngonium podophyllum* 'White Butterfly' after leaf infiltration (a) and leaf petiole pin-prick inoculation (b).

The *X. citri* strains (strains originally isolated from *Aglaonema*, *Xanthosoma* and *Dieffenbachia*)

These strains showed dissimilarity in their virulence to the tested aroids. The strain LMG 7399 from *Dieffenbachia* had the highest virulence (Figure 3.4, Table 3.2) after inoculation by leaf infiltration, but none of the strains caused a systemic infection after pin-prick inoculation in the petiole. Generally, the initial symptoms were a slight yellowing of the infiltrated leaf area and water-soaked spots that subsequently turned necrotic. As the disease progressed, more leaf tissue was killed and the large, brown areas were surrounded by a yellow border. Although symptom expression varied, no correlation could be established between strain/aroid combination.

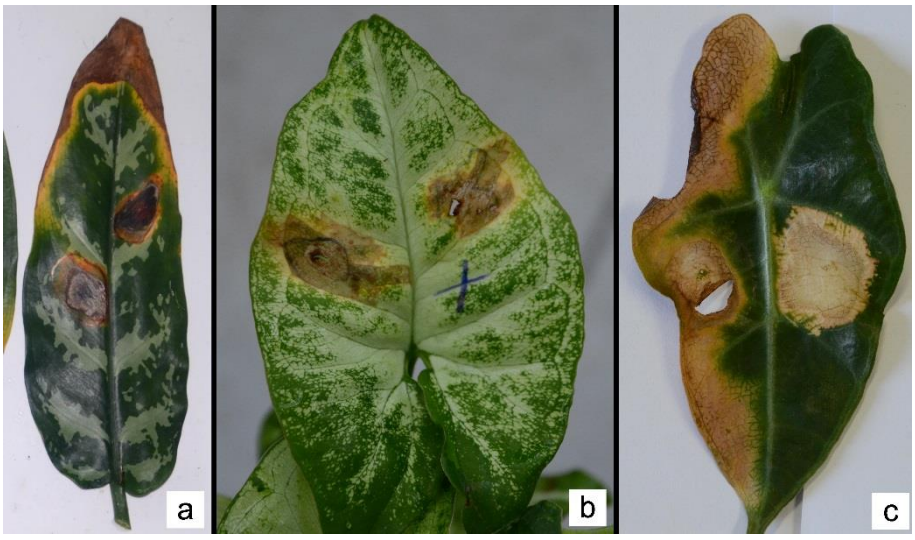

Figure 3.4. Typical leaf blight symptoms caused by the *X. citri* strains. Inoculation of *Aglaonema commutatum* 'Maria' with strain PD 3821 (a), *Syngonium podophyllum* 'White Butterfly' with strain LMG 7399 (b) and *Alocasia* with strain LMG 7399(c).

The *X. euvesicatoria* strains (strains originally isolated from *Philodendron*)

These strains did not show a clear pathogenicity, causing no or very weak symptoms in the aroids tested. In addition, they did not induce a systemic infection in any of the tested aroids after the pin-prick inoculation of petioles. Thus, these strains can be considered as non-pathogenic, even in *Philodendron* from where they have originally been isolated. Tests were performed in *Philodendron scandens*, but the results were also confirmed in *Philodendron 'Xanadu'* (data not shown). Even though no symptoms were observed on inoculated plants during the 2-month observation period, inoculated strains could be re-isolated from the symptomless infiltrated leaf areas indicating that they survived within the leaf tissue (results not shown).

3.3.2 Analyses of draft genome sequences of four strains previously known as Xad

The draft genome sequences of *X. phaseoli* pv. *dieffenbachiae* strain LMG 25940 (hereafter Xpd), *X. phaseoli* pv. *syngonium* strain LMG 9055 (hereafter Xps), *X. citri* pv. *aracearum* strain LMG 7399 (hereafter Xca), and *X. euvesicatoria* strain LMG 12749 (hereafter Xe) were assembled by combining different datasets (a 2x50bp read set from the previous study (Constantin *et al*., 2016) and a 2x91bp read set from this study). The best assemblies were obtained using the following software/dataset combinations for Xpd: NGEN assembly without scaffolding using the 2x91bp reads only; for Xps: CLC GENOMICS WORKBENCH assembly with scaffolding using the 2x91 bp reads only; for Xca: CLC Genomics Workbench assembly without scaffolding using the 2x91bp reads only and for Xe: NGEN assembly without scaffolding using both read sets. After automatic annotation by the PGAAP online annotation pipeline, these draft assemblies were deposited in GenBank WGS database with accession numbers: JPYH02000000 (LMG 7399), JPUN02000000 (LMG 12749), JPYI02000000 (LMG 25940), JPUO02000000 (LMG 9055). RAST annotations are available with accession numbers: LMG7399 (Xca): 92828.12; LMG25940 (Xpd): 6666666.94921; LMG12749 (Xe): 92828.9; LMG9055 (Xps): 270916.4.
Assembly metrics are shown in Table 3.3. The N50 (minimum number of contigs needed to cover 50% of the assembly) values are 146, 52 and 216 for Xpd, Xps and Xe, respectively, and 63 for Xca. All four draft genomes have a high GC content (~65%) as commonly reported for members of the genus *Xanthomonas* (Ryan *et al*., 2011).

The predicted protein sequences of the four strains were compared by reciprocal BLAST searches and the analysis suggested that the core genome consists of 3058 orthologs found in all four genomes. A Venn diagram representing the common and the specific genes of all four genomes is shown in Figure 3.5. Interestingly, 175 genes were specifically shared between the two vascular pathogens (i.e. those able to cause systemic infection after pin-prick inoculation of the petiole) Xpd and Xps (Supplementary Table 3.1). The gene sequences coded for acetyltransferase, ABC transporters, components of type IV

pilus. More than half of the genes shared by Xpd and Xps belonged to hypothetical or unknown proteins.

Table 3.3. General features of the four *Xanthomonas* genome sequences.

	Xca (LMG7399)	**Xe (LMG 12749)**	**Xpd (LMG 25940)**	**Xps (LMG 9055)**
Total contig size (bp)	5.127.485	4.887.792	5.030.124	4.976.908
No. contigs (>200 bp)	205	55	105	232
N50 (bp)[a]	63,001	216,885	146,936	52,766
Largest contig size (bp)	175,936	758,646	587,613	177,161
Average coverage	128.16	117.47	130.3	135.91
Mapped reads (% of total)	99.46	99.98	99.94	99.63
GC content (%)	64.55	65.09	64.89	64.85
Protein coding part (% of contigs)	83	85	84	84
Protein coding genes predicted by RAST	4359	4230	4327	4432
Average ORF length (bp)	981	978	972	939
rRNA operons	3	3	3	5
tRNA genes	54	53	51	48
Insertion sequence elements	20	25	26	21
RAST ID	92828.12	92828.9	6666666.94921	270916.4
GenBank accession number	JPYH02000000	JPUN02000000	JPYI02000000	JPUO02000000

Xca: *Xanthomonas citri* pv. *aracearum*; Xe: *X. euvesicatoria*; Xpd: *X. phaseoli* pv. *dieffenbachiae*; Xps: *X. phaseoli* pv. *syngonii*.
[a] Size of the smallest contig in the N50 set.

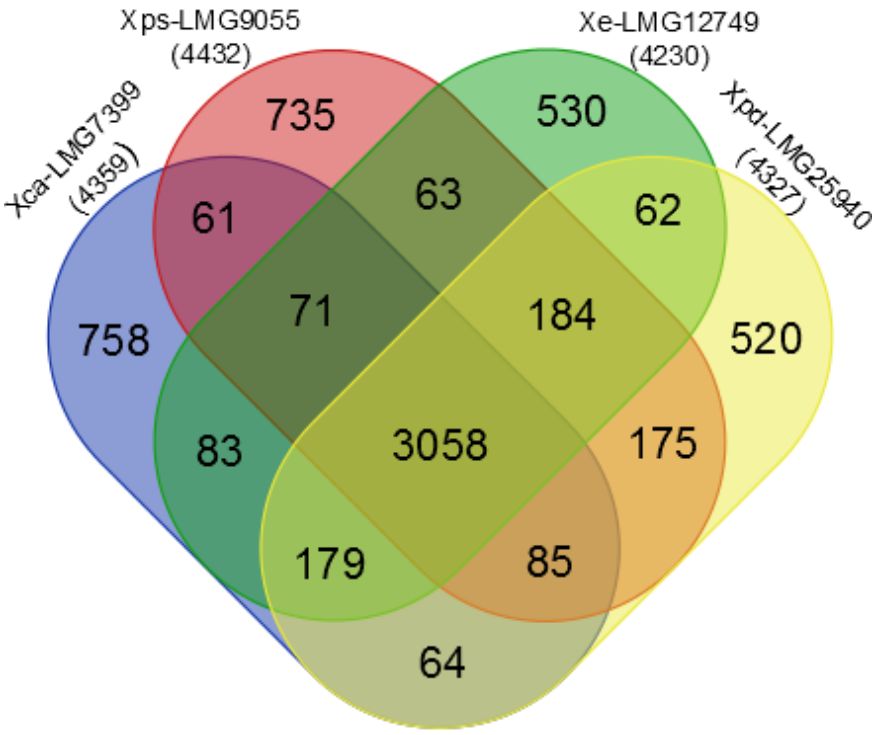

Figure 3.5. Venn diagram representing the common and the specific genes between four genomes of *Xanthomonas* strains from aroids. Yellow = *X. phaseoli* pv. *dieffenbachiae* (Xpd) strain LMG 25940, red = *X. phaseoli* pv. *syngonii* (Xps) strain LMG 9055, green = *X. citri* pv. *aracearum* (Xca) strain LMG 7399 and blue = *X. euvesicatoria* (Xe) strain LMG 12749. Numbers in parentheses represent the amount of protein coding genes found for each strain; they are divided in the diagram as specific or shared between strains.

3.3.3 Genes of the type III secretion system (T3SS)

Annotation of the T3SS gene cluster showed that all four strains have an almost identical *hrp* cluster organization (Figure 3.6). The gene order is similar and consistent with the previously characterized *Xanthomonas hrp* cluster (Jalan *et al*., 2011).

The cluster is composed of 27 genes in Xpd and Xca, 29 genes in Xps, and 30 genes in Xe, extending from *hpa2* to *hrpF* in a region of approximately 27 kb. The three extra genes in Xe are two mobile genetic elements, located between *hpa1* and *hpa2,* and a gene for a hypothetical protein; the extra genes in Xps encode two hypothetical proteins. It has been suggested that loci flanking the *hrp* cluster on both sides are predisposed to the insertion of mobile genetic elements carrying virulence genes and being part of pathogenicity islands (Darrasse *et al*., 2013).

Comparison of the type III secretion effector (T3E) repertoire between the four genome sequences (Table 3.4) allowed the identification of effectors that are either conserved in all four strains, that are strain-specific, or that are common only to the aroid pathogens, (thus differentiating them from the Xe strain that was shown to be nonpathogenic to aroids. Together, a total of 39 out of the 64 T3E gene families identified in other *Xanthomonas* genomes (www.xanthomonas.org; White *et al*., 2009) were found over the four strains (Table 3.4). Three T3Es (*xopE2*, *xopG*, *xopAM*) were found specific for the aroid pathogens and are missing from Xe LMG 12749 (Table 5). These three effectors are important pathogenicity candidates on aroids. Overall, the most aggressive pathogens on aroid plants (*X. phaseoli* strains LMG 25940 and LMG 9055) had the least number of T3E genes that were estimated functional (20 and 22 genes for LMG 25940 and LMG 9055, respectively), while Xe strain LMG 12749 which proved nonpathogenic on the aroids tested, had the greatest T3E repertoire with 27 genes. The strain isolated from *Syngonium* had more T3E genes than the strain isolated from *Anthurium* which was also observed by Robéne *et al*. (2016) for LMG 695 from *Anthurium* and LMG 9055 from *Syngonium*. Moreover, the *XopJ5*, *XopAF* and *XopAJ* genes which were only present in Xe strain LMG 12749 had low GC content (*XopJ5* (*AvrXccB)* - 59%, *XopAF* (*AvrXv3*) - 53%, *XopAJ* (*AvrRxo1*) - 50%) compared to an average genome GC content of 65%. Also, non-functionality of T3 effectors due to gene truncations and frameshifts was higher in the strains that were pathogens on aroids (8, 6, and 6 genes affected in LMG 25940, LMG 9055, and LMG 7399, respectively) than in Xe strain LMG 12749 (4 genes affected).

To check for occurrence of possible sequencing errors that may be responsible for the results, these gene regions (*XopI*, *XopK*, *XopL* and *XopV*) were PCR-amplified and Sanger sequenced using standard conditions. All original sequences were confirmed, and thus supported the correctness of the above findings.

Figure 3.6. Comparison of type III secretion system clusters of the four sequenced strains of *Xanthomonas*. HP = hypothetical protein; ME = mobile element; LMG 12749 = *X. euvesicatoria* strain isolated from *Philodendron*, LMG 25940 = *X. phaseoli* pv. *dieffenbachiae* strain isolated from *Anthurium*, LMG 7399 = *X. citri* pv. *aracearum* strain isolated from *Dieffenbachia*, LMG 9055 = *X. phaseoli* pv. *syngonii* strain isolated from *Syngonium*.

Table 3.4. Overview of the type III secretion effector repertoire in each of the four *Xanthomonas* genomes.

	Gene	Xca - LMG 7399 (*Dieffenbachia*)	Xpd - LMG 25940 (*Anthurium*)	Xe - LMG 12749 (*Philodendron*)	Xps - LMG 9055 (*Syngonium*)
1	*avrBs1*	0	0	0	1
2	*avrBs2*	1	1	1	1
3	*hpa2*	1	1	1	1
4	*hpaA*	1	1	1	1
5	*xopA*	1	1	1	1
6	*xopB*	1	0	0	0
7	*xopC2*	1	1	1	1
8	*xopE1*	1	1	1	0
9	*xopE2*	2	1	0	1
10	*xopF1*	1	1	ΨF	ΨF
11	*xopF2*	ΨT	ΨT	1	1
12	*xopG*	1	1	0	1
13	*xopI*	ΨF	0	1	1
14	*xopJ5*	0	0	1	0
15	*xopK*	ΨF	1	1	ΨF
16	*xopL*	ΨT	1	1	1
17	*xopN*	1	1	1	1
18	*xopP*	1	1	1	2
19	*xopQ*	1	1	1	1
20	*xopR*	1	1	1	0
21	*xopT*	0	0	0	Seq
22	*xopV*	1	ΨF	1	1
23	*xopW*	0	0	ΨF	0
24	*xopX*	1	ΨF	1	1
25	*xopZ1*	1	1	1	1
26	*xopAD*	0	ΨT	1	ΨT
27	*xopAE*	1	1	1	1
28	*xopAF*	0	0	1	0
29	*xopAG*	1	1	1	0
30	*xopAJ*	0	0	1	0
31	*xopAK*	1	ΨF	1	1
32	*xopAL2*	1	0	0	0
33	*xopAM*	1	1	0	1
34	*xopAO*	0	1	0	0
35	*xopAP*	1	ΨT	1	ΨT
36	*xopAU*	1	ΨT	1	1
37	*xopAV*	ΨT	ΨT	ΨT	ΨT

38	*xopAW*	0	0	1	0
39	*xopAX*	ΨT	0	ΨT	ΨT
Total number of functional T3E genes		24	20	27	22

gene regions were PCR-amplified and Sanger sequenced using standard conditions

"1": one homolog present

"2": two homologs present
"0": no homolog found

"ΨT": coding DNA sequence encountered, but protein believed inactive due to truncation

"ΨF": coding DNA sequence encountered, but protein believed to be inactive due to frameshift

"Seq": coding DNA sequence truncated due to incomplete genome-assembly; functional protein assumed present during further processing of data.

Table 3.5. Type III effector genes found in the genomes of the three *Xanthomonas* strains representing the three newly proposed pathovars on aroids.

Strains[b]	***X. phaseoli* pv. *dieffenbachiae* LMG 25940**	***X. phaseoli* pv. *syngonii* LMG 9055**	***X. citri* pv. *aracearum* LMG 7399**	**Pfam domains**
Effector gene [a]				
avrBs2	XPD_4278	XPS_3430	XCA_321	Glycerophosphoryl diester phosphodiesterase
hpa2	XPD_3175	XPS_3246	XCA_2529	-
hpaA	XPD_3157	XPS_3264	XCA_2547	-
xopA	XPD_3173	XPS_3247	XCA_2531	-
xopC2	XPD_376	XPS_3327	XCA_73	Haloacid dehalogenase-like hydrolase
xopE2	XPD_3101	XPS_4455/ XPS_4446[c]	XCA_2864, XCA_2967[d]	Putative transglutaminase
xopG	XPD_4330	XPS_4454	XCA_4362	M27-family peptidase
xopN	XPD_3872	XPS_2688	XCA_1373	ARM/HEAT repeat
xopP	XPD_377	XPS_3326	XCA_74	-
xopQ	XPD_49	XPS_2551	XCA_2652	Inosine uridine nucleoside N-ribohydrolase
xopZ1	XPD_1376	XPS_3104	XCA_871	-
xopAE	XPD_3149	XPS_4456	XCA_2556	LRR protein
xopAM	XPD_405	XPS_3153	XCA_100	-

RAST annotations of the three genomes: LMG 25940: 6666666.94921; LMG9055: 270916.4; LMG 7399: 92828.12.
[a]Bold; type III effector genes not found in *X. euvesicatoria* LMG 12749, which in pathogenicity tests appears not pathogenic to aroids.
[b]Strain names according to the taxonomic study of Constantin *et al*. (2016) and newly proposed pathovar name (this work).
[c]Coding DNA sequence found in two different contigs due to genome assembly, protein assumed functional.
[d]Two homologs present.

3.3.4 Genes of the type II secretion system (T2SS)

The T2SS is important for the secretion of toxins and cell-wall degrading enzymes in several *Xanthomonas* pathogens (Ryan *et al*., 2011). The *xps* gene cluster, present in all the sequenced pathogenic xanthomonads, is known for its contribution to virulence in *X. campestris* pv. *vesicatoria* The *xcs* gene cluster is found only in certain species of *Xanthomonas* and its virulence function is still unclear (Szczesny *et al*., 2010). In the four *Xanthomonas* genomes studied here, both the *xps* and *xcs* gene clusters were found as very conserved, with *xps* being composed of 11 genes and *xcs* of 12 genes (Supplementary Table 3.2).

3.3.5 Genes of the type IV secretion system (T4SS)

The T4SS has been described as an important bacterial factor helping bacterial adaptation to new hosts (Saenz *et al*., 2007). Eleven T4SS genes known to contribute to secretion of virulence factors were identified, but with big differences between the strains (Supplementary Table 3.3). Xpd and Xe strains have the same set of 11 T4SS genes, while Xca and Xps strains have only two but different T4SS genes. Sequence homology between the *vir* genes of Xpd and Xe ranged from 68 to 96%, which makes it unclear if these *vir* genes are all true orthologs. RAST annotated *VirB5*-like genes, but with a low sequence homology (~35%) to *VirB5* of *X. euvesicatoria* 85-10, and thus were interpreted as absent.

3.3.6 Genes of the type VI secretion system (T6SS)

The T6SS is the most recent characterized system implicated in eukaryotic cell targeting and virulence through effector secretion and described in several bacteria among which *Pseudomonas fluorescens* and *P. syringae* (Records & Gross, 2010). Genes of the T6SS were searched in the *Xanthomonas* genomes using the T6SS-related protein class (COG3519). Three T6SS sequence types have been discriminated within *Xanthomonas* (Potnis *et al*., 2011). In this study, two of these types were also found in the genomes of strains LMG 25940, LMG 9055 and LMG 12749, while LMG 7399 had only one type. Cluster analysis based on the sequence of this common T6SS gene, grouped together the three strains proven pathogenic on aroids (LMG 25940, LMG 9055, LMG 7399), clearly separating them from strain LMG 12749 that showed nonpathogenic to aroids (Figure 3.7). Xe strain LMG 12749 grouped with other *X. euvesicatoria* strains (such as strains formerly named *X. perforans* and X*. alfalfae* subsp. *citrumelonis*).

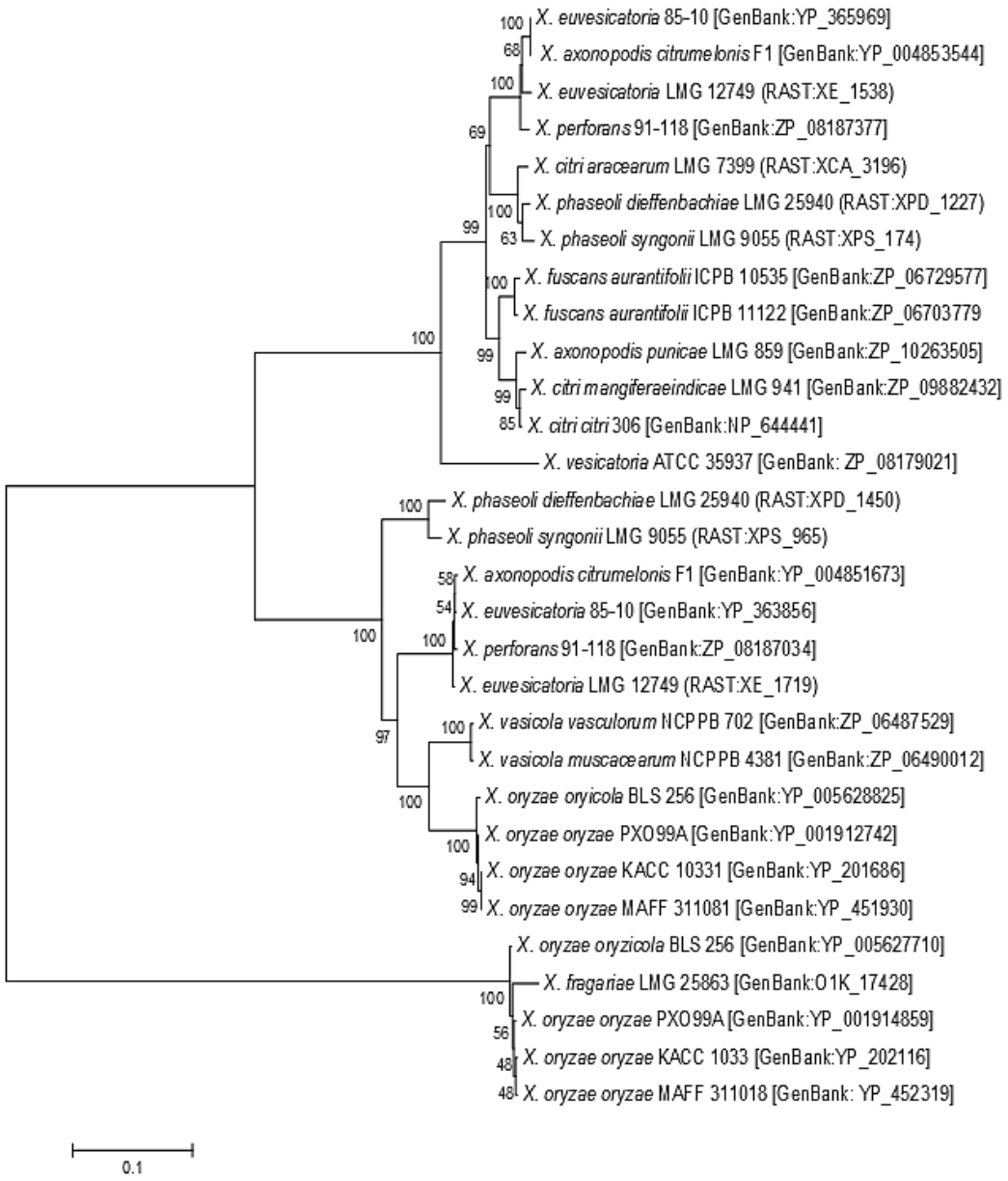

Figure 3.7. Relationship among type VI secretion system-related COG3519-family proteins retrieved from *Xanthomonas* genomes and compared in this study. The tree was constructed using the neighbor joining method and bootstrap values calculated with 1000 replicates are shown on the cluster nodes. Genbank accession numbers are given between rectangular brackets. RAST annotations of the four genomes generated in this study are: *X. citri* pv. *aracearum* strain LMG 7399: 92828.12; *Xanthomonas phaseoli* pv. *dieffenbachiae* strain LMG 25940: 6666666.94921*; X. euvesicatoria* strain LMG 12749: 92828.9; *X. phaseoli* pv. *syngonii* strain LMG 9055: 270916.4; RAST accession numbers are given in round brackets.

3.3.7 Genes of cell-wall degrading enzymes (CWDE)

All four genomes were screened for CWDE by looking for homologs of known *Xanthomonas* CWDE, combined with manual screening of RAST annotations. Each strain seemed to have its own combination of enzymes with pectinolytic, cellulolytic and hemicellulolytic activities (Supplementary Table 3.4). Xpd strain LMG 25940 has the most (40) and Xps strain LMG 9055 the least (27). Noteworthy

was the accumulation of inactive gene homologs due to frameshifts and truncations in the Xps strain LMG 9055.

3.3.8 TonB - dependent transporters

Outer membrane TonB-dependent transporters (TBDT) are involved in the active transport of plant nutrients, mostly carbohydrates (Schauer *et al*., 2008). There is a large variation in the number of TBDT genes among *Xanthomonas* and this number seem to be linked to the ecological niche and lifestyle of the species considered (Schauer *et al*., 2008). The four *Xanthomonas* genomes were screened for homologs of 96 TBDT references (Supplementary Table 3.5). The average TBDT gene repertoire ran up to 55 homologs, with Xca strain LMG 7399 having the most (62) and Xps strain LMG 9055 the least (44). The small TBDT sets in Xps strain LMG 9055 and Xpd strain LMG 25940 may indicate that these strains, like other species of *Xanthomonas* such as *X. oryzae*, *X. albilineans* and *X. fragariae*, are adapted to plant scavenging and to a life in nutrient-poor environments.

3.3.9 Genes involved in bacterial mobility

All four strains contain genes for flagellum synthesis in four similar clusters along the genomes. Clusters 1 and 3, as previously described by Moreira *et al*. (2010), are conserved among all four *Xanthomonas* genomes (Figure 3.8A). Cluster 1 consists of 17 genes from *flgM* to *fliS* and are spread over an approximately 18 kb region. Cluster 3 contains just two genes, coding for flagellar motor proteins A and B (*motA* and *motB*). Cluster 2 consists of 23, 24, 25 and 26 *fli* genes in LMG 7399, LMG 12749, LMG 25940 and LMG 9055, respectively (Figure 3.8B) and these genes are organized in an order similar to those in *X. euvesicatoria* pv *citrumelonis* (formerly *X. axonopodis* pv. *citrumelonis*; data not shown). However, the four genomes differ in the number and location of inserted hypothetical proteins in this cluster. Cluster 4 is part of a cluster identified in *X. citri* pv. *fuscans* (formerly, *X. fuscans* subsp. *fuscans*) (Moreira *et al*., 2010), and in all four draft genomes it contained 7 genes (Figure 3.8C). Furthermore, cluster 4 has several nearly repeated copies of a methyl-accepting chemotaxis protein gene (*mcp*), which is unusual in bacteria, being first reported in *X. campestris* pv. *campestris* and *X. citri* pv. *citri* (formerly *X. axonopodis* pv. *citri*) and suggested to have a remarkable role in chemotaxis (da Silva *et al*., 2002). In addition, multiple copies of the *mcp* gene are scattered throughout the four genomes, not only in the vicinity of the flagellum gene cluster 4.

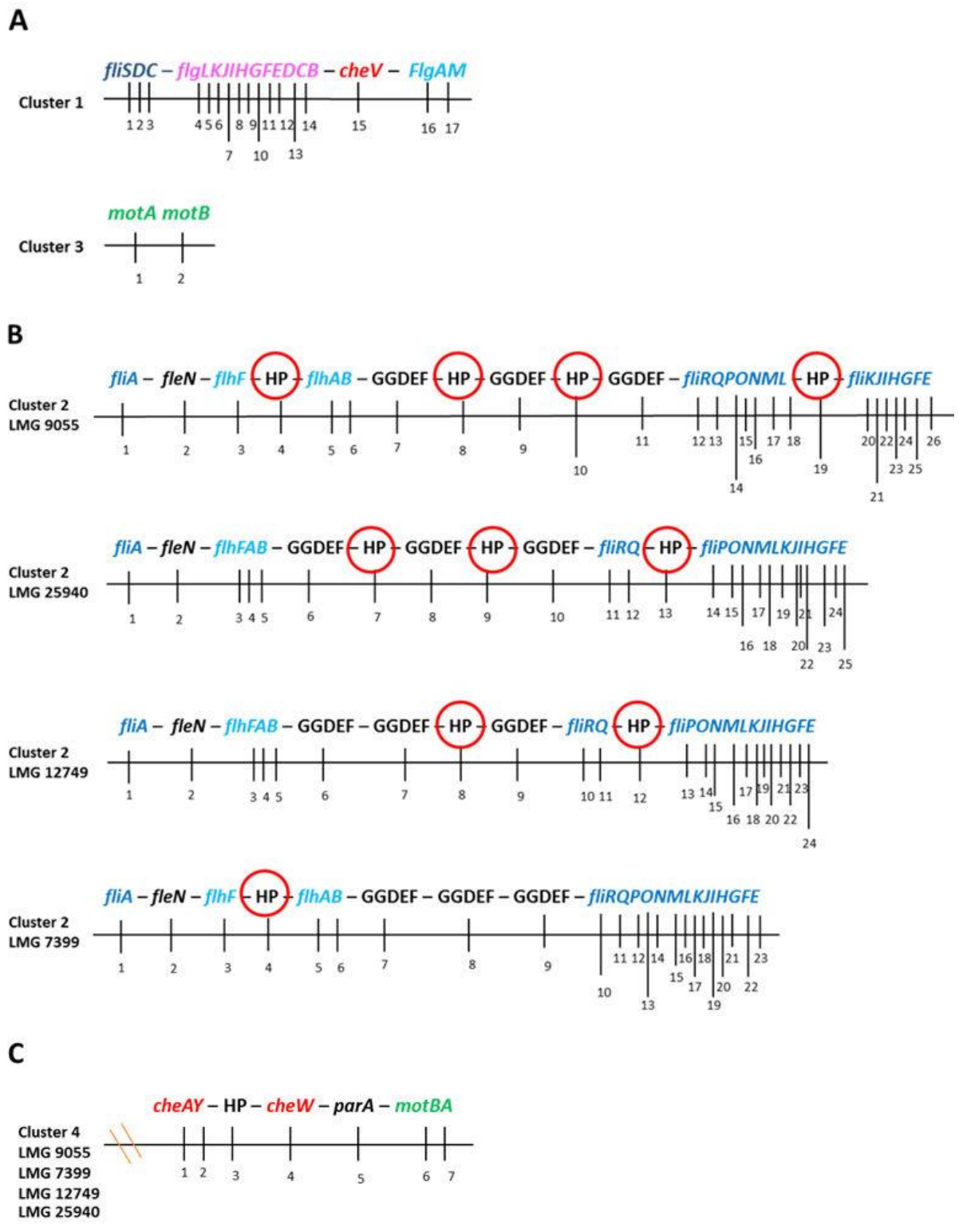

Figure 3.8. Schematic representation of the genomic clusters with flagellar genes (*fli*, *fih*, *mot*) in the four sequenced *Xanthomonas* strains*, Xanthomonas phaseoli* pv. *dieffenbachiae* strain LMG 25940, *X. phaseoli* pv. *syngonii* strain LMG 9055, *X. citri* pv. *aracearum* strain LMG 7399 and *X. euvesicatoria* strain LMG 12749. A) Cluster 1 and cluster 3 found in all four strains, B) Cluster 2 found in all four strains but in different organization, and C) Cluster 4 found in all four strains. GGDEF = protein domain involved in biofilm formation, motility, exopolysaccharide and cell differentiation, HP = hypothetical protein, *che* = chemotaxis protein, *par* = partitioning protein.

3.3.10 Extracellular polysaccharides (EPS)

EPS is regarded as an important pathogenicity factor in plant pathogenic bacteria. Xanthan is a main compound of the EPS in xanthomonads (Rigano *et al*., 2007). It is encoded by a cluster of 12 *gum* genes, *gumCDEFGHIJKLMN* (Supplementary Table 3.6). In the four genomes analysed in this study, this gene cluster was comparable to that of other xanthomonads, showing an identical organization of approximately 15 kb (Jalan *et al*., 2011).

3.3.11 Gene cluster of regulation of pathogenicity factors (*rpf*)

The rpf gene cluster is found in all pathogenic xanthomonads, and was also found in all the four genomes studied here. It encodes a quorum-sensing system that has been associated with regulation of motility, biofilm formation and virulence in several *Xanthomonas* species (Buttner & Bonas, 2010). This cell-to-cell signaling is mediated by the signal molecule DSF (diffusible signaling factor) and RpfF is responsible for the synthesis of DSF, whereas RpfC and RpfG are implicated in DSF perception and signal transduction (Ryan *et al*., 2010). This gene cluster comprises nine genes in *X. campestris* pv. *campestris* 8004 (Tang *et al*. 1991) and eight genes in *X. fuscans* subsp. *fuscans* 4834-R (Darrasse *et al*., 2013). In the four genomes analyzed here, six genes (*rpf BCEFGH*) were predicted (data not shown).

3.3.12 Lipopolysaccharide locus (LPS)

The LPS locus in *Xanthomonas* is a hypervariable cluster which has a role in virulence (Dharmapuri *et al*., 2001). This essential component confers a double role as a physical barrier by protecting bacteria from antibacterial substances and also as an inducer of plant defense-related genes (Newman *et al*., 2000). LPS is an amphipathic molecule consisting of a hydrophobic glycolipid anchor termed lipid A, a hydrophilic polysaccharide portion in the core region and the O-antigen polysaccharide chain (Sperandeo *et al*., 2009). The LPS cluster is involved in systhesis of O-antigen polysaccharide.

The LPS cluster is confined between two highly conserved housekeeping genes, *met* and *etf*, encoding the cystathionine gamma lyase and electron transport flavoprotein, respectively. Comparison of this cluster from draft genomes of the four sequenced xanthomonads revealed high variability in the number of genes and their sequences. The flanking genes of *etfB*, *etfA* and *metB*, *metC* are conserved in all four genomes. Apart from the gene content, the length of the LPS cluster is variable from 19.7 to 25.9 kb, while the GC content varies from 55.9% to 58.6%. The list of genes identified in the four genomes is given in Table 3.6. All genes were manually checked for frameshifts or stop-codons but no mutations were found. The LPS locus of Xpd (LMG 25940) has at least 17 homologs to Xps (LMG 9055), 9 homologs to Xp (LMG 12749) and only 2 to Xca (LMG 7399). The LPS cluster organization in strain

LMG 25940 from *Anthurium* is similar to that in strain LMG 9055 from *Syngonium*, suggesting that they may share a common ancestral LPS cluster (Figure 3.9).

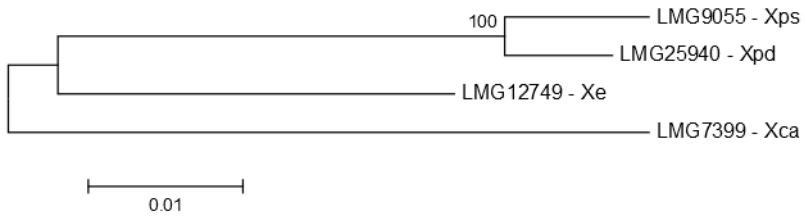

Figure 3.9. Phylogenetic tree based on conserved *metC, metB* and *etfB, etfA* genes that flank the variable lipopolysaccharide (LPS) locus. The tree was generated with MEGA v.6 software using maximum likelihood algorithm with 1000 bootstrap replicates. Branch length is proportional to divergence, the 0,01 scale represents 1% difference. LMG 9055 = *X. phaseoli* pv. *syngonii,* LMG 25940 = *X. phaseoli* pv. *dieffenbachiae,* LMG 12749 = *X. euvesicatoria*, LMG 7399 = *X. citri* pv. *aracearum*.

Table 3.6. LPS locus content and organization in four *Xanthomonas* genomes.

LMG 25940		LMG 9055		LMG 12749		LMG 7399	
Gene	Size (aa)	Gene	Size (aa)	Gene	Size (aa)	Gene	Size (aa)
metC	**456**	**metC**	**456**	**metC**	**456**	**metC**	**456**
metB	**404**	**metB**	**404**	metB	399	metB	397
O-antigen-permease protein RfD	**220**	**O-antigen-permease protein RfD**	**220**	teichoic ATP-binding protein TagH	434	hypothetical protein	72
teichoic ATP-binding protein TagH	**448**	**teichoic ATP-binding protein TagH**	**448**	glycosyltransferase group 2	1415	putative glycosyltransferase	1068
chloramphenicol acethyltransferase	**175**	**chloramphenicol acethyltransferase**	**175**	hypothetical protein	520	O-antigen-permease protein RfD	261
glycosyltransferase	**338**	**glycosyltransferase**	**338**	probable transmembrane protein	660	ATP-binding protein ABC transporter	311
glycosyltransferase	**835**	**glycosyltransferase**	**835**	**phytoene desaturase**	**421**	SAM dependent methyltransferase	453
putative glycosyltransferase	325	putative glycosyltransferase	238	**putative oxidoreductase**	**314**	glycosyltransferase	1125
hypothetical protein	571	hypothetical protein	125	**sorbitol-6-phosphate 2-dehydrogenase**	**242**	GDP-mannose	307
hypothetical protein	534	**hypothetical protein**	**130**	**probable oxidoreductase**	**433**	UDP-glucose epimerase	263
hypothetical protein	501	**phytoene desaturase**	**421**	**putative membrane protein**	**462**	hypothetical protein	686
hypothetical protein	**130**	**putative oxidoreductase**	**314**	**integral membrane protein**	**328**	lipopolysaccharide RfbA	376
phytoene desaturase	**421**	**methyltransferase**	**223**	**etfA**	**314**	etfA	313
putative oxidoreductase	**314**	**sorbitol-6-phosphate 2-dehydrogenase**	**242**	**etfB**	**248**	**etfB**	**248**
methyltransferase	**223**	**probable oxidoreductase**	**433**				
sorbitol-6-phosphate 2-dehydrogenase	**242**	**putative membrane protein**	**462**				
probable oxidoreductase	**433**	**integral membrane protein**	**328**				
putative membrane protein	**462**	**etfA**	**314**				
integral membrane protein	**328**	**etfB**	**248**				
etfA	**314**						
etfB	**248**						

aa, amino acids; bold, proteins with the same length and function in the different genomes.
LMG 25940, *Xanthomonas phaseoli* pv. *dieffenbachiae*; LMG 9055, *X. phaseoli* pv. *syngonii*; LMG 12479, *X. euvesicatoria*; LMG 7399, *X. citri* pv. *aracearum*.

3.4 Discussion

Recently, strains named *Xanthomonas axonopodis* pv. *dieffenbachiae* (Xad) were confirmed to be heterogeneous and were allocated to three distinct species (Constantin *et al*., 2016). This raised questions as whether strains isolated from plants of the family Araceae (aroids) and identified as belonging to one of these distinct species, are equally important as pathogens of aroids. In addition, would a pathovar name be justified to differentiate them from other pathovars of the same species that are pathogens of different hosts? Clarification is also needed in support of the phytosanitary policy towards this heterogeneous group of Xad bacteria that are listed on the A2 list of the European and Mediterranean Plant Protection Organization (EPPO). Therefore, this investigation was carried out, using both plant pathogenicity tests and genome analysis, to identify the true pathogens of aroids and to propose new name combinations using the existing pathovar epithets.

The results of this study showed that there is variability in disease severity dependent on the bacterial strain. This is the first time that the pathogenicity of individual strains within each host-strain group (species) has been presented. Previous studies scored pathogenicity of Xad strains as positive/negative (Berthier *et al*., 1993; Robéne-Soustrade *et al*., 2006), or as a mean severity rating per host-strain group (Chase *et al*., 1992; Lipp *et al.*, 1992); the variation among individual strains within a group was not considered. The results presented here are in agreement with the earlier studies that used a severity rating scale (Chase *et al*., 1992; Lipp *et al*., 1992). From these it can be deduced that a few other strains from hosts other than *Anthurium* (probably *X. citri* pv. *aracearum*) caused symptoms on *Anthurium*.

In general, in the present study, a difference in susceptibility between the tested aroids was observed, with *Aglaonema* and *Philodendron* being respectively the most and least sensitive to infection by the eleven tested strains. This is not fully in agreement with the observation of Chase *et al*. (1992). The strains classified into *X. phaseoli*, originally isolated from *Anthurium* and *Syngonium*, and the strains classified into *X. citri,* originally isolated from a wider host range including *Aglaonema*, *Xanthosoma* and *Dieffenbachia*, were scored and confirmed in the pathogenicity tests as 'real pathogens' on the tested aroids. Within these two species, only the *X. phaseoli* pv. *syngonii* strain LMG 9055 (formerly *X. campestris* pv. *syngonii*) showed a host specialization restricted to its original host *Syngonium*. The other strains tested (classified into *X. phaseoli* and *X. citri*) infected the six tested aroids with a variable degree of aggressiveness and with *X. phaseoli* strains typically being most virulent. In contrast, the strains classified into *X. euvesicatoria*, and originally isolated from *Philodendron*, were only weakly pathogenic on the aroids tested; hence, their pathogenicity could not be confirmed, even after re-inoculation of these strains on two different *Philodendron* cultivars. It is generally accepted that strains isolated from aroids are more virulent on their host of origin than on other aroid hosts; however, the strains from *Philodendron* are atypical as they failed to produce clear symptoms even on their original

host. Althought the number of strains studied and their geographical origin were limited, the data do not support the designation of *X. euvesicatoria* strains from *Philodendron* as a separate pathovar, because of their lack of specificity to aroid hosts. At present, these should be considered as under-studied plant pathogenic strains of unknown plant health-related relevance; further studies are needed to determine whether they should be designated as a separate pathovar of other hosts. Even in *Aglaonema*, shown to be the most susceptible host, these *X. euvesicatoria* strains were not aggressive, although viable cells could be recovered from the places of inoculation and beyond. Multiplication in aroid host tissue is not always a clear indication for a pathogenic interaction, and could equally be an indication for true plant-associated bacteria; nonpathogenic xanthomonads, such as the Xad strains belonging to serogroup 12 or group IV in the study by Robéne-Soustrade *et al*. (2006), have been reported to multiply in aroid leaf tissue after inoculation.

On the other hand, an isolate from a symptomatic aroid plant and identified as *X. citri* or *X. phaseoli* is to be suspected as an important pathogen to this range of host plants. Their allocation to a definitive pathovar allocation within the species, remains to be determined by extra pathogenicity tests on a range of relevant aroid genera. In previous studies, strains isolated from *Syngonium* and identified as *X. phaseoli* pv. *syngonii*, recognized by their aggressive character restricted to *Syngonium*, have been designated as a separate pathovar (Dickey & Zumoff, 1987; Lipp *et al*., 1992).

In the present analysis of four genomes, the pathogenicity-related gene content, (genes for hrp, T2SS, T3SS, T4SS, T6SS, CWDE, Flagella, EPS, GIs, rpf, LPS) enabled the identification of the *X. euvesicatoria* strain LMG 12749 from *Philodendron* as a plant pathogen. It is plausible that these *X. euvesicatoria* strains, originally isolated from *Philodendron*, have another, as yet undefined host range and that their pathogenicity is either not expressed, or is repressed in aroid plants. Although there is variation in functional gene content among the analyzed genomes, especially for specific genes of T3SS, T4SS and CWDE, there may be plausible indications that the *X. euvesicatoria* strain from *Philodendron* has a different host range. For example, the combination of the T3 effector genes *XopE2*, *XopG* and *XopAM* is only found in the genomes of strains that are pathogenic on aroids and classified into *X. phaseoli* and *X. citri*. Also, the T6SS gene sequences of *X. euvesicatoria* strain were clearly differentiated from those of the other strains pathogenic on aroid. Of course, more genomes need to be analyzed to support this interpretation. Based on the phylogenetic differentiation of Xad strains published earlier (Constantin *et al*., 2016) and the results of the pathogenicity tests in this study, it is proposed to name the pathogens on aroids as *X. phaseoli* pvs *dieffenbachiae* and *syngonii* comb. nov., and *X. citri* pv. *aracearum* comb. nov.

In addition to the genes that were scored as present/absent in the genomes, a considerable number of pathogenicity genes were found that were not functional due to frameshift or truncation. Up to eight dysfunctional genes for cell wall degradation were scored in *X. phaseoli* pv. *syngonii* strain LMG

9055, as well as eight dysfunctional T3E genes in *X. phaseoli* pv. *dieffenbachiae* strain LMG 25940. These two strains, which were the most aggressive on aroids, have the least functional T3 effectors of the four strains; in contrast, *X. euvesicatoria* strain LMG 12749, which is not pathogenic to aroids, has 27 T3 effectors. It is yet unclear how these genomic evolutionary changes relate to pathogenicity or virulence of the strains towards aroids. T3 effectors can indeed function as pathogenicity factors, but in other associations can trigger defence in the plant.

Bacterial leaf blight of aroids has the highest impact when the pathogen is not restricted to leaf spots, but expands and spreads in the plant tissue and even further into the vascular system. This also represents a high risk for trade of infected plants or planting material in which visual symptoms are not always readily expressed. The *X. phaseoli* pvs *dieffenbachiae* and *syngonii* strains and the *X. citri* pv. *aracearum* strains were able to cause severe and extended leaf symptoms, depending on the strain-aroid species combination. In addition, *X. phaseoli* pvs *dieffenbachiae* and *syngonii* strains could also affect the vascular tissue. Althought the *X. phaseoli* pv. *syngonii* strain induced a systemic infection only in its original host *Syngonium*, the strains from *Anthurium* (*X. phaseoli* pv. *dieffenbachiae*) caused a systemic infection in *Anthurium* and/or *Dieffenbachia* plants. Thus, strains from *Anthurium* are of major interest for quarantine regulation status.

Detailed comparative genome analyses provide insights into the differentiation and unique pathogenicity-related gene profile of each strain. The four genomes are quite conserved with respect to the different gene families studied. However, in addition to common content, each of these strains has specificities. A unique gene, the effector *XopAO*, was found for *X. phaseoli* pv. *dieffenbachiae* strain LMG 25940, a representative strain of the pathogen typically infecting *Anthurium*. This effector has only been identified in two other xanthomonads, *X. gardneri* and *X. axonopodis* pv. *manihotis*, and an origin from *Pseudomonas* via horizontal gene transfer events has been proposed (Potnis *et al*., 2011). This gene should be searched for in other strains and relatives of *X. phaseoli* pv. *dieffenbachiae* to check it as a potential marker for this important pathogen of *Anthurium* in Europe. Another important gene region is the LPS cluster. Phylogenetic insight, based on conserved *metC*, *metB* and *etfB*, *etfA* genes that flank the LPS locus, suggests that the two vascular pathogens (LMG 25940 and LMG 9055) have retained an ancestral type of LPS gene cluster (Figure 3.9). In contrast, LMG 7399 and LMG 12749 have acquired new genes in the LPS gene cluster during the course of evolution so that the cluster is completely different in gene organization and the number of genes that are encoded. Although it was suggested that there is no obvious correlation of the content of the LPS gene cluster with host specificity (Lu *et al*., 2008), the variation in the LPS gene cluster among the four strains might contribute to their differences in virulence or symptom development in different plant hosts.

As the scientific community is still searching for insight into genomic features that specify host pathogenicity range, it is expected that future research will lead to the development and

implementation of simpler tests for these and other *Xanthomonas* pathovars, several of which are EU-regulated and belong to *X. citri* and *X. phaseoli*, e.g. some important pathogens on citrus and on bean. The work presented should contribute to this aim.

In conclusion, the previously reported phylogenetic heterogeneity of Xad strains (Constantin *et al*., 2016) is also reflected in a pathogenic heterogeneity, justifying the installation of three pathovars for the pathogens on aroids: *X. phaseoli* pv. *dieffenbachiae* comb. nov., *X. phaseoli* pv. *syngonii* comb. nov., and *X. citri* pv. *aracearum* comb. nov.

Supplementary material

Supplementary Table 3.1. Genes common to LMG 9055 and LMG 25940.

LMG9055	LMG25940	Gene name
fig|270916.4.peg.1892	fig|6666666.94921.peg.102	Integrase
fig|270916.4.peg.4278	fig|6666666.94921.peg.1037	Heavy metal RND efflux outer membrane protein, CzcC family
fig|270916.4.peg.4279	fig|6666666.94921.peg.1038	Probable Co/Zn/Cd efflux system membrane fusion protein
fig|270916.4.peg.4280	fig|6666666.94921.peg.1039	Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA
fig|270916.4.peg.4281	fig|6666666.94921.peg.1040	Cobalt-zinc-cadmium resistance protein CzcD
fig|270916.4.peg.4282	fig|6666666.94921.peg.1041	inner membrane protein
fig|270916.4.peg.3890	fig|6666666.94921.peg.1047	hypothetical protein
fig|270916.4.peg.1276	fig|6666666.94921.peg.1056	FIG01210241: hypothetical protein
fig|270916.4.peg.1274	fig|6666666.94921.peg.1057	Porphobilinogen synthase (EC 4.2.1.24)
fig|270916.4.peg.1265	fig|6666666.94921.peg.1065	toxin secretion ABC transporter, ATP-binding subunit/permease protein, putative
fig|270916.4.peg.1264	fig|6666666.94921.peg.1066	hypothetical protein
fig|270916.4.peg.1886	fig|6666666.94921.peg.107	hypothetical protein
fig|270916.4.peg.249	fig|6666666.94921.peg.1104	hypothetical protein
fig|270916.4.peg.474	fig|6666666.94921.peg.1138	hypothetical protein
fig|270916.4.peg.2133	fig|6666666.94921.peg.1152	hypothetical protein
fig|270916.4.peg.3570	fig|6666666.94921.peg.1156	hypothetical protein
fig|270916.4.peg.4207	fig|6666666.94921.peg.1189	hypothetical protein
fig|270916.4.peg.593	fig|6666666.94921.peg.1205	hypothetical protein
fig|270916.4.peg.3590	fig|6666666.94921.peg.1276	hypothetical protein
fig|270916.4.peg.1208	fig|6666666.94921.peg.1294	hypothetical protein
fig|270916.4.peg.2940	fig|6666666.94921.peg.13	hypothetical protein
fig|270916.4.peg.75	fig|6666666.94921.peg.1425	hypothetical protein
fig|270916.4.peg.74	fig|6666666.94921.peg.1426	hypothetical protein
fig|270916.4.peg.3291	fig|6666666.94921.peg.1522	hypothetical protein
fig|270916.4.peg.1287	fig|6666666.94921.peg.1546	hypothetical protein
fig|270916.4.peg.1660	fig|6666666.94921.peg.1550	outer membrane hemolysin activator protein
fig|270916.4.peg.2557	fig|6666666.94921.peg.1554	hypothetical protein
fig|270916.4.peg.2576	fig|6666666.94921.peg.1571	hypothetical protein
fig|270916.4.peg.2582	fig|6666666.94921.peg.1579	hypothetical protein
fig|270916.4.peg.2943	fig|6666666.94921.peg.16	putative; ORF located using Glimmer/Genemark
fig|270916.4.peg.4007	fig|6666666.94921.peg.1644	putative; ORF located using Glimmer/Genemark
fig|270916.4.peg.2023	fig|6666666.94921.peg.1670	hypothetical protein
fig|270916.4.peg.840	fig|6666666.94921.peg.168	Histone acetyltransferase HPA2 and related acetyltransferases

fig|270916.4.peg.3877	fig|6666666.94921.peg.1688	phenol hydroxylase
fig|270916.4.peg.3626	fig|6666666.94921.peg.1703	Carbonic anhydrase (EC 4.2.1.1)
fig|270916.4.peg.1979	fig|6666666.94921.peg.1708	FIG01210955: hypothetical protein
fig|270916.4.peg.4056	fig|6666666.94921.peg.1741	hypothetical protein
fig|270916.4.peg.4060	fig|6666666.94921.peg.1746	hypothetical protein
fig|270916.4.peg.4404	fig|6666666.94921.peg.1785	hypothetical protein
fig|270916.4.peg.853	fig|6666666.94921.peg.180	FIG01213901: hypothetical protein
fig|270916.4.peg.122	fig|6666666.94921.peg.1818	hypothetical protein
fig|270916.4.peg.111	fig|6666666.94921.peg.1828	Transcriptional regulator, AraC family
fig|270916.4.peg.110	fig|6666666.94921.peg.1829	Peptidase, S41 family
fig|270916.4.peg.109	fig|6666666.94921.peg.1830	hypothetical protein
fig|270916.4.peg.4027	fig|6666666.94921.peg.1876	hypothetical protein
fig|270916.4.peg.752	fig|6666666.94921.peg.1910	FIG01210386: hypothetical protein
fig|270916.4.peg.2487	fig|6666666.94921.peg.1929	FIG01212773: hypothetical protein
fig|270916.4.peg.2496	fig|6666666.94921.peg.1937	hypothetical protein
fig|270916.4.peg.2501	fig|6666666.94921.peg.1943	hypothetical protein
fig|270916.4.peg.3754	fig|6666666.94921.peg.2000	hypothetical protein
fig|270916.4.peg.3732	fig|6666666.94921.peg.2023	hypothetical protein
fig|270916.4.peg.1920	fig|6666666.94921.peg.203	Aspartyl-tRNA(Asn) amidotransferase subunit A (EC 6.3.5.6) @ Glutamyl-tRNA(Gln) amidotransferase subunit A (EC 6.3.5.7)
fig|270916.4.peg.1921	fig|6666666.94921.peg.204	Transcriptional regulator, TetR family
fig|270916.4.peg.327	fig|6666666.94921.peg.2076	hypothetical protein
fig|270916.4.peg.332	fig|6666666.94921.peg.2081	hypothetical protein
fig|270916.4.peg.345	fig|6666666.94921.peg.2094	FIG01209735: hypothetical protein
fig|270916.4.peg.1931	fig|6666666.94921.peg.214	hypothetical protein
fig|270916.4.peg.3210	fig|6666666.94921.peg.2140	hypothetical protein
fig|270916.4.peg.3980	fig|6666666.94921.peg.2155	Acyl carrier protein
fig|270916.4.peg.1937	fig|6666666.94921.peg.220	hypothetical protein
fig|270916.4.peg.2172	fig|6666666.94921.peg.2210	hypothetical protein
fig|270916.4.peg.2173	fig|6666666.94921.peg.2211	hypothetical protein
fig|270916.4.peg.2196	fig|6666666.94921.peg.2234	hypothetical protein
fig|270916.4.peg.2214	fig|6666666.94921.peg.2251	putative translation initiation factor IF-2
fig|270916.4.peg.2232	fig|6666666.94921.peg.2268	FIG01213917: hypothetical protein
fig|270916.4.peg.2235	fig|6666666.94921.peg.2272	O-antigen export system permease protein RfbD
fig|270916.4.peg.2236	fig|6666666.94921.peg.2273	Teichoic acid export ATP-binding protein TagH (EC 3.6.3.40)
fig|270916.4.peg.2237	fig|6666666.94921.peg.2274	Chloramphenicol acetyltransferase (EC 2.3.1.28)
fig|270916.4.peg.2238	fig|6666666.94921.peg.2275	Glycosyltransferase (EC 2.4.1.-)
fig|270916.4.peg.2239	fig|6666666.94921.peg.2276	Glycosyltransferase (EC 2.4.1.-)

fig|270916.4.peg.963	fig|6666666.94921.peg.2280	hypothetical protein
fig|270916.4.peg.3650	fig|6666666.94921.peg.2282	FIG01212275: hypothetical protein
fig|270916.4.peg.3653	fig|6666666.94921.peg.2285	Methyltransferase type 11
fig|270916.4.peg.1947	fig|6666666.94921.peg.230	putative autotransporter protein
fig|270916.4.peg.3679	fig|6666666.94921.peg.2311	hypothetical protein
fig|270916.4.peg.3681	fig|6666666.94921.peg.2313	hypothetical protein
fig|270916.4.peg.430	fig|6666666.94921.peg.2346	hypothetical protein
fig|270916.4.peg.414	fig|6666666.94921.peg.2363	Uncharacterized protein
fig|270916.4.peg.3792	fig|6666666.94921.peg.2405	MloA
fig|270916.4.peg.3798	fig|6666666.94921.peg.2412	acetyltransferase, GNAT family
fig|270916.4.peg.3939	fig|6666666.94921.peg.2449	hypothetical protein
fig|270916.4.peg.1518	fig|6666666.94921.peg.2499	hypothetical protein
fig|270916.4.peg.4164	fig|6666666.94921.peg.2519	hypothetical protein
fig|270916.4.peg.4147	fig|6666666.94921.peg.2536	hypothetical protein
fig|270916.4.peg.1029	fig|6666666.94921.peg.2546	hypothetical protein
fig|270916.4.peg.2158	fig|6666666.94921.peg.255	hypothetical protein
fig|270916.4.peg.1048	fig|6666666.94921.peg.2564	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)
fig|270916.4.peg.1062	fig|6666666.94921.peg.2579	FIG01210012: hypothetical protein
fig|270916.4.peg.1102	fig|6666666.94921.peg.2625	hypothetical protein
fig|270916.4.peg.1120	fig|6666666.94921.peg.2643	hypothetical protein
fig|270916.4.peg.1150	fig|6666666.94921.peg.2677	hypothetical protein
fig|270916.4.peg.1164	fig|6666666.94921.peg.2691	FIG01211514: hypothetical protein
fig|270916.4.peg.1363	fig|6666666.94921.peg.2720	hypothetical protein
fig|270916.4.peg.3013	fig|6666666.94921.peg.2793	hypothetical protein
fig|270916.4.peg.3023	fig|6666666.94921.peg.2804	FIG01212583: hypothetical protein
fig|270916.4.peg.961	fig|6666666.94921.peg.2808	hypothetical protein
fig|270916.4.peg.948	fig|6666666.94921.peg.2821	Beta-galactosidase (EC 3.2.1.23)
fig|270916.4.peg.944	fig|6666666.94921.peg.2825	hypothetical protein
fig|270916.4.peg.2753	fig|6666666.94921.peg.2836	hypothetical protein
fig|270916.4.peg.1236	fig|6666666.94921.peg.2911	FIG01210924: hypothetical protein
fig|270916.4.peg.2061	fig|6666666.94921.peg.2914	FIG01211600: hypothetical protein
fig|270916.4.peg.2064	fig|6666666.94921.peg.2917	hypothetical protein
fig|270916.4.peg.2387	fig|6666666.94921.peg.2962	TonB-dependent receptor
fig|270916.4.peg.2386	fig|6666666.94921.peg.2963	Flavin monoamine oxidase-related protein
fig|270916.4.peg.4260	fig|6666666.94921.peg.3062	hypothetical protein
fig|270916.4.peg.555	fig|6666666.94921.peg.3091	Chlorogenate esterase
fig|270916.4.peg.3388	fig|6666666.94921.peg.311	hypothetical protein

fig|270916.4.peg.2452	fig|6666666.94921.peg.3123	hypothetical protein
fig|270916.4.peg.3267	fig|6666666.94921.peg.3154	Type III secretion protein HrpE
fig|270916.4.peg.3239	fig|6666666.94921.peg.3181	Ubiquinone biosynthesis monooxygenase UbiB
fig|270916.4.peg.3227	fig|6666666.94921.peg.3193	Biopolymer transport protein ExbD/TolR
fig|270916.4.peg.287	fig|6666666.94921.peg.3219	hypothetical protein
fig|270916.4.peg.1759	fig|6666666.94921.peg.3266	hypothetical protein
fig|270916.4.peg.1843	fig|6666666.94921.peg.3351	hypothetical protein
fig|270916.4.peg.1854	fig|6666666.94921.peg.3362	hypothetical protein
fig|270916.4.peg.1859	fig|6666666.94921.peg.3366	motif=eukaryotic putative RNA-binding region RNP-1 signature
fig|270916.4.peg.1324	fig|6666666.94921.peg.3396	YciL protein
fig|270916.4.peg.3895	fig|6666666.94921.peg.3435	hypothetical protein
fig|270916.4.peg.2019	fig|6666666.94921.peg.3485	putative secreted protein
fig|270916.4.peg.371	fig|6666666.94921.peg.3517	N-carbamoylputrescine amidase (EC 3.5.1.53)
fig|270916.4.peg.362	fig|6666666.94921.peg.3527	hypothetical protein
fig|270916.4.peg.2926	fig|6666666.94921.peg.3646	hypothetical protein
fig|270916.4.peg.3642	fig|6666666.94921.peg.3683	Uncharacterized protein ImpJ/VasE
fig|270916.4.peg.2764	fig|6666666.94921.peg.3747	Type IV fimbrial biogenesis protein PilY1
fig|270916.4.peg.2766	fig|6666666.94921.peg.3749	Type IV fimbrial biogenesis protein PilW
fig|270916.4.peg.2767	fig|6666666.94921.peg.3750	Type IV fimbrial biogenesis protein PilV
fig|270916.4.peg.3127	fig|6666666.94921.peg.379	hypothetical protein
fig|270916.4.peg.2826	fig|6666666.94921.peg.3812	cath1
fig|270916.4.peg.3130	fig|6666666.94921.peg.382	hypothetical protein
fig|270916.4.peg.2729	fig|6666666.94921.peg.3832	methylated-DNA-protein-cysteine S-methyltransferase related protein
fig|270916.4.peg.3135	fig|6666666.94921.peg.386	hypothetical protein
fig|270916.4.peg.2682	fig|6666666.94921.peg.3878	Transcriptional regulator
fig|270916.4.peg.2681	fig|6666666.94921.peg.3879	FIG01211170: hypothetical protein
fig|270916.4.peg.2661	fig|6666666.94921.peg.3895	hypothetical protein
fig|270916.4.peg.3139	fig|6666666.94921.peg.390	hypothetical protein
fig|270916.4.peg.1726	fig|6666666.94921.peg.3943	hypothetical protein
fig|270916.4.peg.1731	fig|6666666.94921.peg.3949	hypothetical protein
fig|270916.4.peg.201	fig|6666666.94921.peg.4046	FIG01213181: hypothetical protein
fig|270916.4.peg.193	fig|6666666.94921.peg.4056	LptA, protein essential for LPS transport across the periplasm
fig|270916.4.peg.992	fig|6666666.94921.peg.4075	hypothetical protein
fig|270916.4.peg.1609	fig|6666666.94921.peg.4107	Histidine kinase/response regulator hybrid protein
fig|270916.4.peg.1641	fig|6666666.94921.peg.4136	hypothetical protein
fig|270916.4.peg.3070	fig|6666666.94921.peg.4155	hypothetical protein
fig|270916.4.peg.1377	fig|6666666.94921.peg.4233	FIG01209684: hypothetical protein

fig|270916.4.peg.1687	fig|6666666.94921.peg.4251	L-Proline/Glycine betaine transporter ProP
fig|270916.4.peg.1697	fig|6666666.94921.peg.4260	hypothetical protein
fig|270916.4.peg.3424	fig|6666666.94921.peg.4273	Death on curing protein, Doc toxin
fig|270916.4.peg.4203	fig|6666666.94921.peg.4292	hypothetical protein
fig|270916.4.peg.2038	fig|6666666.94921.peg.4300	hypothetical protein
fig|270916.4.peg.2048	fig|6666666.94921.peg.4310	hypothetical protein
fig|270916.4.peg.2051	fig|6666666.94921.peg.4312	hypothetical protein
fig|270916.4.peg.2547	fig|6666666.94921.peg.44	hypothetical protein
fig|270916.4.peg.2855	fig|6666666.94921.peg.448	hypothetical protein
fig|270916.4.peg.2842	fig|6666666.94921.peg.460	hypothetical protein
fig|270916.4.peg.682	fig|6666666.94921.peg.479	hypothetical protein
fig|270916.4.peg.680	fig|6666666.94921.peg.481	hypothetical protein
fig|270916.4.peg.2552	fig|6666666.94921.peg.50	HigA protein (antitoxin to HigB)
fig|270916.4.peg.647	fig|6666666.94921.peg.517	hypothetical protein
fig|270916.4.peg.636	fig|6666666.94921.peg.527	hypothetical protein
fig|270916.4.peg.609	fig|6666666.94921.peg.554	hypothetical protein
fig|270916.4.peg.3822	fig|6666666.94921.peg.568	hypothetical protein
fig|270916.4.peg.3867	fig|6666666.94921.peg.611	hypothetical protein
fig|270916.4.peg.2125	fig|6666666.94921.peg.621	FIG01209811: hypothetical protein
fig|270916.4.peg.2112	fig|6666666.94921.peg.634	hypothetical protein
fig|270916.4.peg.2107	fig|6666666.94921.peg.639	Mlr6622 protein
fig|270916.4.peg.2246	fig|6666666.94921.peg.697	FIG01211604: hypothetical protein
fig|270916.4.peg.487	fig|6666666.94921.peg.710	putative; ORF located using Glimmer/Genemark
fig|270916.4.peg.514	fig|6666666.94921.peg.735	hypothetical protein
fig|270916.4.peg.3953	fig|6666666.94921.peg.783	hypothetical protein
fig|270916.4.peg.4223	fig|6666666.94921.peg.812	Type IV fimbrial assembly, ATPase PilB
fig|270916.4.peg.4222	fig|6666666.94921.peg.813	Type IV fimbrial assembly, ATPase PilB
fig|270916.4.peg.65	fig|6666666.94921.peg.843	hypothetical protein
fig|270916.4.peg.162	fig|6666666.94921.peg.907	Lactoylglutathione lyase and related lyase
fig|270916.4.peg.155	fig|6666666.94921.peg.913	hypothetical protein
fig|270916.4.peg.927	fig|6666666.94921.peg.985	hypothetical protein

Supplementary Table 3.2. Type II secretion system (T2SS).

Gene name	CDS name			
	LMG 7399 (Xca)	LMG 25940 (Xpd)	LMG 12749 (Xe)	LMG 9055 (Xps)
xcsC	Xca_3877	Xpd_662	Xe_2003	Xps_2277
xcsD	Xca_3876	Xpd_666	Xe_2004	Xps_2278
xcsE	Xca_3875	Xpd_665	Xe_2005	Xps_2279
xcsF	Xca_3874	Xpd_664	Xe_2006	Xps_2280
xcsG	Xca_334	Xpd_663	Xe_2007	Xps_2281
xcsH	Xca_335	Xpd_662	Xe_2008	Xps_2282
xcsI	Xca_336	Xpd_661	Xe_2009	Xps_2283
xcsJ	Xca_337	Xpd_660	Xe_2010	Xps_2284
xcsK	Xca_338	Xpd_659	Xe_2011	Xps_2285
xcsL	Xca_339	Xpd_658	Xe_2012	Xps_2286
xcsM	Xca_340	Xpd_657	Xe_2013	Xps_2287
xcsN	Xca_341	Xpd_656	Xe_2014	Xps_2288
xpsD	Xca_3694	Xpd_1733	Xe_2536	Xps_873
xpsE	Xca_3704	Xpd_1722	Xe_2525	Xps_862
xpsF	Xca_3703	Xpd_1724	Xe_2527	Xps_864
xpsG	Xca_3702	Xpd_1725	Xe_2528	Xps_865
xpsH	Xca_3701	Xpd_1726	Xe_2529	Xps_866
xpsI	Xca_3700	Xpd_1727	Xe_2530	Xps_867
xpsJ	Xca_3699	Xpd_1728	Xe_2531	Xps_868
xpsK	Xac_3698	Xpd_1729	Xe_2532	Xps_869
xpsL	Xac_3697	Xpd_1730	Xe_2533	Xps_870
xpsM	Xac_3696	Xpd_1731	Xe_2534	Xps_871
xpsN	Xac_3695	Xpd_1732	Xe_2535	Xps_872

RAST annotations of the four genomes: LMG7399: 92828.12; LMG25940: 6666666.94921; LMG12749: 92828.9; LMG9055: 270916.4.
Xca = *X. citri* pv. *aracearum*; Xpd = *X. phaseoli* pv. *dieffenbachiae*; Xe = *X. euvesicatoria*; Xps = *X. phaseoli* pv. *syngonii*; CDS = Coding DNA sequence.

Supplementary Table 3.3. Type IV secretion system (T4SS).

		Xca - LMG 7399	Xpd - LMG 25940	Xe - LMG 12749	Xps - LMG 9055
1	*virD4*	Seq	1	1	0
2	*virB1*	0	1	1	0
3	*virB2*	0	1	1	0
4	*virB3*	0	1	1	0
5	*virB4*	0	1	1	0
6	*virB5*	0	0	0	0
7	*virB6*	1	1	1	0
8	*virB7*	0	1	1	0
9	*virB8*	0	1	1	0
10	*virB9*	0	1	1	0
11	*virB10*	0	1	1	1
12	*virB11*	0	1	1	1
Total		**2**	**11**	**11**	**2**

"1": one homolog present; "0": no homolog found; "Seq": coding DNA sequence truncated due to incomplete genome-assembly; functional protein assumed present during further processing of data.

Supplementary Table 3.4. Genes of cell-wall degrading enzymes (CWDE).

Pectinolytic enzymes

			Xca - LMG 7399 (*Dieffenbachia*)	Xpd - LMG 25940 (*Anthurium*)	Xe - LMG 12749 (*Philodendron*)	Xps - LMG 9055 (*Syngonium*)
Polygalacturonases	*X. campestris campestris* ATCC 33913	[Genbank:NP_637621]	0	1	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_638805]	1	1	0	ΨT
Pectate Lyases	*X. campestris campestris* ATCC 33913	[Genbank:NP_635517]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_636036]	ΨF	1	1	Seq
	X. campestris campestris ATCC 33913	[Genbank:NP_636037]	0	0	0	0
	X. campestris raphani 756C	[Genbank:YP_005635831]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_638163]	1	1	1	Seq
	X. citri citri 306	[Genbank:NP_642689]	0	0	0	ΨT
	X. euvesicatoria 85-10	[Genbank:YP_364009]	0	0	0	0
Pectin Methylesterases	*X. campestris campestris* ATCC 33913	[Genbank:NP_635516]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_637620]	ΨF	1	0	0
Rhamnogalacturonan acetylesterase	*X. campestris campestris* ATCC 33913	[Genbank:NP_635549]	1	1	1	1
Rhamnogalacturonases	*X. citri citri* 306	[Genbank:NP_643812]	1	1	1	ΨF
	X. sacchari NCPPB 4393	[Genbank:ZP_09854047]	0	0	0	0
Beta-galactosidases	*X. axonopodis* pv. citrumelo F1	[Genbank: AEO42072]	1	1	1	1
	X. fuscans	[Genbank: WP_007971476]	1	1	1	1
	X. axonopodis	[Genbank: WP_046736031]	1	1	1	1
	X. fuscans	[Genbank: WP_007970973]	1	1	1	1
	X. fuscans	[Genbank: WP_007971487]	1	0	0	0
	X. fuscans	[Genbank: WP_042676235]	0	0	0	0
	X. euvesicatoria	[Genbank: WP_042841330]	1	1	1	ΨF

Arabinogalactan galactosidase	*X. axonopodis*	[Genbank: WP_039568305]	0	1	0	1
	X. axonopodis	[Genbank:WP_039567878]	ΨF	ΨF	ΨF	1
			10	**13**	**9**	**9**
		Cellulolytic enzymes				
Cellulases	*X. campestris campestris* ATCC 33913	[Genbank:NP_635421]	2	2	2	0
	X. campestris campestris ATCC 33913	[Genbank:NP_635422]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_635423]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_637119]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_637741]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_638726]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_638727]	1	1	ΨT	1
	X. campestris campestris ATCC 33913	[Genbank:NP_638867]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_638881]	1	1	0	0
	X. citri citri 306	[Genbank:NP_643823]	1	1	1	1
	X. citri citri 306	[Genbank:NP_640702]	1	ΨF	1	1
Cellobiosidases	*X. campestris campestris* ATCC 33913	[Genbank:NP_638506]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_638880]	1	1	0	0
Beta-glucosidases	*X. campestris campestris* ATCC 33913	[Genbank:NP_636465]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_637141]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_638240]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_639445]	1	1	0	ΨF
	X. campestris campestris ATCC 33913	[Genbank:NP_636777]	1	1	1	0
	X. campestris campestris ATCC 33913	[Genbank:NP_639159]	1	1	1	1
	X. axonopodis	Genbank:WP_029829302]	1	0	0	0
			17	**15**	**12**	**10**
		Hemicellulolytic enzymes				
Xylanases	*X. campestris campestris* ATCC 33913	[Genbank:NP_635539]	0	0	0	1
	X. campestris campestris ATCC 33913	[Genbank:NP_636248]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_638385]	0	0	0	0
	X. campestris campestris ATCC 33913	[Genbank:NP_639454]	1	1	1	0
	X. campestris campestris ATCC 33913	[Genbank:NP_639457]	1	1	1	ΨF
	X. citri citri 306	[Genbank:NP_644551]	1	1	1	1
Xylosidases/arabinosidases	*X. campestris campestris* ATCC 33913	[Genbank:NP_635544]	ΨF	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_636552]	1	1	1	1

	X. campestris campestris ATCC 33913	[Genbank:NP_637122]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_637752]	1	1	1	0
	X. campestris campestris ATCC 33913	[Genbank:NP_639314]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_639403]	1	1	1	1
	X. campestris campestris ATCC 33913	[Genbank:NP_639444]	1	1	1	ΨF
	X. campestris campestris ATCC 33913	[Genbank:NP_639461]	1	1	1	ΨT
			11	**12**	**12**	**8**
	Total		**38**	**40**	**33**	**27**

"1": at least one homolog present.
"2": two homologs present.
"0": no homolog found.
"ΨT": coding DNA sequence encountered, but protein believe inactive due to truncation.
"ΨF": coding DNA sequence encountered, but protein believed inactive due to frameshift.
"Seq": coding DNA sequence truncated due to incomplete genome-assembly; functional protein assumed present during further processing of data.

Supplementary Table 3.5. Occurrence of TonB-dependent transporters among the four sequenced *Xanthomonas* strains.

Reference retrieved from	Reference Locus	Xca - LMG 7399 (*Dieffenbachia*)	Xpd - LMG 25940 (*Anthurium*)	Xe - LMG 12749 (*Philodendron*)	Xps - LMG 9055 (*Syngonium*)
X. campestris campestris ATCC 33913	[Genbank:NP_635445]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_635493]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_635514]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_635515]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_635553]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_635788]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_635791]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_635923]	1	ΨT	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_636066]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_636150]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_636159]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_636332]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_636412]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_636416]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_636553]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_636632]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_636714]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_636765]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_637089]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_637116]	1	1	1	ΨT
X. campestris campestris ATCC 33913	[Genbank:NP_637257]	ΨT	1	1	0
X. campestris campestris ATCC 33913	[Genbank:NP_637355]	1	0	1	0
X. campestris campestris ATCC 33913	[Genbank:NP_637411]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_637564]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_637739]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_637749]	1	1	1	0
X. campestris campestris ATCC 33913	[Genbank:NP_637754]	Seq	ΨF	1	0
X. campestris campestris ATCC 33913	[Genbank:NP_637821]	1	1	1	Seq
X. campestris campestris ATCC 33913	[Genbank:NP_637847]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_637920]	1	1	ΨT	Seq
X. campestris campestris ATCC 33913	[Genbank:NP_637921]	0	1	0	1
X. campestris campestris ATCC 33913	[Genbank:NP_638006]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638013]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_638120]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638176]	1	ΨT	1	Seq
X. campestris campestris ATCC 33913	[Genbank:NP_638215]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_638235]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638292]	0	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638383]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638390]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638392]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638393]	1	1	1	1

X. campestris campestris ATCC 33913	[Genbank:NP_638397]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638414]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638426]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638507]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638523]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638555]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638625]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_638662]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_638704]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638751]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_638754]	Seq	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_638773]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_638820]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_638864]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_638941]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_638981]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_639060]	1	0	1	0
X. campestris campestris ATCC 33913	[Genbank:NP_639302]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_639391]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_639459]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_639470]	1	1	1	ΨF
X. campestris campestris ATCC 33913	[Genbank:NP_639471]	1	ΨT	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_639496]	0	0	0	0
X. campestris campestris ATCC 33913	[Genbank:NP_639556]	0	0	1	0
X. campestris campestris ATCC 33913	[Genbank:NP_639569]	1	1	1	1
X. campestris campestris ATCC 33913	[Genbank:NP_639571]	0	0	0	0
X. fragariae LMG 25863	[Genbank:O1K_06422]	0	0	0	0
X. fragariae LMG 25863	[Genbank:O1K_11570]	ΨF	ΨF	1	1
X. campestris campestris B100	[Genbank:YP_001901736]	0	0	0	0
X. citri citri 306	[Genbank:NP_640647]	1	0	0	0
X. citri citri 306	[Genbank:NP_641043]	ΨF	1	1	1
X. citri citri 306	[Genbank:NP_641059]	0	0	0	0
X. citri citri 306	[Genbank:NP_642097]	1	1	1	1
X. citri citri 306	[Genbank:NP_642502]	1	0	0	0
X. citri citri 306	[Genbank:NP_643082]	1	ΨF	0	ΨT
X. citri citri 306	[Genbank:NP_643359]	0	0	0	0
X. citri citri 306	[Genbank:NP_643386]	1	0	0	0
X. citri citri 306	[Genbank:NP_643677]	1	1	1	1
X. citri citri 306	[Genbank:NP_643725]	1	1	1	1
X. citri citri 306	[Genbank:NP_643751]	1	1	1	ΨF
X. citri citri 306	[Genbank:NP_643755]	1	1	1	1
X. citri citri 306	[Genbank:NP_643805]	0	0	0	0
X. citri citri 306	[Genbank:NP_643836]	0	0	0	0
X. citri citri 306	[Genbank:NP_643920]	1	0	0	0
X. citri citri 306	[Genbank:NP_643927]	1	0	0	0
X. citri citri 306	[Genbank:NP_644430]	1	1	1	1
X. gardneri ATCC 19865	[Genbank:ZP_08182603]	ΨT	1	0	ΨT
X. gardneri ATCC 19865	[Genbank:ZP_08183237]	0	0	0	0
X. gardneri ATCC 19865	[Genbank:ZP_08185761]	0	0	0	0
X. sacchari NCPPB 4393	[Genbank:ZP_09853472]	0	0	0	0
X. sacchari NCPPB 4393	[Genbank:ZP_09854914]	0	0	0	0
X. sacchari NCPPB 4393	[Genbank:ZP_09856367]	0	0	0	0
X. sacchari NCPPB 4393	[Genbank:ZP_09856515]	0	0	0	0
X. sacchari NCPPB 4393	[Genbank:ZP_09856761]	0	0	0	0
Total		**62**	**55**	**60**	**44**

"1": one homolog present.
"0": no homolog found.
"ΨT": coding DNA sequence encountered, but protein believe inactive due to truncation.
"ΨF": coding DNA sequence encountered, but protein believed inactive due to frameshift.
"Seq": coding DNA sequence truncated due to incomplete genome-assembly; functional protein assumed present during further processing of data.

Supplementary Table 3.6. The xanthan gum gene cluster in the four sequenced *Xanthomonas* strains.

Gene name	**CDS name**			
	LMG 7399 (Xca)	**LMG 25940 (Xpd)**	**LMG 12749 (Xe)**	**LMG 9055 (Xps)**
gumC	Xca_2779	Xpd_1263	Xe_77	Xps_3603
gumD	Xca_2780	Xpd_1264	Xe_78	Xps_3602
gumE	Xca_2781	Xpd_1265	Xe_79	Xps_3601
gumF	Xca_2782	Xpd_1266	Xe_80	Xps_3600
gumG	Xca_2783	Xpd_1267	Xe_81	Xps_3599
gumH	Xca_2784	Xpd_1268	Xe_82	Xps_3598
gumI	Xca_2785	Xpd_1269	Xe_83	Xps_3597
gumJ	Xca_2786	Xpd_1270	Xe_84	Xps_3596
gumK	Xca_2787	Xpd_1271	Xe_85	Xps_3595
gumL	Xca_2788	Xpd_1272	Xe_86	Xps_3594
gumM	Xca_2789	Xpd_1273	Xe_87	Xps_3593
HP	Xca_2790	Xpd_1274	Xe_88	Xps_3592
gumN	Xca_2791	Xpd_1275	Xe_89	Xps_3591

RAST annotations of the four genomes: LMG7399: 92828.12; LMG25940: 6666666.94921; LMG12749: 92828.9; LMG9055: 270916.4.
Xca = *X. citri* pv. *aracearum*; Xpd = *X. phaseoli* pv. *dieffenbachiae*; Xe = *X. euvesicatoria*; Xps = *X. phaseoli* pv. *syngonii*; CDS = Coding DNA sequence; HP = hypothetical protein.

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Chapter 4:

General discussion, conclusions and perspectives

4.1 Introduction

Bacterial blight of aroids caused by bacteria named as *Xanthomonas axonopodis* pv. *dieffenbachiae* (Xad) is a devastating disease and responsible for significant crop losses. Difficulties in controlling the spread of the pathogen, as well as the severity of the disease led to the classification of Xad as an A2 quarantine organism on the EPPO list (EPPO, 2009). Within the EU, this is especially relevant in order to protect and control the disease in the production of *Anthurium* which is an economically important host plant. Based on *gyrB* and *avrBs2* sequences generated in the EU-FP7 project QBOL ('Development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health'), the ILVO partner has revealed three different sequence groups within Xad and a correlation of this grouping with the host plant from which they had been isolated (generally *Anthurium*, *Dieffenbachia* and *Philodendron*). This indicates that the Xad strains do not reflect one genetic entity, and consequently, it also implicated further research on the pathogenic group known as Xad and on its regulated status. Clarifying the taxonomic position of Xad strains, investigating the pathogenic capacity of Xad strains on a range of aroids and exploring whole genome sequences for sets of pathogenicity genes were the three main objectives of this PhD study.

4.2 Clarifying the taxonomic position of Xad strains within *X. axonopodis* species complex

The genus *Xanthomonas* has been subject to numerous taxonomical and phylogenetic studies. Since 1990, a major overhaul of *Xanthomonas* taxonomy was undertaken by Vauterin *et al*. (1990), a report that was followed by many other studies. The need for this revision arose due to the extensive use of the pathovar classification system for newly isolated *Xanthomonas*, which is a classification with no taxonomic relevance that is used as a matter of convenience. The use of this system, with disregard for an accurate taxonomic positioning, led to more than 100 different pathovars being included in *Xanthomonas campestris*. Although the pathovar designation should be based on the type of symptom it produces on the host plant from which it was isolated and also on the host plant range (Dye, 1980), strains of *X. campestris* were often assigned to the pathovar only on the basis of the host plant they were isolated from without regarding pathogenicity (Starr, 1981). The DNA-DNA hybridization (DDH) assays (Vauterin *et al*., 1995) and more recently, Multi Locus Sequence Analysis (MLSA) and Amplified Fragment Length Polymorphism (AFLP) (Ah-You *et al*., 2009; Young *et al*., 2010) led to a major rearrangement of the taxonomic status of several *Xanthomonas* species within the genus. A comprehensive DNA-DNA hybridization study resulted in the recognition of 20 species (Vauterin *et al*., 1995) with *X. axonopodis* (DNA-DNA homology group 9) being the least homogeneous species of the

genus (Schaad *et al*., 2000). Based on AFLP and rep-PCR data six genetic clusters were described within this species (Rademaker *et al*., 2000; Rademaker *et al*., 2005).

X. axonopodis has been recently modified by several proposals that renamed some pathovars as species (Jones *et al*., 2004; Schaad *et al*., 2005). These proposals did not put forward a comprehensive review of this species, but several name changes were made in studies that focused on a few target pathogens (e.g. those on tomato and pepper, bean, citrus), of which the nomenclature was changed, leaving unmodified the nomenclature of phylogenetically very closely related pathovars. Moreover, it has been intensely debated that *X. axonopodis* is in fact a species complex and that the different genetic groups identified within it may represent different species. The proposals made were not validated as they did not fulfil the taxonomic and nomenclatural rules (Young *et al*., 1991; Ah-You *et al*., 2009). Clearly, a more robust taxonomic investigation of the *X. axonopodis* species complex was needed. In this context, our study was very timely. Besides clarifying the taxonomic position of Xad strains, it also resolved an unclear taxonomic situation with several parallel and sometimes non-comprehensive nomenclatures and led to a better understanding of the relationships between strains of *X. axonopodis sensu* Vauterin *et al*. (1995).

Because for practical diagnosis it is important that quarantine organisms are unambiguously identified, the first objective of this dissertation was to clarify the taxonomic allocation of Xad strains known to be heterogeneous. Therefore, an extensive study has been performed as detailed in chapter 2. To date, DDH, 16S rRNA gene sequence analyses and MLSA analysis are the preferred techniques for delineating bacterial species. However, DDH and 16S rRNA gene sequence analysis have some limitations including the impossibility of assembling cumulative databases based on DDH and the inadequate resolution for species differentiation of the 16S rRNA gene sequences due to the conservative nature of this gene.

Therefore, often the application of additional molecular techniques is needed. More recently, the average nucleotide identity (ANI) analysis of conserved and shared genes between two strains has been proposed to delineate bacterial species (Richter & Rosselló-Mora, 2009). Although the method is probably the most accurate for species delination, comparative analysis of whole genome data remains still limited accessible and therefore not yet fully exploited in the context of new species description. However, this is changing fast. Recently, taxonomic journals such as Systematic and Applied Microbiology have started to list the complete genome sequence of the type strain and calculation of ANI values with close relatives as requirements for new species descriptions (Richter & Rosselló-Mora, 2009). It therefore seems complete genomes will soon become indispensable for taxonomy.

As the taxonomic resolution of 16S rRNA gene is too low within *Xanthomonas*, this gene was not investigated for the strains included in our study. First, MLSA based on seven genes was used to investigate a set of Xad strains together with a considerable number of phylogenetically related strains.

Our MLSA data was congruent with those of previous studies (Ah-You *et al*., 2009; Parkinson *et al*., 2009). Moreover, the four phylogenetic groups from our study correspond to the clades previously described by Parkinson *et al*. (2009) (*X. fuscans* clade, *X. euvesicatoria* species complex, *X. euvesicatoria* species complex sister clade and *X. axonopodis* clade) and the subgroups identified by Rademaker *et al*. (2005), subgroups 9.5 & 9.6, subgroups 9.2, 9.3, and 9.4 (Table 4.1). Second, a well-chosen set of strains was examined using DDH, ANI calculations and biochemical analysis. Based on the data generated in this study, we proved that each of the four groups represent different species. Moreover, the MLSA data was congruent with the ANI data and proved to be suitable for accurate identification of strains of *X. axonopodis sensu* Vauterin *et al*. (1995) at the species level. So, for the Xad strains we were able to show that they belong to three different species outside *X. axonopodis*. Based on this data, and in compliance with the rules of the International Code of Nomenclature of Prokaryotes (the "Code") (Parker *et al*., 2015) and the International Standards for Naming Pathovars (the "Standards") (Dye *et al*., 1980; Young *et al*., 1992), new taxonomic proposals are made. Due to the huge genotypic diversity within these taxa, the taxonomic revision was challenging, especially because some phylogenetic groups (PGs) contained multiple type strains. In these cases the question was which species name to maintain for these phylogenetic groups. PG I included the type strains of *X. citri* subsp. *citri*, *X. citri* subsp. *malvacearum*, *X. fuscans* subsp. *fuscans* and *X. fuscans* subsp. *aurantifolii*, while PG II included the type strains of *X. euvesicatoria*, *X. perforans*, *X. alfalfae* subsp. *alfalfae* and *X. alfalfae* subsp. *citrumelonis*. The combination of genotypic and phenotypic data together with the rules of the International Code of Nomenclature of Prokaryotes (Rule 42: "In the case of subspecies, species, subgenera, and genera, if two or more of those taxa of the same rank are united, the oldest legitimate name or epithet is retained") allowed for reclassification of *X. perforans* and *X. alfalfae* as *X. euvesicatoria* and emendations of the descriptions of *X. citri*, *X. phaseoli* and *X. axonopodis*. PG III contains the 'long forgotten' *X. phaseoli* type strain. The species was described by Gabriel *et al*. (1989), and validly published however Young *et al*. (1991) and the subcommittee of the taxonomy of *Pseudomonas* found the proposal to reinstate *X. phaseoli* as species of the genus *Xanthomonas* insufficient. During the last 20 years this species was forgotten and not included in the major taxonomic studies on *Xanthomonas* (Vauterin *et al*. 1995; Rademaker *et al*., 2005; Parkinson *et al*., 2009; Young *et al*., 2008) and not even in papers on *X. axonopodis* pv. *phaseoli* (Mkandawire *et al*., 2004; Alavi *et al*., 2007; 2008). However *X. phaseoli* it is still on the list of Prokaryotic Names with Standing in Nomenclature so it has to be considered; therefore the strains from PG III were named *X. phaseoli*. PG IV is the only one remaining to be called *X. axonopodis* as it contains the type strain of *X. axonopodis*.

As already mentioned, the taxonomic proposals from this study were made according to the "Code" and the "Standards" although the bacterial classification and nomenclature based on the "Code" is

often conflicting with the pathovar classification applied to the phytopathogenic diversity within *Xanthomonas*. The use of pathovars is well established for the genus *Xanthomonas* and clearly indicates differences in pathogenic ability within a species, but has no taxonomic value. However, for some pathogenic *Xanthomonas*, subspecies have been created instead of pathovars (*X. fuscans* subsp. *fuscans*, *X. fuscans* subsp. *aurantifolii*, *X. citri* subsp. *citri*, *X. citri* subsp. *malvacearum*, *X. alfalfae* subsp. *alfalfae* and *X. alfalfae* subsp. *citrumelonis*). It seems unapproriate to create subspecies for groups of strains that are meant to be distinguished on the basis of their pathogenicity on a certain host. Therefore, we proposed to change the investigated subspecies to pathovars. The existing pathovars have been reclassified according to the classification proposed here (Table 4.2). Although 113 strains were investigated in our study, it is clear that still more pathovars of *X. axonopodis* are misclassified at the species level and require further revision (i.e. *X. axonopodis* pv. *begoniae*).

Thus, a first taxonomic framework for the *X. axonopodis* species complex was proposed in this study, removing subspecies and describing pathovars instead, allowing more extended studies that tackle the host–pathogen relation in more depth. The proposed classification system is a combination of a rational taxonomy and a convenient pathovar classification system for phytosanitary management.

In conclusion, our study not only confirmed earlier research regarding the heterogeneity within this group of xanthomonads, but also took the decisive step to make a reclassification of strains formerly named as Xad, including phylogenetically closely related pathovars. Another merit of our study was that for the first time, Xad strains have been affiliated to three species, and remarkably they do not belong to *X. axonopodis*. Moreover, only Xad strains grouping with the pathotype strain, can maintain the pathovar epithet “dieffenbachiae” in a new combination as *X. phaseoli* pv. *dieffenbachiae* comb. nov. according to the International Standards for Naming Pathovars (Rule 27: “Division of a pathovar. When a pathovar is divided into two or more new pathovars the original pathovar epithet must be-retained for that new pathovar which contains the designated pathotype strain”). This new classification has consequences not only for these aroid pathogens on the EPPO A2 quarantine list but also for other EU regulated *Xanthomonas* plant pathogens (Council Directive 2000/29/EC). For instance, the citrus pathogen on the EPPO A1 quarantine list, *X. citri* causes citrus canker – a disease which results in heavy economic losses to the citrus industry worldwide. The names have to be adjusted based on the new classification in the List of Accepted Species Names. According to the bacterial code (Parker *et al*., 2015), a proposed name is validly published when it appears in the International Journal of Systematic and Evolutionary Microbiology (IJSEM). Therefore we sent a validation request together with our published paper on the revision of *X. axonopodis* species complex (Constantin *et al*., 2016) to the IJSEM. The emendations of *Xanthomonas* species descriptions will be listed in List of Changes in Taxonomic Opinion no 26, to be published in the July issue of the IJSEM.

Table 4.1. The position of the four emended species (*X. citri*, *X. euvesicatoria*, *X. phaseoli*, *X. axonopodis*) in previous studies

Constantin *et al*. (2016)	**Parkinson *et al*. (2009)**	**Rademaker *et al*. (2005)**
X. citri PG I	*X. fuscans*	RG 9.5 & 9.6
X. euvesicatoria PG II	*X. euvesicatoria*	RG 9.2
X. phaseoli PG III	*X. euvesicatoria* sister clade	RG 9.4
X. axonopodis PG IV	*X. axonopodis*	RG 9.3

Tabel 4.2 Reclasification of strains that belonged to *X. axonopodis* species complex

Strain no	**Former name (below species level) according to Bull *et al*. (2010 & 2012)**	**Proposed name**
LMG 9322	*X. citri* subsp. *citri*	*X. citri* pv. *citri*
306, AW 12879	*X. axonopodis* pv. *citri*	*X. citri* pv. *citri*
GSPB 1386, GSPB 2388, LMG 11726, X 18	*X. citri* subsp. *malvacearum*	*X. citri pv. citri*
12-2	*X. axonopodis* pv. *glycines*	*X. citri* pv. *glycines*
LMG 859	*X. axonopodis* pv. *punicae*	*X. citri* pv. *punicae*
CFBP 2938, LMG 941	*X. axonopodis* pv. *mangiferaeindicae*	*X. citri* pv. *mangiferaeindicae*
LMG 826, LMG 8039, LMG 8130	*X. fuscans* subsp. *fuscans*	*X. citri* pv. *fuscans*
ICPB 10535, ICPB 11122, LMG 8655, LMG 9179, LMG 9181, LMG 9182, LMG 9654, LMG 25933, LMG 25937	*X. fuscans* subsp. *aurantifolii*	*X. citri* pv. *aurantifolii*
LMG 7399, LMG 7400, PD 3821	*X. axonopodis* pv. *dieffenbachiae*	*X. citri* pv. *aracearum*
CFBP 2914	*X. axonopodis* pv. *anacardii*	*X. citri* pv. *anacardii*
LMG 8021, LMG 8022	*X. axonopodis* pv. *rhynchosiae*	*X. citri* pv. *rhynchosiae*
LMG 867	*X. axonopodis* pv. *sesbaniae*	*X. citri* pv. *sesbaniae*
LMG 936	*X. axonopodis* pv. *vignaeradiatae*	*X. citri* pv. *vignaeradiatae*
LMG 828, LMG 8136	*X. axonopodis* pv. *vignicola*	*X. citri* pv. *vignicola*
LMG 532	[*X. campestris*] pv. *aracearum*	*X. citri* pv. *aracearum*
LMG 872	[*X. campestris*] pv. *thirumalacharii*	*X. citri* pv. *thirumalacharii*
LMG 495, LMG 497	*X. alfalfae* subsp. *alfalfae*	*X. euvesicatoria* pv. *alfalfae*
F1, LMG 9325	*X. alfalfae* subsp. *citrumelonis*	*X. euvesicatoria* pv. *citrumelonis*
85-10, LMG 668, LMG 909, LMG 910, LMG 913, LMG 922, LMG 926, LMG 932, LMG 25943, LMG 25945, LMG 27970	*X. euvesicatoria*	*X. euvesicatoria* pv. *euvesicatoria*
LMG 28258, LMG 904, NCPPB 4322	*X. perforans*	*X. euvesicatoria* pv. *perforans*
LMG 580, LMG 9489, LMG 21894, LMG 25669, LMG 25670, LMG 25671, LMG 25672, LMG 25674, LMG 25675, LMG 25676, LMG 25677, LMG 25910, LMG 25911	*X. axonopodis* pv. *allii*	*X. euvesicatoria* pv. *alii*
LMG 686	*X. axonopodis* pv. *coracanae*	*X. euvesicatoria* pv. *coracanae*
CFBP 5693, LMG 12749, LMG 12752, LMG 12894, LMG 12895	*X. axonopodis* pv. *dieffenbachiae*	*X. euvesicatoria*
LMG 698	*X. axonopodis* pv. *erythrinae*	*X. euvesicatoria* pv. *erythrinae*
LMG 845	*X. axonopodis* pv. *physalidicola*	*X. euvesicatoria* pv. *physalidicola*
LMG 470	[*X. campestris*] pv. *alangii*	*X. euvesicatoria* pv. *alangii*
LMG 499	[*X. campestris*] pv. *amorphophalli*	*X. euvesicatoria* pv. *amorphophalli*
LMG 534	[*X. campestris*] pv. *argemones*	*X. euvesicatoria* pv. *argemones*
LMG 9040	[*X. campestris*] pv. *betae*	*X. euvesicatoria* pv. *betae*
LMG 846	[*X. campestris*] pv. *physalidis*	*X. euvesicatoria* pv. *physalidis*
LMG 865	[*X. campestris*] pv. *sesami*	*X. euvesicatoria* pv. *sesami*
LMG 873	[*X. campestris*] pv. *tribuli*	*X. euvesicatoria* pv. *tribuli*
GBBC 922, LMG 695, LMG 12708, LMG 12716, LMG 12734, LMG 12738, LMG 12739, LMG 12741, LMG 25938, LMG 25939, LMG 25940, NCPPB 3380, PD 2797, PD 3413, PD 4015, PD 4394, PD 4485	*X. axonopodis* pv. *dieffenbachiae*	*X. phaseoli* pv. *dieffenbachiae*
AT6B, CFBP 1851, CIO1	*X. axonopodis* pv. *manihotis*	*X. phaseoli* pv. *manihotis*
LMG 29033, LMG 7455	*X. axonopodis* pv. *phaseoli*	*X. phaseoli* pv. *phaseoli*
LMG 9055	[*X. campestris*] pv. *syngonii*	*X. phaseoli* pv. *syngonii*
LMG 982	*X. axonopodis* pv. *axonopodis*	*X. axonopodis* pv. *axonopodis*
LMG 895, LMG 901	*X. axonopodis* pv. *vasculorum*	*X. axonopodis* pv. *vasculorum*

All *X. campestris* pathovars not examined by Vauterin *et al*. (1995) and that are shown here not to belong to *X. campestris* are placed in parentheses [ ].

4.3 Pathogenicity and virulence gene content of the *Xanthomonas* pathogens on Araceae

Bacterial blight of aroids is caused by xanthomonads formerly known as *Xanthomonas axonopodis* pv. *dieffenbachiae*. Prior to 1995, xanthomonads causing bacterial blight of aroids were classified under a single species, *X. campestris* and then transferred to *X. axonopodis* (Vauterin *et al*., 1995). As a consequence of our taxonomic study (Chapter 2, Constantin *et al*., 2016), Xad strains are now allocated in three other species namely, *X. citri*, *X. phaseoli* and *X. euvesicatoria*.

It is clear that bacterial classification and nomenclature based on the bacterial code (Parker *et al*., 2015) is not appropriate for pathovar infrasubspecies classification, which is however needed to identify a specific pathogen in some *Xanthomonas* species that harbor different pathovars, and which is the case for the three species to which the Xad strains have been allocated. Pathogenicity tests to determine host range are an essential part of the pathovar classification. Consequently, our first paper on the taxonomic revision clearly needed a continuation in order to identify these strains as groups of aroid pathogens within their respective species. Therefore, in Chapter 3 we studied their pathogenicity to aroid hosts by in planta bioassays, and whole genome sequence analysis has been performed to supplement the knowledge concerning genomic features related to pathogenicity in *Xanthomonas*.

There is still insufficient information available in the literature about the actual phytopathogenic specialization of the former Xad strains. In contrast to the numerous studies reporting on the Xad genetic diversity, only few studies included pathogenicity tests on a range of aroid hosts (Lipp *et al*., 1992; Chase *et al*., 1992; Berthier *et al*., 1993; Robéne-Soustrade *et al*., 2006). Moreover, these studies described the pathogenicity as mean values per host-strain group, not showing variation among individual strains. Lipp *et al*. (1992) and Chase *et al*. (1992) used a “virulence rating scale” for scoring pathogenicity but the other studies scored the symptoms only as “positive or negative”, in which case a general conclusion was made based on the majority of strains within each host-strain group, hence strain variation within each group cannot be recognized. Our study is the first to present the pathogenicity of individual strains within each host-strain group (species), and shows a strain dependent variation in virulence to aroid host genera.

We performed pathogenicity tests with eleven strains on six different aroid species. The first important findings was that *Aglaonema* plants were the most susceptible and *Philodendron* plants the least susceptible to infection by the eleven tested strains. Two different *Philodendron* cultivars were tested with the same results. The symptoms on *Philodendron* were weak and appeared later than on the other plants. Besides that the strain isolated from *Syngonium* only infects *Syngonium* inducing severe and systemic infection, confirming the findings of Chase *et al*. (1992). Another remark was that the strains isolated from *Anthurium* which now belong to *X. phaseoli* were aggressive and able to cause

systemic infections on *Anthurium* and *Dieffenbachia*, as also reported by previous studies (Lipp *et al*., 1992; Robéne-Soustrade *et al*., 2006). Also the strains isolated from *Aglaonema*, *Xanthosoma* and *Dieffenbachia* which now belong to *X. citri* were virulent, they produced leaf spots, but not systemic infections. In contrast, the *X. euvesicatoria* strains isolated from *Philodendron* showed only a weak and doubtful pathogenicity. Overall, our tests have demonstrated that except for the pathogen isolated from *Syngonium*, which is restricted to this host, strains had some overlapping host ranges, although severity of disease can vary between host species.

In order to get insights into the host range specificity and diversity among these former Xad strains now belonging to three species, we sequenced whole genomes of four representative strains belonging to the three groups. Comparison of the predicted protein sequences of the four strains by reciprocal blast search showed that *X. phaseoli* pv. *dieffenbachiae* (isolated from *Anthurium*) is closely related to *X. phaseoli* pv. *syngonii* (isolated from *Syngonium*), which was an extra confirmation for both strains now designated to the same *X. phaseoli* species. We compared different clusters of pathogenicity-related genes in the draft genomes and interestingly found similar profiles, also for the *X. euvesicatoria* strain isolated from *Philodendron* which was scored as non-pathogenic to the tested aroid hosts. So based on its gene content, this strain, and by extention also the other *X. euvesicatoria* strains isolated from *Philodendron* that were previously in the big Xad pool, are probably to be regarded as pathogens but to yet unknown plant hosts. Although the four genomes are quite conserved with respect to the different gene families studied, each strain has its specificities. Certain pathogenicity genes are possibly associated with strain preference to a particular host range or virulence. We discerned differences in gene content between the strains restricted to mesophyll (causing leaf symptoms) and the strains that cause systemic infections, but also differences in gene content among the strains that cause systemic infections. For the time being, the meaning of these gene differences, is not clear-cut, it stays hypothetical as the function of several genes is still unknown. In this respect, our work did not provide clear interpretations on the relation between gene presence and symptomatology or host range, but is rather to be regarded as a contribution to building data and knowledge in this complex matter. Type II and type III secretion clusters were present in the four strains, and they were similar in terms of genetic organization and sequence identity. Type III secretion effectors are regarded as important pathogenicity factors and host range determinants. They are translocated into the plant cell and interfere with the plant immune response. We screened the four draft genomes for effectors and found interesting similarities and differences in the effector repertoires. Common effectors (13) of the three strains representing the three newly proposed pathovars on aroids are important pathogenicity candidates on aroids. Three of these 13 effectors namely *XopE2*, *XopG* and *XopAM* were found absent from the *X. euvesicatoria* strain identified as a non-pathogen for the aroid hosts. Although these effectors are also reported in other *Xanthomonas*

genomes (da Silva *et al*., 2002; Potnis *et al*., 2011), this effector combination, might contribute to disease on aroid plants. These genes need to be functionally characterized to understand their roles in virulence and host specificity. This can be done creating mutants for these genes and testing their pathogenic capacity (Büttner *et al*., 2002; Lorenz & Büttner, 2009).

Based on our previous study (Constantin *et al*., 2016) and the results of the pathogenicity tests in this study, we propose to name the pathogens on aroids as *X. phaseoli* pvs *dieffenbachiae* and *syngonii* comb. nov., and *X. citri* pv. *aracearum* comb. nov. We also propose to reconsider the current quarantine status of the pathogen within the EU and to restrict phytosanitary regulations for xanthomonads on aroids to these three pathovars. The name *X. axonopodis* pv. *dieffenbachiae* has to be removed from the A2 list and be replaced by the three pathovars mentioned above. The EPPO Lists are reviewed every year by the Working Party on the Phytosanitary Regulations and approved by Council. These pathovar names have also to be adjusted based on the classification proposed in this study by the Committee on Taxonomy of Phytopathogenic Bacteria of the International Society for Plant Pathology (ISPP-CTPPB). Therefore our published paper (Constantin *et al*., 2017) will be sent to the ISPP-CTPPB and in consequence the new pathovar names proposed here will be published further in the List of New Names of Plant Pathogenic Bacteria in the Journal of Plant Pathology.

It is clear that pathovars have been created for a practical reason and that they are linked with pathogenicity characteristics, in consequence with the quarantine system. It would be interesting to create a quarantine system that is based on the presence of well documented pathogenicity determinants (e.g. the effectors). With enough information on the causative genes that are determinative for infection, the presence of these genes would then be the “quarantine criteria”.

Although this dissertation leaves many questions unanswered, all methods applied in this PhD study painted a consistent picture of Xad as a complex of pathogens. The work presented shows a global overview of mechanisms involved in virulence but further studies are needed to test and improve the understanding of the role of all the virulence-related features mentioned here in repect to the pathogenicity groups correlating to the three Xanthomonas species in which the Xad strains are now allocated. Final conclusions on the species-specific virulence factors would yet be premature and incorrect since virulence on the same host plants showed to be also strain dependend.

In any case, the results of this PhD study can be included in an reassessment of the regulatory status of Xad within the EU, and may present interesting insights to researchers studying plant pathogenic *Xanthomonas* in general as well as those developing future control measures for Xad on aroids. The work outlined in this thesis has demonstrated that the phylogenetic heterogeneity of Xad strains is mirrored in pathogenic heterogeneity justifying the installation of three pathovars for the pathogens on aroids, and omitting the *X. euvesicatoria* strains.

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Curriculum Vitae

Personal information

Name: Elena Corina Constantin

Nationality: Romanian

Date of birth: 30 March, 1986

Address: Avenue Henri Pauwels 7, Woluwe - Saint - Lambert, Bruxelles

E-mail: elena.constantin@ilvo.vlaanderen.be; elenagav@yahoo.com

Educational background

2012 Present

PhD – Ghent University, Faculty of Sciences; Institute for Agricultural and Fisheries Research (ILVO); Dissertation "Taxonomic revision of *Xanthomonas axonopodis* pv. *dieffenbachiae* strains and pathogenicity on Araceae plants".
Promoters: Dr. M. Maes, Prof. Dr. P. De Vos & Prof. Dr. Anne Willems

2009 – 2011

Master's degree – University of Craiova, Faculty of Horticulture; Dissertation "Isolation, identification, quantification of *Xanthomonas arboricola* pv. *juglandis* populations, causing walnut blight".
Promoter: Prof. Dr. Mihai Botu

2005 – 2009

Bachelor's degree – University of Craiova, Faculty of Horticulture; Dissertation "Identification and evaluation of plum genetic resources in the introduction to conservation".
Promoter: Dr. Mihai Botu

Research experience

July 2011

Short - Term Scientific Mission, Cost 873 program; University of Girona, Spain; Institute of Food and Agricultural Technology; Topic "In vitro activity of antibacterial peptides against *Xanthomonas arboricola* pv. *juglandis*".
Supervisor: Dr. Concepcio Moragrega Garcia

July 2010

Short - Term Scientific Mission, Cost 873 program; University of Girona, Spain; Institute of Food and Agricultural Technology; Topic "Field and laboratory techniques for isolation, identification and quantification of *Xanthomonas arboricola* pv *juglandis* populations, causing walnut blight".

Supervisor: Dr. Concepcio Moragrega Garcia

February-May 2010

Erasmus training on Phytopathology; Technological Education Institute of Larissa, Greece; Topic "Sampling, isolation, evaluation and monitoring techniques for the microorganisms associated with Brown apical Necrosis (BAN) in walnuts".
Supervisor: Dr. Alexandros Papachatzis

Courses

Microbial evolution and diversity; Ghent University; 2012.

Scientific output

A1 publications

E. C. Constantin, I. Cleenwerck, M. Maes, S. Baeyen, C. Van Malderghem, P. De Vos, and B. Cottyn, 2016. Genetic characterization of strains named as *Xanthomonas axonopodis* pv. *dieffenbachiae* leads to a taxonomic revision of the *X. axonopodis* species complex. *Plant Pathology* **65**, 792-806, doi: 10.1111/ppa.12461.

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[1] Last name changed from Gavrila to Constantin in 2014

B. Cottyn, P. De Vos, M. Holterman, J. Zaluga, E. Gavrila and M. Maes. Barcode identification of quarantine bacteria: strategy and results. QBOL-EPPO Conference on DNA barcoding and diagnostic methods for plant pests, Haarlem-The Netherlands, May 23, 2012.

E. Gavrila. Isolation, identification, quantification of *Xanthomonas arboricola* pv. *juglandis* populations, causing walnut blight, Annual Conference & MCM, Jurmala-Latvia, September 13-15, 2010.

Conferences attendance without contribution

64th International Symposium on Crop Protection. Ghent, May 22, 2012

67th International Symposium on Crop Protection. Ghent, May 19, 2015.