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Molecular characterization of *Xanthomonas* species isolated from Araceae and the development of a triplex TaqMan assay for detection of *Xanthomonas phaseoli* pv. *dieffenbachiae*

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Abstract In total 58 *Xanthomonas* strains isolated from Araceae worldwide, together with 13 other phylogenetically-related *Xanthomonas* strains, were characterized using multilocus sequence analysis based on concatenated sequences of seven single copy orthologous genes, extracted from whole genome sequences. The analysis revealed a monophyletic clade of 48 strains, 44 isolated from *Anthurium*, identified as *X. phaseoli* pv. *dieffenbachiae* (Xpd) confirmed by nucleotide identity analysis. The other strains from aroids were identified as *Xanthomonas euvesicatoria* (2 strains), *X citri* (5 strains) and *Xanthomonas sacchari* (3 strains). Two TaqMan assays were designed for specific detection of Xpd, one targeting sequences of a hypothetical protein and one targeting a type I restriction endonuclease subunit S. The two assays showed similar reaction kinetics and were merged with an assay comprising an amplification and extraction control into a triplex assay. The assay was able to detect minimally 100 copies of a target sequence delivered as a gBlock, 100 fg of genomic DNA and 10^4 cells per mL in an *Anthurium* leaf extract.

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Introduction

Anthurium bacterial blight and leaf spot is caused by bacteria belonging to the genus *Xanthomonas*. In 1995 the pathogens from aroids were classified as *Xanthomonas axonopodis* pv. *dieffenbachiae* on the basis of DNA-DNA hybridization studies (Parkinson et al., 2009; Vauterin et al., 1995). The pathogen is not restricted to *Anthurium*, but can infect a number of aroids, amongst which *Dieffenbachia*, *Philondendron*, *Aglaonema* and *Syngonium* (Anonymous, 2009). The pathogen causes wet, chlorotic or necrotic leaf spots, blight and decay of plant tissues. Infections usually occur through hydathodes and therefore symptoms often start at the leaf margins and at the abaxial side of leaves. The pathogen is widely distributed and has been found on all continents (www.cabi.org/isc/datasheet/56931). *X. axonopodis* pv. *dieffenbachiae* is listed on EPPO alert list (A2).

Strains of xanthomonads pathogenic in ornamental aroids are highly heterogeneous as shown with various genetic techniques which comprised RAPD-PCR (Khoodoo & Jaufeerally-Fakim, 2004), rep-PCR (Rademaker et al., 2005), AFLP (Rademaker et al., 2005), rRNA restriction patterns (Berthier et al., 1993), single locus (*gyrB*) sequencing (Parkinson et al., 2009),

multilocus sequence analysis (Constantin et al., 2016; Donahoo et al., 2013; Young et al., 2008), DNA-DNA-hybridization (Constantin et al., 2016; Donahoo et al., 2013) and Average Nucleotide Identity calculations (ANI) based on whole genome sequence analysis (Constantin et al., 2016).

Using rep-PCR techniques (BOX-, ERIC- and REP-PCR), *X. axonopodis* pv. *dieffenbachiae* strains were clustered in two subgroups of *X. axonopodis*, subgroup 9.4 comprising pathovars *manihotis* and *phaseoli*, and subgroup 9.6 comprising pathovars *phaseoli fuscans*, *aurantifolii*, *vignaeradiatae*, *rhynchosia* (cajani), *bauhinae* and *sesbaniae* (Rademaker et al., 2005). A polyphasic taxonomic approach, including multilocus sequence analysis, ANI values, DNA-DNA hybridization data and phenotypic analysis of strains resulted in a taxonomic revision of the *X. axonopodis* complex (Constantin et al., 2016). Strains isolated from *Dieffenbachia*, *Philondendron* and *Anthurium* were found to cluster within three different groups, each representing a different species. PG1 clustered with type strains of *Xanthomonas citri* and *Xanthomonas fuscans*, PGII with type strains of *Xanthomonas euvesicatoria, X. perforans* and *Xanthomonas alfalfae*, and PGIII with the type strain of *X. phaseoli.* No strains were found in aroids that clustered with the type strain of *X. axonopodis*. Taxonomic proposals were made to encompass strains of PGI as *X. citri*, PGII, comprising strains formerly designated *X. perforans* and *X alfalfae* as *X. euvesicatoria*, PGIII as *X. phaseoli* and PGIV as *X. axonopodis*.

X. phaseoli pv. *dieffenbachiae* (Xpd, PGIII) is the predominant causative agent of bacterial blight and leaf spot in *Anthurium* species (Constantin et al., 2017. Bioassays with some of these strains showed that they can be highly aggressive on *Anthurium*, although strains from *X. citri* and *X. euvesicatoria* could also cause symptomatic infections albeit with mild symptoms (Constantin et al., 2017)(unpublished results WUR, Wageningen, the Netherlands).

The primary aim of this study was to develop a specific triplex TaqMan assay for reliable detection of Xpd in various substrates. For this, studies on the taxonomic position and the genetic diversity of Xpd were undertaken. *Xanthomonas* strains isolated from various aroids (*Anthurium, Aglaonema*, *Philodendron* and *Dieffenbachia*) worldwide, but with an emphasis on *Anthurium* strains from the Netherlands, were included in the studies. Sequence information of the strains was generated or collected from public databases and analysed together with sequences of relevant type or pathotype strains using multilocus sequence analysis (MLSA). Specific primers and probes were selected for detection of Xpd using whole genome sequence information of target and non-target strains. A triplex TaqMan protocol was designed comprising assays against two target loci and including an extraction - amplification control. The specificity and sensitivity of the assay were determined and the assay was evaluated using Anthurium leaf material supplemented with various densities of Xpd.

Materials and methods

Bacterial strains and growth conditions

Strains were collected from international culture collections and work collections at Naktuinbouw (Roelofarendsveen, the Netherlands), the Dutch National Plant Protection Organization (NVWA, Wageningen, NL) and Wageningen University & Research (WUR, Wageningen, NL) (Table 1). Strains from Araceae were isolated from symptomatic plants, but in most cases the pathogenicity of strains was not studied. The bacterial names were used as proposed by Constantin et al. (2016). Bacteria were grown for 48 h on TSA at 27 °C prior to use.

DNA extraction

Bacteria were collected from the agar surface of 9 cm diameter TSA plates in approximately 0.5 mL of water and stored at −20 °C until DNA extraction. The DNA was extracted using the Wizard Magnetic DNA purification System for Food (Promega, Leiden, The Netherlands). DNA yield was determined by using the Pico® Green I dye (Invitrogen, Bleiswijk, NL) and an Infinite® M200 pro microplate reader (Tecan, Männedorf, Switzerland) to measure fluorescence.

Illumina DNA sequencing, genome assembly and annotation

DNA concentration was brought to a concentration of 4 ng/μL for sequencing. Library preparation was

Table 1 *Xanthomonas* strains used in this study and the specificity of TaqMan assays Xpd866 and Xpd4494 against *X. phaseoli* pv. *dieffenbachiae*

Strain nr.	(Tentative) identification	host	Other collection numbers	Country of origin	(patho)type strain	year of isolation	Taqman (Ct-values)		Accession number
							Xpd866	Xpd4494	
LMG 695	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	PD 992, PVRS, CFBP 3133, IPO 1104, NCPPB 1833	Brazil	PT	1965	22,0	21.4	JPYB00000000
IPO 1823	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	NAKB 87	The Netherlands		1996	23.2	22.4	JAGHVS000000000
NBC 10	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	NAKB 108	The Netherlands		1998	15.01	16.12	JAGHVT000000000
NBC 54	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		Poland		2002	23.3	22.1	JAGHVU000000000
IPO 1838	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	nd	nd	JAGHVV000000000
IPO 1839	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	nd	nd	JAGHVW000000000
IPO 1842	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	22.5	22,0	JAGHVX000000000
IPO 1843	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	nd	nd	JAGHVY000000000
IPO 1847	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	nd	nd	JAGHVZ000000000
IPO 1848	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	nd	nd	JAGHWA000000000
IPO 1849	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	nd	nd	JAGHWB000000000
IPO 1850	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	22.8	22.2	JAGHWC000000000
NBC 11	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1997	24.5	23.3	JAGHWD000000000
NBC 547	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		Spain		2004	nd	nd	JAGHWE000000000
PD 1483	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1860	The Netherlands		1989	nd	nd	JAGHWF000000000
PD 2179	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1861	The Netherlands		1993	21.9	21.3	JAGHWG000000000
PD 3185	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1862	The Netherlands		1997	nd	nd	JAGHWH000000000
PD 3186	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1863	Italy		1997	22.3	21.9	JAGHWI000000000
PD 3208	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1864	The Netherlands		1998	nd	nd	JAGHWJ000000000
PD 3347	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1865	The Netherlands		1998	nd	nd	JAGHWK000000000
PD 3413	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1866	The Netherlands		1998	nd	nd	JAGHWL000000000
PD 2170	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1867	The Netherlands		1992	nd	nd	JAGHWM000000000
IPO 1878	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		1999	22.6	21.5	JAGHWN000000000
NBC 703	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		Dominican Republic		2006	14.41	15.53	JAGHWO000000000
NBC 785	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		Ghana		2006	14.9	16.2	JAGHWP000000000
D-27-2-L	*X. phaseoli* pv. *dieffenbachiae*	*Aglaonema sp.*	IPO 1906	USA, Hawaii		1984	21.2	21.5	JAGHWQ000000000
D-92	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1908	USA, Kaneohe		1985	nd	nd	JAGHWS000000000
NBC 978	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	nd	nd	JAGHWW000000000
D-115	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1913	USA, Hawaii		1981	22.4	21.7	JAGHWX000000000
A2108	*X. phaseoli* pv. *dieffenbachiae*	*Dieffenbachia sp.*	IPO 1916	USA, Kauai		1982	22.1	21.6	JAGHXA000000000
A2650	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1917	Jamaica		1990	nd	nd	JAGHXB000000000
D-102	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1919	USA, Hawaii		1980	nd	nd	JAGHXC000000000
D-114	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1920	USA, Hawaii		1981	22.5	22,0	JAGHXD000000000
D-15-3	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1921	USA, Kauai		1984	nd	nd	JAGHXE000000000
D-16-2	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*	IPO 1922	USA, Kauai		1984	nd	nd	JAGHXF000000000
NBC 1112	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	nd	nd	JAGHXH000000000
NBC 1141	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	nd	nd	JAGHXI000000000
NBC 1180	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	nd	nd	JAGHXJ000000000
NBC 1181	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	23.8	22.7	JAGHXK000000000
NBC 1193	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	nd	nd	JAGHXL000000000
NBC 1211	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	23.6	22.4	JAGHXM000000000
NBC 1421	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		-		2008	nd	nd	JAGHXN000000000
NBC 1741	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		China		2009	nd	nd	JAGHXO000000000
LMG 12752	*X. phaseoli* pv. *dieffenbachiae*	*Philodendron sp.*	NBC 4674	USA		1992	22,0	21.4	JAGHXQ000000000
LMG 25939	*X. phaseoli* pv. *dieffenbachiae*	*Dieffenbachia sp.*	NBC 4675	Brazil		1995	24.5	23.3	JAGHXR000000000
NBC 5028	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		France		2015	nd	nd	JAGHXS000000000
NBC 5884	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		China		2019	23.5	22.5	JAGHXT000000000
NBC 977	*X. phaseoli* pv. *dieffenbachiae*	*Anthurium sp.*		The Netherlands		2007	15.03	16.28	
NBC 1192	*X. phaseoli* pv. *manihotis*	*Manihot esculenta*	LMG 765	Malaysia		1980	-	38.9	JAGHXV000000000
NBC 1264	*X. phaseoli* pv. *manihotis*	*Euphorbia pulcherrima*		-		2008	-	-	JAGHXW000000000
NBC 1194	*X. phaseoli* pv. *manihotis*	*Manihot esculenta*	LMG 767	Zaire		1973	-	39,0	JAGHXX000000000
NBC 1265	*X. phaseoli* pv. *manihotis*	*Euphorbia pulcherrima*		-		2008	-	-	JAGHXY000000000
CFBP 7153	*X. phaseoli* pv. *manihotis*	*Manihot esculenta*		-		1965	-	-	JAGHYD000000000
LMG 5274	*X. phaseoli* pv. *manihotis*	*Manihot esculenta*	NBC 1201, IPO 103	USA		1941	-	-	
CFBP 2534	*X. phaseoli* pv. *phaseoli*	*Phaseoli vulgaris*		USA	T	1986	-	-	JAGHYC000000000
NCPPB 1811	*X. phaseoli* pv. *phaseoli*	*Phaseoli vulgaris*		Romania		1966	-	-	JAGHYE000000000
LM G982	*X. axonopodis* pv. *axonopodis*	*Axonopus scoparius*		Columbia	PT	1949	nd	nd	JPYE00000000
NBC 626	*X. axonopodis* pv. *poinsettiicola*	*Euphorbia pulcherrima*		Italia		2005	-	-	
NBC 716	*X. axonopodis* pv. *poinsettiicola*	*Euphorbia pulcherrima*		Africa		2006	-	-	
NBC 1002	*X. axonopodis* pv. *poinsettiicola*	*Euphorbia pulcherrima*		The Netherlands		2007	-	-	
A1962	*X. euvesicatoria*	*Philodendron sp.*	IPO 1914	USA, Florida		1982	-	-	JAGHWY000000000
LMG 12749	*X. euvesicatoria*	*Philodendron sp.*	NBC 4672	USA		1992	-	-	JAGHXP000000000
LMG 495	*X. euvesicatoria* pv. *alfalfae*	*Medicago sativa*		India		1954	nd	nd	JPYG00000000
NBC 95	*X. axonopodis* pv. *begoniae*	*Begonia sp.*	NAKB 170	The Netherlands		2000	-	-	
NBC 96	*X. axonopodis* pv. *begoniae*	*Begonia sp.*		The Netherlands		2000	-	-	JAGHXU000000000
CFBP 2524	*X. axonopodis* pv. *begoniae*	*Begonia sp.*		New Zealand		1962	-	-	JAGHYB000000000
LMG 9322	*X. citri* pv. *citri*	*Citrus aurantifolia*		USA	PT	1989	nd	nd	JPYD00000000
D-36-1	*X. citri* pv. *aracearum*	*Syngonium sp.*	IPO 1907	USA, Honolulu		1985	-	-	JAGHWR000000000
D-99	*X. citri* pv. *aracearum*	*Xanthosoma sp.*	IPO 1910	USA, Florida		1982	-	-	JAGHWU000000000
CFBP 7667	*X. citri* pv. *aracearum*	*Dieffenbachia sp.*		USA		1950	-	-	JAGHXZ000000000
CFBP 7666	*X. citri* pv. *aracearum*	*Dieffenbachia sp.*		USA		1950	-	-	JAGHYA000000000
LMG 7399	*X. citri* pv. *aracearum*	*Dieffenbachia sp.*	NBC 294	USA		1950	-	-	
LMG 826	*X. citri* pv. *fuscans*	*Phaseolus vulgaris*		Canada	T	1957	-	nd	JPYF00000000
A1809	*X. euvesicatoria*	*Caladium sp.*	IPO 1915	USA, Florida		1983	-	-	JAGHWZ000000000
LMG 27970	*X. euvesicatoria*	*Capsicum frutescens*		USA	T	1991	-	nd	JPYC00000000
D-93	*X. sacchari*	*Spathiphyllum*	IPO 1909	USA, Waimanalo		1985	-	-	JAGHWT000000000
D-109	*X. sacchari*	*Anthurium sp.*	IPO 1911	USA, Hawaii		1981	-	-	JAGHWV000000000
A2111	*X. sacchari*	*Colocasia sp.*	IPO 1923	USA, Oahu		1986	-	-	JAGHXG000000000
LMG 4641	*X. sacchari*	*Saccharum officinarum*		France, Guadeloupe	T	1995	-	-	MDEK00000000
LMG 958	*Stenotrophomonas maltophilia*	*Homo sapiens*		?		1981	-	-	MTGD00000000
NBC 405	*X. hortorum pv.carotae*	*Daucus* sp		?		?	-	-	
NBC 55	*X. hortorum pv.pelargonii*	*Pelargonium* sp.		The Netherlands		1988	-	-	
NCPPB 1469	*X. fragariae*	*Fragaria chiloensis*	LMG 708, IPO 1675	USA		1978	-	-	nd
PD 2659	*X. fragariae*	*Fragaria sp.*	IPO 3053	USA		1962	-	-	nd
Xv0056	*X. vesicatoria*	*Lycopersicon esculentum*	NBC 1059, IPO 3663	Brasil		1987	-	-	nd
JBJones 56	*X. vesicatoria*	?	NBC 373	?		?	-	-	nd
JBJones 444	*X. gardneri*	?	NBC 375	?		?	-	-	
Xv0444	*X. gardneri*	*Lycopersicon esculentum*	NBC 1073, IPO 3661	Costa Rica		1991	-	-	nd
Xv0938	*X. perforans*	*Lycopersicon esculentum*	NBC 1067, IPO 3662	Greece		1991	-	-	nd
xv0938 J.Jones	*X. perforans*	*Solanum lycopersicum*	NBC 1067	Greece		1991	-	-	
Xv0980	*X. euvesicatoria*	*Lycopersicon esculentum*	NBC 1088, IPO 3660	Mexico		1992	-	-	nd
IPO 923	*X. euvesicatoria*	?	NBC 292	?		?	-	-	
PD2814	*X. dyei*	*Lobelia sp.*	NBC 98	The Netherlands		1998	-	-	
DM 071	*X. campestris* pv. *raphani*	?	NBC 269	USA		?	-	-	
IPO 102	*X. campestris* pv. *campestris*	*Brassica oleracea*	NBC 275	?		?	-	-	
A4	*X. campestris* pv. *campestris*	?		USA		?	-	-	
NCPPB 528	*X. campestris* pv. *campestris*	*Brassica oleracea*	IPO 3076	United Kingdom		1957	-	-	nd
PD 2693	*X. arboricola*	*Fragaria sp.*	IPO 3085	Italy		1994	-	-	nd

nd = not determined; - = TaqMan negative (no reaction after 40 cycles); ? = information unknown; Data marked in black were derived from DNA purified from a bacterial culture, in red from suspensions boiled in 50 mM NaOH

IPO strain are from the work collection plant pathogenic bacteria of Wageningen University & Research

A and D strain are from the work collection of University of Hawaii (USA), strains were kindly provided by Prof. Dr. A.M. Alvarez

PD strain are from the culture collection of the Dutch National Plant Protection Service (NVWA, Wageningen, NL) kindly provided by Dr M. Bergsma-Vlami

NBC strain are from the the Naktuinbouw bacteria collection (Roelofarendsveen, NL)

NCPPB strain are from the National Collection of Plant Pathogenic Bacteria (FERA, UK)

CFBP strains are from the French Culture Collection of Plant Pathogenic Bacteria (Angers, FR)

LMG strain are from the BCCM/LMG Bacteria Collection (Genth, Belgium)

Xv and JB strain nrs are from the collection of Prof. Dr J.B. Jones (University of Florida, USA)

performed on 50 μL of 4 ng/μL DNA using the protocol as recommended by Illumina and sequenced with the Novaseq technology at BaseClear B.V. (Leiden, NL). A paired-end DNA library with 2 X 150 bp reads was constructed for each strain. Subsequently, samples were demultiplexed and reads, containing adaptors were removed. FASTQ files were imported in CLC genomic workbench version 12.03 (Qiagen, Aarhus, Denmark). Reads were quality trimmed in CLC using a quality score of 0.05 and a maximum of two ambiguous nucleotides per read. Reads were filtered in length with a minimum number of nucleotides per read of 45.

De novo assembly was performed with the trimmed Paired-End data using automatic bubble size and a minimum contig length of 500 pb. A mapping mode of mapping the reads back to contigs was used with a length and similarity fraction of 0.8. Sequencing reads are available at NCBI under project number BioProject PRJNA716613. The accession numbers are provided in Table 1.

Genome assemblies were exported annotated using Prokka (version 1.14.6) (Seemann, 2014), with default settings for the bacteria.

Multilocus sequence analysis

Sequences of the seven housekeeping genes *atpD* (1407 bp), *dnaK* (1926 bp), *efp* (567 bp), *glnA* (1410 bp), *gyrB* (2445 bp), *lrp* (480 bp) and *rpoD* (1878 bp) were used. Sequences of these seven genes were used as previously described (Constantin et al., 2016), but the full length sequences were analyzed in our studies. Sequences were extracted from the annotated contigs using CLC. The genes were concatenated in alphabetic order into a total sequence of 10.113 bp. An alignment was made in CLC Work Bench and imported into MEGA 7.0 software for constructing a phylogenetic tree. A phylogenetic tree was inferred by using the Maximum Likelihood method based on the General Time Reversible model with 500 bootstrapping replications. Strains of *X. citri* pv. *phaseoli* LMG826 *X. citri* pv. *citri* LMG 9322 representing Phylogenetic Group I, *X. euvesicatoria* LMG27970 representing Phylogroup II, *Xpd* LMG 695 (in the collection catalogue listed as *X. axonopodis* pv. *dieffenbachiae*) representing Phylogenetic Group III, *X. axonopodis* pv. *axonopodis* LMG 982 representing Phylogenetic Group IV were used (Constantin et al., 2016) as a references. *Stenotrophomonas maltophilia* LMG 958 was used as an out-group.

Average nucleotide identity

Average Nucleotide Identity (ANI) scores between genomes of (patho)type strains and other *Xanthomonas* strains in the same clade or related clades were estimated using JSpeciesWS (online service offered by Ribocon, https://www.ribocon.com).

Designing of a TaqMan assay specific for Xpd

To design a TaqMan assay that only detects Xpd, the pathotype strain LMG 695 was used as a reference. Potential target sites were identified using CLC genomic workbench in a two-step filtering process: first identifying sequences that are conserved in all strains of the target group (Xpd) and removing sequences that are similar to non-target strains. In more detail, the genome sequence of pathotype strain LMG 695 (NZ_CP014347.1; 5.04 Mb) was dissected in 500 bp-long sequences (10074). These sequences were stringently mapped to all Xpd strains analyzed in the current study, and only those sequences (4694) were kept that mapped to all Xpd strains with 100% similarity. Subsequently this narrowed down set of sequences was mapped (CLC mapping settings: length fraction: 0.85, similarity fraction: 0.85, global alignment: no) to all other strains included in this study, removing sequences that mapped to any of the non-target strains. To assist the final selection and design step, these sequences were added as a track list to the genome of LMG 695. Before proceeding to the design step, the candidate regions were checked for sequence similarity with non-target organisms using BLAST in the nucleotide database of the NCBI Genbank.

Two sets of primers/probe combinations were designed on 500 bp target-specific fragments using primer quest tool of Integrated DNA Technologies (IDT, Leuven, Belgium) with default settings (Table 2). The first set (Xpd866) was derived from a gene encoding for a hypothetical protein and consists of the forward primer FwXpd866 (5'-TACCTGCCTCGCCTCTT3′), reverse primer RvXpd866 (5'-GGATCGTCGGTCTT GTGTTT −3′) and the FAM labelled probe pXpd866 (5'CAACAGCGTGAGAAAGAAACTCGGCA-3′).

Table 2 Primers and probes used in this study

Primer name	Sequence	Label
Set 1 against a hypothetical protein of *Xanthomonas phaseoli* pv. *dieffenbachia* (Xpd)		
FwXpd866	5'-TACCTGCCTCGCCTCTT-3′	
RvXpd866	5'-GGATCGTCGGTCTTGTGTTT −3′	
pXpd866	5'-CAACAGCGTGAGAAAGAAACTCGGCA-3′*	FAM
Set 2 against a type I restriction endonuclease subunit S of Xpd		
fwXpd4494	5'-GTATAGATGTACTGACGGCTCAC-3′	
RvXpd4494	5'-CGCGATCATTCCCGATACTT-3′	
pXpd4494	5'-CGCTTGATTGCAGTTCCACTCAGGA-3*	ATTO532
Set for detection of *Acidovorax cattleya* used as extraction and amplification control (Bonants et al., 2019)		
Acat 2-F	5'-TGTAGCGATCCTTCACAAG- 3	
Acat 2-R	5'-TGTCGATAGATGCTCACAAT - 3′	
Acat 2-Pr	5'-CTTGCTCTGCTTCTCTATCACG - 3′	Texas Red

Fw forward primer, *Rv* reverse primer, *p* probe

* Probes were double quenched with ZEN/Iowa Black FQ

The other set (Xpd4494) was derived from a gene encoding for a type I restriction endonuclease subunit S and consists of the forward primer fwXpd4494 (5'-GTATAGATGTACTGACGGCTCAC3′), reverse primer RvXpd4494 (5'-CGCGATCATTCCCG ATACTT-3′) and an ATTO532 labelled probe pXpd4494 (5'-CGCTTGATTGCAGTTCCACT CAGGA-3′). Probes of TaqMan assays were double quenched with ZEN/Iowa Black FQ (IDT, Leuven, Belgium).

Specificity of the TaqMan assays

The specificity of the assays was tested using genomic DNA of 22 Xpd strains, 46 non-target Xanthomonas strains belonging to different species and a strain of *S. maltophilia* (Table 1). For most strains 0.2 ng of purified DNA was used, but for some strains a suspension of 10^9–10^{10} cells/mLwas boiled in 50 mM NaOH prior to testing. In Table 1 these results are marked with red.

For each TaqMan assay 5 μL of DNA or the boiled suspensions was mixed with 20 μL reaction mix containing 5 μL PerfeCTa multiplex qPCR ToughMix 5x (Quantabio, Beverly, USA), 100 nM probe and 300 nM of each forward and reverse primer. The reactions were performed in a Biorad CFX touch Real-Time PCR detection system (BioRad, Hercules, USA) using the following conditions: 95 °C for 2 min; 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s. Analysis of the data was done by automatic threshold calculation within the Biorad system software. A Ct value ≤35 was considered positive.

Triplex TaqMan design

The two assays for Xpd were combined with an assay that quantify *Acidovorax cattleya* (Acat) (Bonants et al., 2019) into a triplex TaqMan (Table 2). The Acat assay consists of a forward primer Acat 2-F (5'-TGTAGCGA TCCTTCACAAG- 3′), reverse primer Acat 2-R.

(5'-TGTCGATAGATGCTCACAAT - 3′) and a TexasRed labelled probe Acat 2-Pr (5'-CTTGCTCT GCTTCTCTATCACG - 3′). The triplex TaqMan was performed using the same PCR conditions and materials as the simplex tests. For the assays a primer concentrations of 300 nM was used and 100 nM probe in a total volume of 25 μL.

Sensitivity triplex TaqMan assay

The analytical sensitivity (detection threshold) of the triplex TaqMan assay was determined using gBlocks, genomic DNA and target bacteria administered to an *Anthurium* leaf extract. The gBlock, synthetic oligonucleotides (Integrated DNA Technologies, USA), contained concatenated sequences of the amplicons of the two assays for Xpd, an UTC

Universal Template Control (UTC), as a control on the quality of the gBlock, interspaced with some extra flanking nucleotides (Table S1). A ten-fold serial dilution from 10^6 to 1 copies of the gBlocks DNA was used to determine the sensitivity of the triplex Taqman. Per reaction, 1 μL of 10^4 copies of a gBlock of Acat served as an internal control (Bonants et al., 2019).

For determining the detection threshold for genomic DNA, a ten-fold serial dilution of 1 ng-1 fg of genomic DNA from strain LMG 695 (IPO 1104) was used. Each sample was supplemented with 1 μL of 2 pg gDNA of Acat as an amplification control.

In addition the sensitivity was determined with Xpd supplemented to an *Anthurium* leaf extract. Six whole leaves (12 g) of cultivar 'White Champignon' in a BioReba bag with a synthetic intermediate layer (BioReba, Kanton Reinach, Swiss) were crushed using a sample crusher (AAA lab equipment B.V. Roelofarendsveen). Subsequently, 24 mL of 0.05 M PBS (2.5 g of NaH_2PO_4, 2.52 g Na_2HPO_4 and 8.2 g NaCl in 1 Liter water, pH 7.2) was added and after homogenization a serial dilution of bacterial cells of strain IPO1104 was supplemented in a ten-fold serial density of 10^7–10^1 cells/mL. From each dilution 1 mL was pipetted in a strip of Qiagen tubes (Qiagen, Aarhus, Denmark) in replicates. The tubes were centrifuged for 10 min at 6000 g and the supernatant discarded. The pellet was supplemented with 50 μL of a 1000-fold diluted suspension of Acat cells with an optical density at 600 nm of 0.8 (ca. 8. 10^8 cells/ml). DNA extraction was performed with the AGOWA maxi kit (Nucleics, Woollahra, Australia) according to the manufacturer's instructions.

Data analysis

Ct-values were imported from the QuantStudio software (ThermoFisher Scientific, Waltham, USA). Ct-values were plotted against the 10log number of gBlock or gDNA copies, or the density of bacterial cells. The log of the densities in the leaf extract, and (linear) trend lines were determined and the coefficient of determination (R^2) were calculated using MS Excel. The amplicon efficiency was calculated from the slope of the calibration curves, as $10^{1/slope} - 1$ (Kubista et al., 2006). To compare the two assays in the triplex TaqMan, the Ct-values were plotted against each other, to calculate the coefficient of determination (R^2).

Effect of the concentration of the leaf extract on the sensitivity of the TaqMan.

To determine the maximum weight of material that can be used in a composite sample, DNA was extracted from increasing amounts of the central parts of the leaves including part of the petiole in fixed volume of buffer, i.e. 6.2–27.1 g in 100 mL of 0.01 M PBS which were homogenized in, representing different pooling strategies. A suspension of Acat (NBC 430) was supplemented in a density of approximately 10^5 cells per mL before the DNA extraction as extraction and amplification control, to obtain a Ct-value between 27 and 30 after 40 cycles. DNA extraction was carried out using the mag maxi DNA extraction kit (LGC Genomics, Berlin, Germany) on a KingFisher KF96 system following the manufacturer's instructions.

Results

Strain characterization

In the multilocus sequence analysis (MLSA) based on concatenated sequences of seven housekeeping genes (*atpD, dnaK, efp*, *glnA*, *gyrB*, *lrp* and *rpoD)*, strains isolated from *Anthurium* in different parts of the world (the Netherlands, Poland, Spain, Italy, Dominican Republic, Ghana and the USA), together with four strains from other aroids (*Aglaonema*, *Philodendron* and two of *Dieffenbachia*), clustered in a monophyletic group with the pathotype LMG 695 of *X. phaseoli* pv. *dieffenbachiae* (Xpd) (Fig. 1). Seven SNP's were found to be unique and specific for the set of 48 strains in the 10.113 nt sequence analysed. In addition, a 6 bp long deletion in *rpoD* was found to be specific for six strains (PD 2179, PD 2170, LMG 695, LMG 12752, NBC 785 and A2108).

Not surprisingly, the cluster most related to Xpd comprised two strains of *X. phaseoli* pv. *phaseoli* isolated from bean (CFBP 2534 and NCPPB 1811) and five strains of *X. phaseoli* pv. *manihotis* (CFBP 7153, NBC 1192, NBC 1194, NBC 1264 and NBC 1265). *X. axonopodis* pv. *begoniae* (CFBP 2524 and NBC 96) was the closest related species to *X. phaseoli*.

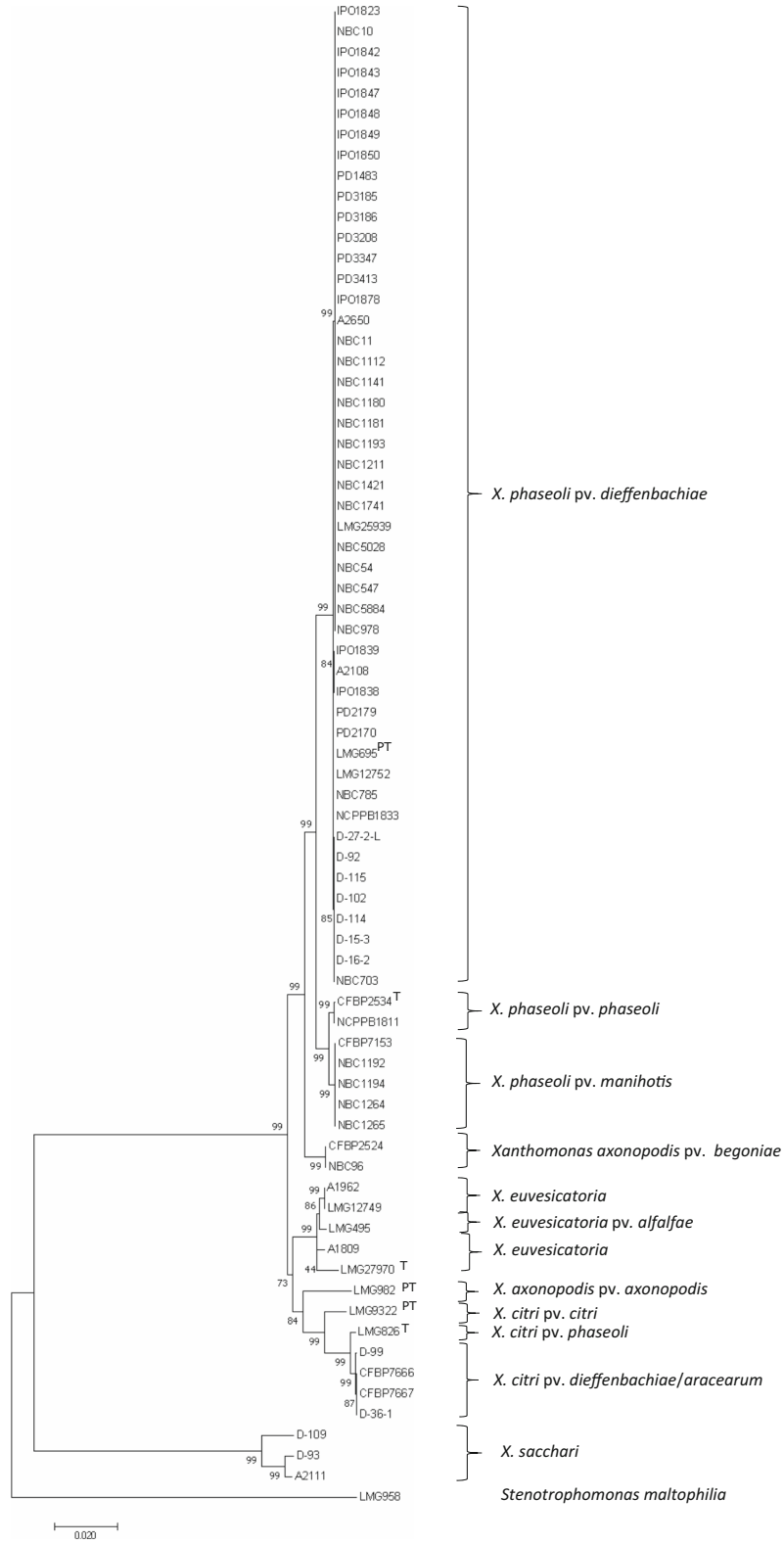

Fig. 1 Maximum-likelihood phylogenetic tree of concatenated nucleotide sequences of the *atpD, dnaK, efp, glnA, gyrB, lrp* and *rpoD* genes from 73 *Xanthomonas* strains. Bootstrap values greater than 50% are shown for 500 replicates. Concatenated sequences of *Stenotrophomonas maltophilia* were used as an out-group. T = type strain; PT = pathotype strain

Using the ANI score, strains isolated from aroids were not only identified as *X. phaseoli*, but also as *X. citri*, *X. euvesicatoria* and *Xanthomonas sacchari* (Table S5–8). All strains of *Anthurium*, however, were identified as Xpd, with the exception of one strain from Hawaii, identified as *X. sacchari.*

Specificity

The specificity of the two assays (Xpd866 and Xpd4494) in the triplex TaqMan was determined on the basis of an in silico analysis using the blastn function in NCBI database. Both assays were highly specific and full matches of the amplicon sequences, 74 nt for Xpd866 and 100 nt for Xpd4494, were found only with target strains LMG 25940 and LM G695. In *X. arboricola* and *Xanthomonas dyei* the closest non-target sequences were found for Xpd866 and Xpd4494, respectively, but only with a low level of homology.

In addition, genomic DNA was tested of 22 strains of Xpd, 46 other *Xanthomonas* strains from which eight belonging to other *X. phaseoli* pathovars and one strain of *S. maltophilia* (Table 1). A positive reaction with low Ct values (21.2–24.5) was found for all 22 Xpd strains in both assays. All non-target strains were negative in both assays. A weak reaction above the detection threshold (Ct values of 38.9 and 39) was only found with two strains (PD 3347 and PD 2170) of the closely-related pathogen *X. phaseoli* pv. *manihotis* in assay Xpd4494.

Analytical sensitivity and linearity of the TaqMan assays

Both assays (Xpd866 and Xpd4494) were able to detect up to 100 copies of the gBlock, 100 fg of genomic DNA and 10^4 cells per mL in *Anthurium* leaf extract (Fig. 2, Table S2–4). If the Ct-values were plotted against the logarithm of the copy number, a regression coefficient higher than 0.98 was found within the dynamic range of the assay (Fig. 2). This was not influenced by the target or the matrix, gBlock, gDNA or cells in *Anthurium* leaf extract. The efficiency of the Xpd866 for gBlocks, gDNA and cells in leaf extract were 103.5, 88.9 and 93.8%, respectively. The estimated efficiency of the Xpd4494 for gBlocks, gDNA and cells in leaf extract were 92.7, 102.2 and 103.1%, respectively. The two assays for Xpd showed highly similar Ct values in experiments with the serially diluted targets. If the Ct-values of the serial dilutions of gBlocks, gDNA or cell-suspensions in leaf extracts for the two assays were plotted against each other, the regression coefficient (R^2) was always higher than 0.99 (Fig. S1). This was despite the difference in the delta Rn values for the assays, caused by the difference in fluorophore (FAM versus Atto532) used in the two assays (Fig. 3). The Ct-values of the Acat TaqMan assay ranged in all samples with the gBlock and gDNA between 27.7 and 29.1 (Table S3 and S4). For the cells in leaf extract (0.5 g/mL), the Ct values for Acat ranged between 31.7 and 34.0 (Table S3).

Effect of the leaf extract concentration

The analytical sensitivity of the triplex TaqMan assay was determined for Anthurium leaf concentrations ranging between 0.062 and 0.271 g per mL (Table 3). Reactions was considered positive if the Ct values of both assays (Xpd866 and Xpd4494) were $\leq$ 35. Detection thresholds were always determined with 5 µl of sample as template. The detection threshold was 10^4 cells per mL for all leaf extract concentrations except for a concentration of 0,171 g/mL in which the threshold was 10^3 cells/mL. Ct values of the Acat TaqMan assay were relatively constant (27.6–31.4), but at the highest density of Xpd (10^8 cells per mL), the reaction of Acat TaqMan was inhibited.

Discussion

A set of 58 Xanthomonas strains of aroids, were characterized using MLSA on concatenated sequences of seven housekeeping genes as described by Constantin et al. (2016), as a first step in the development of a TaqMan assay for specific detection of *Xpd*. All strains isolated from *Anthurium* plus strains of *Aglaonema*, *Philodendron* and two of *Dieffenbachia* grouped into the clade with the type strain of the pathotype of *Xpd* (LMG 695), previously named *X. axonopodis* pv. *dieffenbachiae* (Constantin et al., 2016). In accordance with the work of Constantin et al. (2016), also strains isolated from aroids were identified as *X. euvesicatoria* and *X. citri*. The use of pathovar names for these two species is premature as no pathogenicity data are available.

For the first time, *X. sacchari* is described in association with *Anthurium*, but the pathogenicity has still to

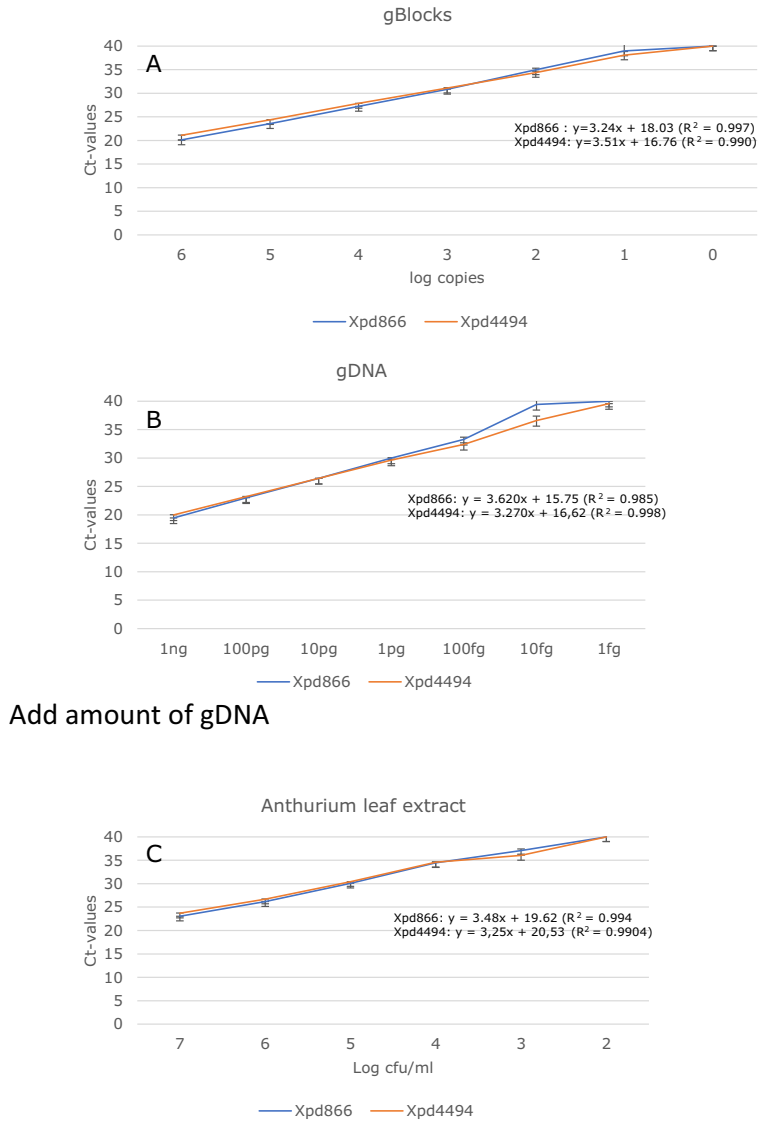

Fig. 2 Calibration curves of TaqMan assays for detection of *Xanthomonas phaseoli* pv. *dieffenbachiae* (Xpd). In A, two TaqMan assays (Xpd866 and Xpd4494) were individually calibrated using a gBlock comprising the amplicon sequences of both assays. The Ct values and the logarithm of the number of gBlock copies were plotted on the x and y axis, respectively. In B, the TaqMan was calibrated in a multiplex format with both assays using genomic DNA (gDNA). The Ct values and the logarithm of the weight of the gDNA of Xpd strain NCPPB 1833 were plotted on the x and y axis, respectively. In C, the TaqMan was also calibrated in a multiplex format with both assays using cell suspensions of Xpd strain NCPPB 1833 in an extract of *Anthurium* leaves. The Ct values and the logarithm of the density of Xpd cells were plotted on the x and y axis, respectively. The error bars represent standard deviations

be determined. In our studies, three strains from *Anthurium* had an ANI score near to 95% with the type strain of *X. sacchari*, one of which (strain D109 from Hawaii, USA) exceeded the threshold of 95% for species delineation. A *Xanthomonas* strain closely-related to *X. sacchari* is known to be able to cause a grain rot of rice (Mirghasempour et al., 2020). The data suggest a genomic species complex for *X. sacchari* and a broad host range as strains were initially found in association with sugar cane, but later also with banana plants (Studholme et al., 2011) and with rice seeds (Bansal et al., 2021; Cottyn et al., 2001; Fang et al., 2015).

The clade of Xpd comprises strains that can differ in their aggressiveness on their host of origin (Constantin et al., 2017), but several were found highly aggressive on *Anthurium* including IPO1838 and 1839 (unpublished results). It has been evidenced that strains isolated from other aroids, including *Dieffenbachia* and *Syngonium* were aggressive on *Anthurium*, but the identity of these strains is not known (Chase et al., 1992). More recently this was also reported by Cottyn et al. (2018).

For detection of Xpd, various methods have been described, based on the use of semi-selective media (Laurent et al., 2009; Norman & Alvarez, 1989),

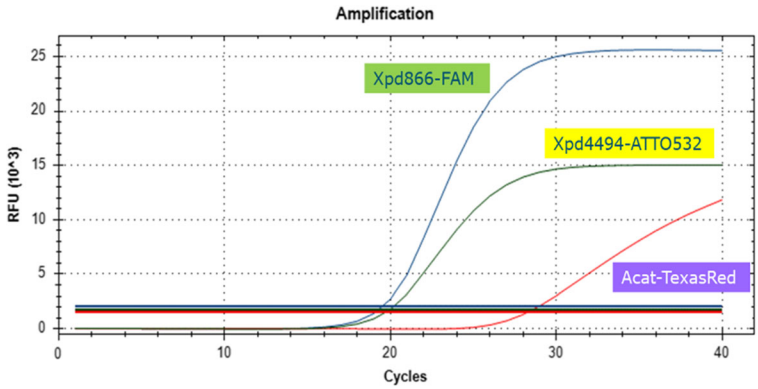

Fig. 3 Amplification plot of the two TaqMan assays, Xpd866 labelled with FAM and Xpd4494 labelled with Atto532 and designed for detection of *Xanthomonas phaseoli* pv. *dieffenbachiae* (Xpd). For the plots a gBlock comprising the amplicon sequences of both assays was used. An assay for *Acidovorax cattleya* (Acat), labelled with Texas Red, was used as amplification control. RFU = relative fluorescence units

serology (Lipp et al., 1992), DNA-based amplification (Lu et al., 2012, Robene-Soustrade et al., 2006, Chabirand et al., 2014,) and methods combining assays of different principles (Khoodoo et al., 2005; Norman & Alvarez, 1994). Immunocapture PCR using Xanthomonas-specific monoclonal antibodies and primers designed from sequence characterized amplified regions (SCARS) allowed detection of strains from Aroids at a level of 10^2–10^3 cfu/mL (Khoodoo et al., 2005). This assay successfully detected Xpd strain D15–3 from Hawaii (USA) which were also included in our study. However, it also detected strain D93, designated in our studies as *X. sacchari*, indicating that the assay is not entirely Xpd specific. For on-site detection of Xpd, a loop-mediated isothermal amplification (LAMP) assay has been developed allowing detection of 10^4 cfu/mL and 1–10 fg of pure genome DNA (Jun-Hai et al., 2015). We presume that the assay has been developed for Xpd, but only Chinese strains were included and a (pathovar) type strain was lacking in their studies.

In 2019, a duplex quantitative (TaqMan) real-time PCR assay for detection of Xpd was described, targeting sequences of a gene coding for an ABC transporter (Jouen et al., 2019). The authors used an assay based on sequences encoding for chalcone synthase in *Anthurium andreanum* as an extraction and amplification control. The assay was able to detect as low as 18 bacterial cells per reaction. The specificity of the assay was evaluated both in situ and in silico with a representative panel of 50 Xpd strains from different regions worldwide and with closely related *Xanthomonas* strains. Only a strain of *X. euvesicatoria*, not pathogenic on *Anthurium*, was found positive.

In this paper, a second TaqMan assay for detection of *Xpd* has been described. TaqMan assays are the method of choice in many inspection laboratories for routine detection of plant pathogens, due to its specificity, sensitivity, robustness and the possibilities for multiplexing and quantification (Martinelli et al., 2015; Mirmajlessi et al., 2015). We developed a triplex TaqMan, including assays detecting Xpd specific sequences of two loci and an assay for *Acidovorax cattleya* to check for extraction and amplification efficiency, similarly as described by (Bonants et al., 2019). In comparison with the assay described by Jouen et al. (2019), the added value of the triplex assay describes in this paper lies primarily in its double check for the presence of Xpd, by targeting Xpd specific sequences of two different genes. In addition, the use of *Acidovorax cattleya* as in internal control has the advantage that it is useful for detection of Xpd in any matrix, not only in plant material. The specificity was high according to the in silico analysis and this was confirmed using DNA from 22 target and 47 non target strains. The analytical sensitivity of the assay for gBlocks (100 copies) and genomic DNA (100 fg) was high but for detection of the target in *Anthurium* leaf extracts a minimum of 10^4 cells per mL was required to exceed the detection threshold (Ct 35). In case of a low bacterial density in symptomless plant material, an

Table 3 Influence of the concentration of Anthurium leaf material on the detection of *Xanthomonas phaseoli* pv. *dieffenbachia* (Xpd) in the TaqMan assays Xpd866 and Xpd4494. A suspension of Acidovorax cattleya (Acat) with a fixed density of approximately 10^5 cells/ml was supplemented to the extracts before DNA extraction as extraction and amplification control

	0,062 g/ml[1]			0,136 g/ml			0,171 g/ml			0,258 g/ml			0,271 g/ml		
Cells/ml	Xpd866	Xpd4494	Acat	Xpd866	Xpd4494	Acat	Xpd866	Xpd4494	Acat	Xpd866	Xpd4494	Acat	Xpd866	Xpd4494	Acat
10^8	18,7	18,9	ND	19,2	19,4	ND	18,9	19,0	ND	20,0	19,9	ND	20,5	20,5	ND
10^7	21,9	22,1	29,8	22,3	22,3	30,4	21,9	22,0	29,4	23,0	23,1	30,6	23,2	23,1	30,0
10^6	25,2	25,3	28,6	25,5	25,6	29,0	25,4	25,5	29,0	25,9	25,8	29,4	26,8	27,1	30,2
10^5	28,7	29,1	29,1	29,2	29,0	29,5	28,5	28,6	29,2	29,2	29,2	30,0	29,5	29,5	29,6
10^4	**32,2**[2]	**31,9**	29,3	**32,3**	**31,8**	29,8	31,9	31,4	29,0	**32,9**	**32,4**	30,2	**32,7**	**33,2**	30,1
10^3	35,1	35,3	29,2	34,6	35,5	29,9	**34,9**	**34,8**	29,5	36,0	ND	30,5	34,9	ND	30,3
10^2	ND	36,3	29,8	ND	ND	30,1	36,5	35,2	29,3	ND	37,9	30,5	ND	ND	29,9
0	ND[3]	ND	29,6	ND	37,2	30,5	ND	ND	30,0	ND	ND	31,4	ND	ND	30,6

[1] Concentration anthurium leaf extract

[2] In bold: Minimum density of Xpd required for a positive result in both TaqMan assays (Ct-value <35)

[3] ND = no signal detected after 40 cycles

enrichment in or on a selective growth medium may be required before TaqMan analysis similarly as used for other plant pathogenic bacteria (Schaad et al., 1995; Schaad et al., 1999; Song et al., 2004). The assays for the two target specific loci resulted in very similar reaction values. The diagnostic sensitivity and specificity were 100%.

In practice, the triplex TaqMan assay was highly robust. Only minor effects were found of the concentration leaf material between 0.6 and 0.27 g per mL on the analytical sensitivity of the triplex TaqMan assay. In addition, the cultivar type had no noticeable influence on the TaqMan values. Results were independent for cut or potting plants and independent of the color of leaves or flowers (data not shown).

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Data availability statement Data available on request from the authors.

Compliance with ethical standards The authors herewith declare that they have no conflict of interest. This study does not involve studies with human participants or animals performed by any of the authors.

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