The two-faced *Xanthomonas euroxanthea:* from DNA markers to genomic determinants of pathogenicity

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Mestrado em Biologia Celular e Molecular
Departamento de Biologia
2022

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"The only real mistake is the one from which we learn nothing."

— John Powell

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Acknowledgements

Ao fim deste último ano de muito trabalho, aprendizagem, bons momentos e crescimento pessoal; tenho de agradecer a todos aqueles que para tal contribuíram.

Professor Fernando, muitíssimo obrigada por tudo. Desde os exemplos caricatos às introspeções brilhantes. O professor certificou-se sempre que nenhum dia no laboratório era aborrecido, nem passava sem aprender algo novo! O professor foi sempre um bom mentor – sugeria sem impor, promovia o debate, levantava o ânimo no laboratório e foi sempre muito "humano" e empático. É admirável a confiança que o professor deposita nas experiências e projetos; e as suas perspetivas sobre determinada situação laboratorial (ou pessoal) são, quase sempre, algo que até ao momento ninguém tinha pensado. Obrigada por passar no laboratório para confirmar que estava tudo bem, quando sabia que lá estava sozinha até mais tarde; pela resposta a infinitas dúvidas e pela perspetiva prática que costuma ter. Obrigada por aceitar a minha escrita mais literária, e fazer correções que ainda assim permitissem manter essa essência.

Leonor, poderia agradecer e agradecer e de certeza que ainda me faltaria algo! Foste uma orientadora excecional em todos os aspetos. Para além de orientadora, também fomos uma boa equipa - foi uma honra ser tua coautora no meu primeiro artigo científico. Vou ter saudades do teu bom humor, saídas inesperadas e devaneios sobre a vida que, às vezes, as longas horas no laboratório promoviam. O teu entusiamo pela ciência, o querer saber mais e mais, a dedicação, a capacidade de comunicação, o olho para pormenores fazem de ti uma admirável investigadora. Para além de tudo isto, és uma pessoa impecável, "uma pessoa de pessoas", a tua empatia permite-o. Admiro o facto de tomares iniciativa para mudar aquilo que à tua volta te impacienta. Só me falta perguntar mais uma vez…"Queres um salgadinho?".

Mafalda Pereira, és uma querida, desde a sabedoria infinita, à boa disposição, permanente positividade e perseverança, e classe constante! Obrigada por me teres orientado também no MDE, e me teres ajudado a resolver cada problema mais inesperado e caótico. Fizeste com que o laboratório fosse um lugar seguro e organizado. Obrigada por partilhares, por estares sempre pronta a ouvir uma amiga, pelo teu entusiamo com a vida em geral e com as suas pequenas coisas – o cafezinho, os livros, os doggies, as caminhadas na praia e bolachinhas boas. E por esta frase icónica: "A cookie a day keeps the meltdown away".

Mafalda Pinto, não há palavras que te façam justiça! Disse-to já pessoalmente que és um raio-de-sol, porque és mesmo. És mesmo já tendo passando por adversidades, e talvez o sejas mesmo por isso. Escolhes continuar a ver o positivo em tudo, a nunca desistir, a acreditar que vai dar certo. Durante este ano estiveste sempre pronta a ajudar e, inclusive, cedeste-me os teus tabaquinhos, obrigada. Os momentos de descontração não teriam sido

os mesmos sem a tua personalidade vibrante, riso contagioso e intervenções hilariantes. Em cima de tudo isto, és também uma brilhante e muito curiosa cientista!

Rita Fernanades, obrigada pelas noções e introspeções científicas, e pelos separadores abertos sobre curvas de crescimento. Obrigada também por acreditares em mim e pelas palavras encorajadoras! Desde os livros, passando pelo kpop, à ciência, a tua perspetiva foi sempre valorizada. “try-error-try. try-error-try. try-error-try. try-error-try-it.”.

A todos os membros do MDE (com os quais contactei), professor Fernando, Leonor, Mafalda Pereira, Mafalda Pinto, Rita, Sofia, Sara, Rafael, Miguel e Nuno. A vossa paixão pela investigação, disponibilidade e amabilidade é de destacar! O MDE foi mais do que investigação e do que um desafio, foi um lugar que me fazia levantar da cama com verdadeiro entusiamo – não só pela ciência, mas também pelas pessoas que aqui encontrei.

Aos meus coautores, Leonor, Miguel Teixeira, Joël Pothier e professor Fernando – escrever o artigo e continuar a estudar o mundo da fitopatologia e genómica foi desafiante, recompensador e, sobretudo, muito divertido.

A todos aqueles que me aconselharam e cederam material no percurso desta dissertação, professor Fernando, Leonor, Mafalda Pereira, Mafalda Pinto, Rita, Sara, Maria João, Jessy, Ana Marta, Diana, Rafael, Nuno, Joana, D. Helena, Teresa, Lili, professor Luís e Cristiana.

Às meninas do doutoramento e companheiras de almoço, Leonor, Mafalda Pereira, Mafalda Pinto, Sara Pinto, Maria João, Jessy, obrigada por me incluírem e terem tornado os almoços divertidos! O péssimo café do bar valia a pena pelos momentos de convívio!

Ao quarteto de Biologia, Telma, Sofia e João. Telma, a minha gémea, fazíamos que qualquer minuto roubado aqui e ali desse para falarmos no corredor, obrigada pela sabedoria e tranquilidade infinita desses momentos. Sofia, a mais prática de nós todos, obrigada por me dares a perspetiva maior quando me sentia mais em dúvida. João, és um exemplo de como a calma é amiga da perfeição, obrigada pelas palavras de apoio e constante preocupação.

À Adriana, amiga de todas as loucuras e grande fã de “partir pratos”. Obrigada pelas conversas intermináveis, apoio, e viagens mágicas e inesperadas!

À Susana e Mariana, obrigada pelo ânimo dado aquando dos momentos mais difíceis. Desde a comidinha boa, a ir ao mar à meia-noite, às conversas introspetivas, e pela perspetiva de que o crescimento envolve algum desconforto (“growing gains”).

À minha família, mãe, pai, Nadia, Nuno, Keanu, avó Almira, avó Maria e avô Agostinho. Pelo apoio, comida boa, paciência para relatos científicos, ensaios de apresentações, intervenções humorísticas, boleias, ajuda em formatações e normalização dos mutantes. São o meu mais que tudo, e são o motivo pelo qual sou quem sou.

A todos os pores-do-sol, ensaios bem-sucedidos, livros emprestados, bolachinhas partilhadas, chás, memes do laboratório, pessoas amigas e profissionais excelentes.

Kayla, conseguimos! Obrigada por teres trabalhado até este momento; nunca te esqueças de parar, e comer a noz.

Resumo

Xanthomonas euroxanthea é uma nova espécie bacteriana que engloba estirpes patogénicas e não-patogénicas e é frequentemente encontrada a colonizar as mesmas plantas hospedeiras que *Xanthomonas arboricola*. Estes dados apelam a estudos de distribuição e do papel desempenhado por estas duas espécies co-colonizadoras nas plantas hospedeiras. Em particular, torna-se essencial desenvolver um método de deteção e genotipagem capaz de rastrear essas bactérias em consórcios microbianos com outras *Xanthomonas*; e desvendar o processo infecioso, ainda não descrito para *X. euroxanthea*, através da avaliação do papel de genes putativos de patogenicidade e virulência, nomeadamente, os genes *hrcT*, *hrpG* e *hrpX* do sistema de secreção tipo III (T3SS).

Oito marcadores de DNA específicos para *X. euroxanthea* (XEA1–XEA8) foram selecionados por genómica comparativa e validados *in silico* quanto à sua especificidade e consistência através de BLASTn, análises de sintenia, conteúdo de CG, preferência de codões (valores CAI/eCAI) e proximidade cromossómica a determinantes de plasticidade genómica. Primers específicos para estes marcadores de DNA foram desenhados e utlizados na otimização de um ensaio de PCR multiplex.

Nas análises *in silico*, verificou-se que cinco marcadores de DNA (XEA4, XEA5, XEA6, XEA7 e XEA8) foram infalivelmente encontrados nos genomas de estires de *X. euroxanthea*. Um PCR multiplex tendo como alvo os marcadores XEA1 (819 pb), XEA8 (648 pb) e XEA5 (295 pb) permitiu a identificação com sucesso de *X. euroxanthea*. A topologia do dendrograma obtido pelas sequências concatenadas de três marcadores (XEA5, XEA6 e XEA8) mostrou-se ser tão informativa como o dendrograma obtido a partir das sequências concatenadas de quatro genes de *housekeeping* (*gyrB*, *rpoD*, *fyuA* e *acnB*) na discriminação de linhagens de *X. euroxanthea*. Esta metodologia capaz de conciliar marcadores de deteção com genotipagem pode instruir sobre como selecionar outros marcadores de DNA de deteção espécie-específicos.

Para abordar o papel desempenhado por putativos determinantes de patogenicidade, mutantes knock-out de *X. euroxanthea* foram obtidos através da introdução do construto do plasmídeo não replicativo pUC57 contendo um fragmento interno do gene alvo, contando-se com o evento de cruzamento único por recombinação homóloga para interromper o gene alvo - genes *hrcT*, *hrpG* e *hrpX*. Os mutantes foram confirmados por colony PCR e ensaio da resposta hipersensitiva (HR) em tabaco. A aptidão de crescimento dos mutantes foi avaliada por ensaios de curva de crescimento.

Ao contrário da estirpe selvagem, o mutante 7622ΔhrcT é incapaz de induzir HR no tabaco, sugerindo a sua incapacidade de transferir efetores tipo III (T3Es) para os tecidos da planta, por não possuir proteínas estruturais do T3SS para fazê-lo. Os genes alvo de T3SS

são cruciais para a patogenicidade e virulência de *X. euroxanthea*, assim, a patogenicidade de *X. euroxanthea* depende fortemente da secreção dos T3Es. Os ensaios de curvas de crescimento mostram que os mutantes do T3SS entram na fase logarítmica de crescimento mais precocemente do que as estirpes selvagens utlizadas, sugerindo uma otimização metabólica mais rápida.

Em suma, este estudo contribuiu com ferramentas moleculares (PCR multiplex e mutantes no T3SS) capazes de abordar a ecologia de *X. euroxanthea* e monitorizar a sua presença em habitats também colonizados por *X. arboricola*. Adicionalmente, com este trabalho também foi possível contribuir para avaliar o papel desempenhado pelo T3SS no processo de infeção por *X. euroxanthea*.

Palavras-chave: *Xanthomonas euroxanthea*; marcadores de DNA taxa-específicos; PCR multiplex; genómica comparativa; genotipagem; *hrcT; hrpG*; *hrpX*; T3SS

Abstract

Xanthomonas euroxanthea is a novel bacterial species encompassing both pathogenic and non-pathogenic strains and is frequently found colonizing the same host plants as *X. arboricola*. This calls for an investigation of the distribution and role played by these two co-colonizing species within the plant hosts. Specifically, it is essential to develop a detection and genotyping assay able to track these bacteria in microbial consortia with other xanthomonads; and to unearth the yet undescribed infectious process by *X. euroxanthea*, through the assessment of the role of supposedly key pathogenicity and virulence genes, namely, *hrcT*, *hrpG* and *hrpX* of the type III secretion system (T3SS).

Eight *X. euroxanthea*-specific DNA markers (XEA1–XEA8) were selected by comparative genomics and validated *in silico* regarding their specificity and consistency using BLASTn, synteny analysis, CG content, codon usage (CAI/eCAI values), and genomic proximity to plasticity determinants. Primers with an affinity for these DNA markers were used to optimize a multiplex PCR assay.

In silico analysis showed that five DNA markers (XEA4, XEA5, XEA6, XEA7 and XEA8) were unfailingly found in the genomes of *X. euroxanthea* strains. A multiplex PCR targeting markers XEA1 (819 bp), XEA8 (648 bp) and XEA5 (295 bp) was shown to successfully detect *X. euroxanthea*. The tree topology obtained from the concatenated sequences of three markers (XEA5, XEA6 and XEA8) was equally informative as the tree obtained by the concatenated sequences of four housekeeping genes (*gyrB*, *rpoD*, *fyuA*, and *acnB*) in the discrimination of *X. euroxanthea* lineages. The workflow herein utilized that conciliates detection and genotyping might be applied in the selection of other species-specific detection DNA markers.

To address the role played by putative pathogenicity determinants, knock-out mutants of *X. euroxanthea* were obtained by introducing the construct of the non-replicative plasmid pUC57 containing an internal fragment of the target gene, relying in the single cross-over event by homologous recombination to disrupt the target gene - *hrcT*, *hrpG* and *hrpX* genes. Mutants were confirmed by colony PCR and hypersensitive response assay on tobacco. The growth fitness of the mutants was evaluated by growth curve assays.

Contrary to the wild-type strains, the mutant 7622ΔhrcT is incapable of causing hypersensitive response in tobacco, indicative of the inability to translocate type III effectors (T3Es) into the plant tissues as it lacks the structural proteins to do so. T3SS target genes are crucial for the pathogenicity and virulence of *X. euroxanthea*, hence the pathogenicity of *X. euroxanthea* heavily depends on the secretion of the T3Es. The growth curve assays showed that T3SS-mutants enter the logarithmic phase of growth earlier than the wild-type strains used, which suggests a faster metabolic adaptation.

Overall, this study contributed with molecular tools (multiplex PCR and T3SS-mutants) capable to address the ecology of *X. euroxanthea* and monitoring for its presence in *X. arboricola*-colonizing habitats. In addition, this work sheds some light on the role played by the T3SS in the infectious process of *X. euroxanthea*.

Keywords: *Xanthomonas euroxanthea*; taxa-specific DNA markers; multiplex PCR; comparative genomics; genotyping*; hrcT; hrpG; hrpX*; T3SS

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List of abbreviations and acronyms

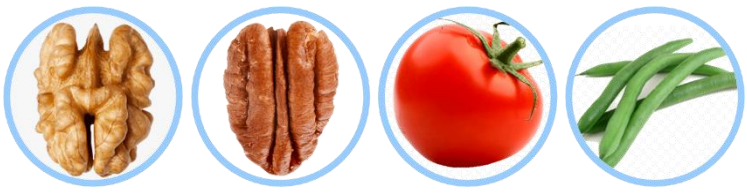
acnB	ACONITATE HYDRATASE B
ANI	AVERAGE NUCLEOTIDE IDENTITY
BAN	BROWN APICAL NECROSIS
BLAST	BASIC LOCAL ALIGNMENT SEARCH TOOL
BLASTn	NUCLEOTIDE BLAST
BP	BASE PAIRS
BRIP	PLANT PATHOLOGY HERBARIUM
C-	NEGATIVE CONTROL
CAI	CODON ADAPTATION INDEX
CDS	CODING DNA SEQUENCE
CFBP	COLLECTION FRANÇAISE DE BACTÉRIES PHYTOPATHOGÈNES (FRENCH COLLECTION OF PHYTOPATHOGENIC BACTERIA)
CFU	COLONY-FORMING UNIT
CPBF	COLEÇÃO PORTUGUESA DE BACTÉRIAS FITOPATOGÉNICAS (PORTUGUESE COLLECTION OF PHYTOPATHOGENIC BACTERIA)
dDDH	DIGITAL DNA-DNA HYBRIDIZATION
DNA	DEOXYRIBONUCLEIC ACID
dNTP	DEOXYNUCLEOTIDE TRIPHOSPHATE
eCAI	EXPECTED VALUE OF CODON ADAPTATION INDEX
EDGAR	EFFICIENT DATABASE FRAMEWORK FOR COMPARATIVE GENOME ANALYSES USING BLAST SCORE RATIOS
EPPO	EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION
EPS	EXOPOLYSACCHARIDE
ETI	EFFECTOR-TRIGGERED IMMUNITY
fyuA	FERRIC YERSINIABACTIN UPTAKE RECEPTOR
GC	GUANINE-CYTOSINE
GM	GENTAMYCIN
gyrB	DNA GYRASE SUBUNIT B
HGT	HORIZONTAL GENE TRANSFER
HPA	HRP-ASSOCIATED
HR	HYPERSENSITIVE RESPONSE

HRC	HRP-CONSERVED
HRP	HYPERSENSITIVE RESPONSE AND PATHOGENICITY
IPM	INTEGRATED PEST MANAGEMENT
IS	INSERTION SEQUENCE
KM	KANAMYCIN
LAMP	LOOP-MEDIATED ISOTHERMAL AMPLIFICATION
LB	LYSOGENY BROTH
LMG	BACTERIA COLLECTION LABORATORIUM VOOR MICROBIOLOGIE UNIVERSITEIT GENT (BELGIAN COORDINATED COLLECTION OF MICROORGANISMS)
LOD	LIMIT OF DETECTION
MaGe	MAGNIFYING GENOMES
MEGA	MOLECULAR EVOLUTIONARY GENETICS ANALYSIS
MFP	MEMBRANE FUSION PROTEIN
MLSA	MULTILOCUS SEQUENCE ANALYSIS
MLST	MULTILOCUS SEQUENCE TYPING
NCBI	NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION
NCPPB	NATIONAL COLLECTION OF PLANT PATHOGENIC BACTERIA
NPPO	NATIONAL PLANT PROTECTION ORGANIZATION
NT	NUCLEOTIDES
OD	OPTICAL DENSITY
ORF	OPEN READING FRAME
PAI	PATHOGENICITY ISLAND
PCR	POLYMERASE CHAIN REACTION
PSA	PEPTONE-SUCROSE AGAR
RPM	REVOLUTIONS PER MINUTE
rpoD	RNA POLYMERASE SIGMA FACTOR RpoD
SDW	STERILE DISTILLED WATER
SNP	SINGLE NUCLEOTIDE POLYMORPHISM
T3E	TYPE III EFFECTOR
T3SS	TYPE III SECRETION SYSTEM
T4P	TYPE IV PILUS
VOC	VERTICAL OOZING CANKER

WBB	WALNUT BACTERIAL BLIGHT
WT	WILD TYPE
XA	*XANTHOMONAS ARBORICOLA*
XEA	*XANTHOMONAS EUROXANTHEA*

C H A P T E R I

General introduction



Chapter I: General introduction

1. Production of *Juglans regia* L.

Since time immemorial *Juglans regia* L., commonly known as the common Persian or English walnut, has been cultivated in virtually all continents, making it the most widespread nut tree worldwide and the second-largest crop (19%) of the global share [1–4] (Figure I.1a). As of 2020/2021, China leads in the commercial cultivation of walnut, followed by the USA, Chile, Ukraine, France, and Turkey (Figure I.1b) [3,4]. Over the last decade, world walnut production has increased, reaching over 1.02 million metric tons (kernel basis) in season 2020/2021, a 101.5% rise over 2011/12 (Figure I.1c) [4]. Portugal is no different (in what walnut appreciation is concerned) - walnuts are found in a great portion of its continental territory, and walnut production is a prosperous business (Figure I.1d) [5–7]. In 2020, Portugal produced 5,111 metric tons of walnut kernels using a total surface area of 5,397 hectares; being that the region of Alentejo was the most important for production, 50.4%, and had the greatest tree planting area, 40.7% (Figure I.1e) [6]. These numbers are due to the Portuguese investment made in the last decade towards nut cultivation that directly resulted in a 101.2% increase in the walnut cultivation surface area [6,8]. Portugal used to be mainly an importing country, but with the unmistakable revolution of all the Portuguese nut sector (as statistics illustrate) 96% of domestic consumption is already satisfied, and the president of Portugal Nuts – Associação de Promoção de Frutos Secos, Tiago Costa, predicts that Portugal will be self-sufficient by 2023 [9].

The Portuguese and overall international demand and investment in the common walnut resides in its economically rewarding great-quality timber and the nutritious edible part of the fruit (the seed or kernel) [1–3,6,10]. A diet rich in walnuts has a plethora of health benefits for ever-upsurging health-aware consumers – the reduction of the risk of cancer, cardiovascular symptoms, diabetes, and degenerative disorders [1,3,10–12].

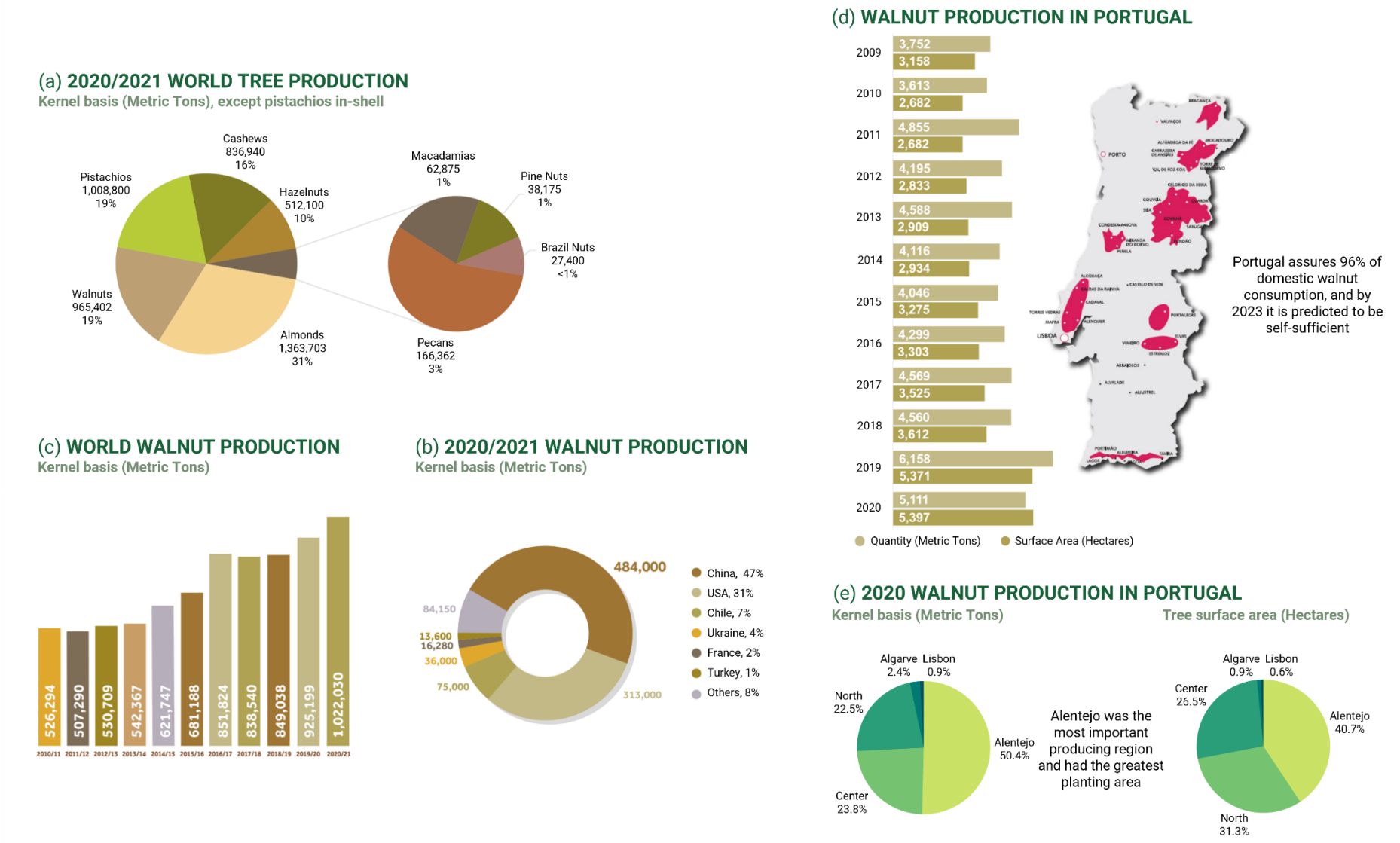


Figure I.1. Global and domestic walnut production statistics [4,6,13].

Walnuts are mainly cultivated to harness the wood and kernels, although efforts are being made to purposely utilize other of its parts – the green husk or hull (epicarp or mesocarp), hard shell of the nut (endocarp), dividing membranes of the kernel (pellicle), flower, root, bark, branch, and leaf (Figure I.2) [11].

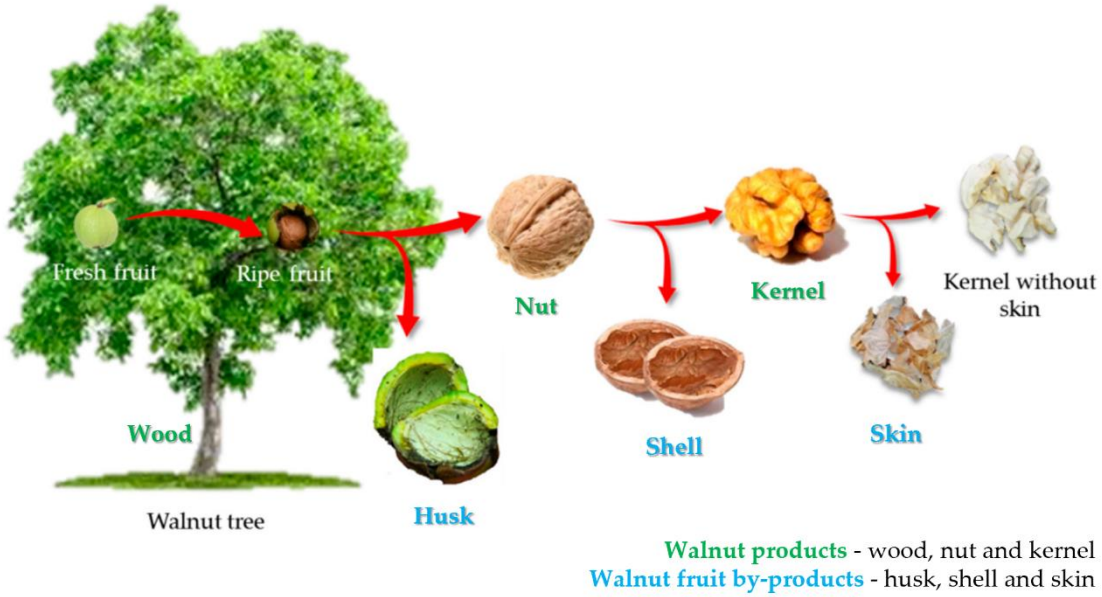


Figure I.2. The phases of development of the walnut fruit (fresh and ripe fruit), walnut commercially interesting products (wood, nut and kernel; in green) and walnut fruit by-products (husk, shell and skin; in blue). Image adapted from Jahanban-Esfahlan et al. 2019 [11].

2. *Xanthomonas arboricola* pv. *juglandis*: a walnut threat

The walnut is susceptible to an array of pests – fungi (*Armillaria mellea*, *Phytophthora cinamomii*, *P. cambivora*, and *Gnomonia leptostyla*), parasites (*Cydia pomonella* and *Amyelois transitella*), viruses and bacteria [14]. Particularly *Xanthomonas arboricola* pv. *juglandis*, first described at the beginning of the 20th century, is considered one of the most serious threats to walnuts, as it is a hemibiotrophic pathogen, that under favorable conditions, is responsible for diseases such as walnut bacterial blight (WBB), brown apical necrosis (BAN), and vertical oozing canker (VOC) (Figure I.3) [15–17]. WBB is characterized by necrosis of immature walnut fruits, leaves, catkins, or flowers and twigs [15–19]. BAN is characterized by necrotic spots close to the blossom end of the nut [15,17,18,20,21]. VOC is associated with brown to black oozing exudates staining the bark, vertical cankers on trunks and branches, and longitudinal severe deformations of trunks [15,17,18,20,21].

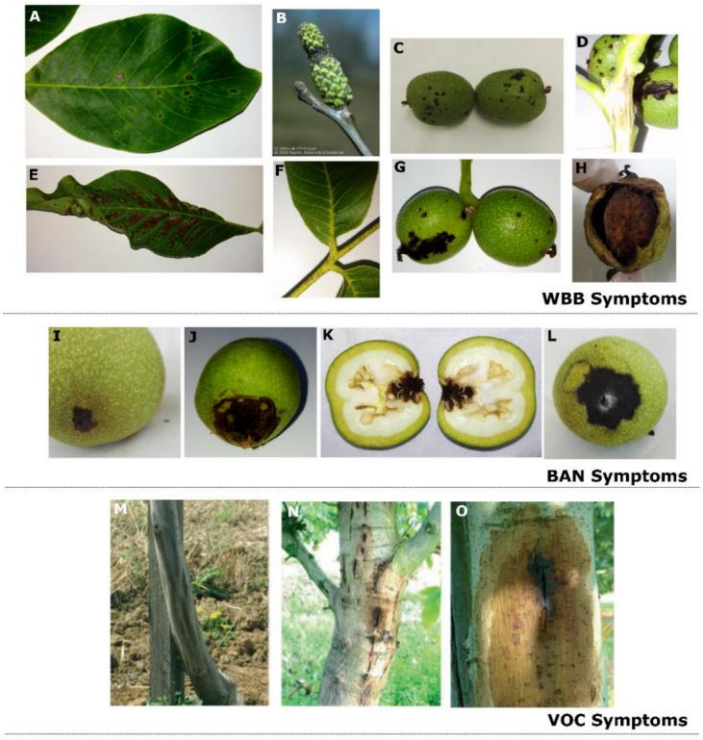


Figure I.3. Symptomology of walnut diseases caused by *Xanthomonas arboricola* pv. *juglandis* – walnut bacterial blight (WBB), brown apical necrosis (BAN), and vertical oozing canker (VOC) [18]. Image from Fernandes *et al*. 2019 [18].

Hence the question arises – if walnut production is such a thriving business, as seen by worldwide statistics, why should scientific efforts be made toward the management of this bacterial illness? The truth is major economic losses arise due to the non-marketability of nuts with necrotic spots and premature fruit drop, the overall reduction of orchard productivity, the augmented nursery production costs, and, in the long run, the death of trees [10,16,20,22]. Therefore, it is of most importance that this problem is solved, solutions must run by adequate early-on detection, accurate diagnosis, and prompt disease management; all of these topics are encompassed by the phytosanitary umbrella that, if applied correctly, will serve its duty as a powerful-protection against an impending phytohealth catastrophe [22].

As the market responds to the continuously increasing demand for these goods, which implies an increase in the number of *Juglans regia* exemplars planted, these pathogens continue to decimate orchards in all major walnut-growing areas (Figure I.4) [10,16,20]. In fact, outbreaks of these diseases on *Juglans regia* have increased in recent years [23].

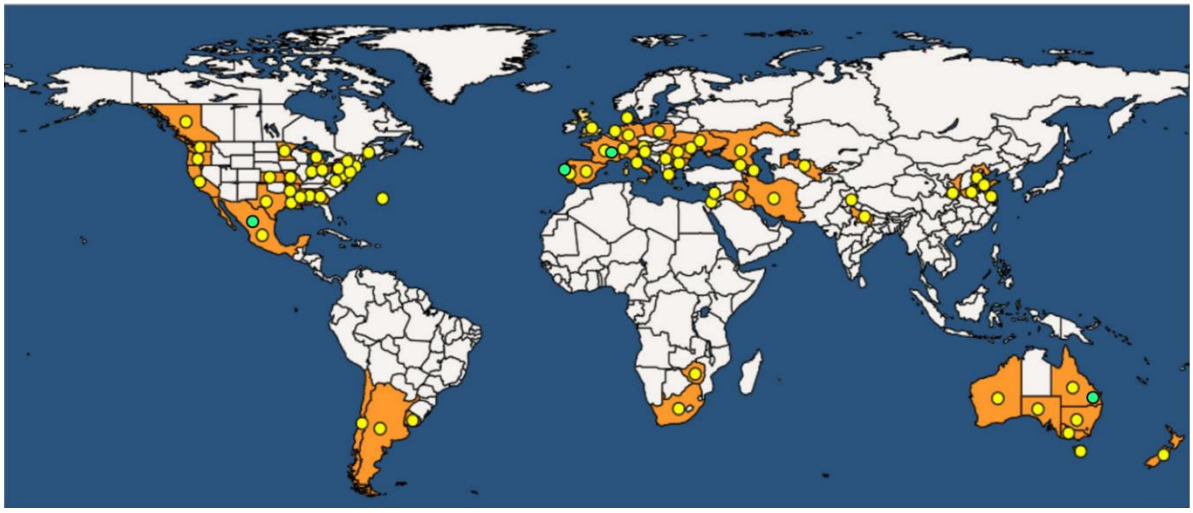


Figure I.4. The global distribution of *Xanthomonas arboricola* pv. *juglandis*, in yellow (●), and *Xanthomonas euroxanthea*, in green (●); based on EPPO Global Database [24].

3. Novel species *Xanthomonas euroxanthea*

On the account of the unmatched genetic diversity of the walnut threat *X. arboricola* pv. *juglandis* when compared to other *X. arboricola* pathovars, a diversity study of walnut-colonizing *X. arboricola* pv. *juglandis* in Portugal was prompted [25–28]. Surprisingly, from a diseased walnut, alongside *X. arboricola* pv. *juglandis*, were recovered the isolates CPBF 367, CPBF 424^{T}, and CPBF 426 with ANI (average nucleotide identity) and dDDH (digital DNA-DNA hybridization) values that allocated them to a novel species [25,26]. Thereby, *Xanthomonas euroxanthea* was welcomed to the scientific world by Martins *et al.* 2020, and it encompasses both non-pathogenic, and pathogenic strains on walnut, namely CPBF 424^{T}, responsible for WBB [26]. Since then, other strains have been allocated to this species, being that as of today *X. euroxanthea* encompasses the strains - CPBF 367, CPBF 424^{T}, CPBF 426, CPBF 761, CPBF 766, CFBP 7622, CFBP 7653, BRIP 62409, BRIP 62411, BRIP 62415, BRIP 62418, 2949, 2955, 2957, 2974, 3338, 3640, and F2 (Table I.1) [23,25,29-34].

Table I.1. Currently available genomes of *Xanthomonas euroxanthea*.

Strain	Pathogenicity [2]	Isolation [3]	Genome accession	Geographic origin	Year of isolation	References
CPBF 367	Non-pathogenic	Walnut buds	NZ_LR824641	Portugal (Loures)	2016	[29]
CPBF 424^T	Pathogenic	Walnut buds	NZ_LR994544	Portugal (Loures)	2016	[30]
CPBF 426	Non-pathogenic	Walnut buds	NZ_LR861805	Portugal (Loures)	2016	[29]
CPBF 761	Pathogenic	Pecan	NZ_HG999363	Portugal (Alcobaça)	2016	[31]
CPBF 766	Pathogenic	Pecan	NZ_HG999364	Portugal (Alcobaça)	2016	[31]
CFBP 7622	Non-pathogenic	Common bean	NZ_MIGF00000000	USA	1985	[25]
CFBP 7653	Non-pathogenic	Walnut	NZ_MIGK00000000	France	2008	[25]
BRIP 62409	Non-pathogenic	Tomato	KY658947 (*gyrB* [4])	Australia	2015	[32]
BRIP 62411	Non-pathogenic	Tomato	KY658948 (*gyrB* [4])	Australia	2015	[32]
BRIP 62415	Non-pathogenic	Tomato	KY658950 (*gyrB* [4])	Australia	2015	[32]
BRIP 62418	Non-pathogenic	Tomato	KY658952 (*gyrB* [4])	Australia	2015	[32]
F2	Unknown	Ground cherry	JACHOL000000000	Unknown	Unknown	[33,34]
2949	Unknown	Rain	NZ_JAASRK000000000	Unknown	Unknown	[33,34]
2955	Unknown	Rain	NZ_JAASRJ000000000	Unknown	Unknown	[33,34]
2957	Unknown	Rain	NZ_JAATIU000000000	Unknown	Unknown	[33,34]
2974	Unknown	Rain	NZ_JACICH000000000	Unknown	Unknown	[33,34]
3338	Unknown	Rain	NZ_JACICI000000000	Unknown	Unknown	[34]
3640	Unknown	Rain	NZ_JACHOI000000000	Unknown	Unknown	[33,34]

[1]Other known names for these strains: CPBF 367=LMG 31036=CCOS 1890; CPBF 424^T=LMG 31037^T=CCOS 1891^T=NCPPB 4675^T; CPBF 426=LMG 31038=CCOS 1892.
[2] Pathogenicity of the strain to walnut
[3] Walnut (*Juglans regia*), pecan (*Carya illinoinensis*), common bean (*Phaseolus vulgaris*), tomato (*Solanum lycopersicum*), and ground cherry (*Physalis peruviana* L.)
[4] Genome accession not available, thus *gyrB* accession number was included

The unearthing of this novel species was possible due to dDDH, multilocus sequence analysis (MLSA), ANI, biochemical analysis, and the improvement of bacterial genome assembly and annotation tools; which altogether allowed for a more confident interspecific discrimination of *Xanthomonas* spp. [26].

As the novel species *X. euroxanthea* includes pathogenic and non-pathogenic strains it is considered a suitable model for investigating genetic determinants of pathogenicity and unraveling the evolution of virulence in *Xanthomonas* spp.; and because these strains are associated to various taxa-hosts *X. euroxanthea* can, as well, be a case study for host-adaptation [15–17,25,28,30,35–39].

There is an hypothesis that *X. euroxanthea* was initially pathogenic, as seen by the CPBF 424^T strain, and that some of its strains (such as CPBF 367, CPBF 426, CFBP 7622, and CFBP 7653) lost their pathogenic ability, as they lack numerous pathogenicity-genetic-determinants [25]. Conversely, the co-localization (in walnut) of the two *Xanthomonas* species suggests a sympatric lifestyle, in which probably occurs horizontal gene transference (HGT) of pathogenicity genes, which presumptively could lead to the emergence of new pathogenic lineages [17,35,36,39].

A whole-genome comparison analysis brought to light the discrepancies between *X. euroxanthea* and *X. arboricola* pv. *juglandis* strains regarding genome size, CDSs repertoire, and richness of IS (insertion sequence) elements (Table I.2) [16,27]. The genomes of *X. euroxanthea* strains are shorter in approximately 400 CDSs, than the general *X. arboricola* pv. *juglandis* genomes, which may be indicative of a genome reduction responsible for the loss of the plant-infection ability of some of these *X. euroxanthea* strains [16].

Table I.2. General genomic features of five *Xanthomonas* sp. strains [16]. This table has been retrieved from Fernandes et al. 2019 [16].

General features	***X. euroxanthea* strains**			***X. arboricola* pv. *juglandis* strains**	
	CPBF 367	**CPBF 424^T**	**CPBF 426**	**CPBF 427**	**CPBF 1521**
Genome size (bp)	4,956,382	4,896,146	4,894,012	5,190,560	5,194,740
No. contigs	22	10	11	57	56
N50 (bp)	687,415	1,029,447	730,188	178,455	173,159
G+C (%)	65.81	65.89	65.87	65.41	65.41
Plasmids	0	0	0	0	0
No. Total CDS	4094	4034	4049	4354	4368
IS elements	5	10	7	61	60
CDS shared with CFBP 2528	3476	3502	3500	3983	3985
tmRNA	1	1	1	1	1
tRNA	56	58	53	53	53
rRNA	4	4	4	4	4
Misc_RNA	48	46	45	71	68
Assembly no.	GCA_900537245	GCA_900476395	GCA_900537265	GCA_900537235	GCA_900476315
WGS accession	UNRN00000000.1	UIHB00000000.1	UNRM00000000.1	UNRO00000000.1	UIHD00000000.1

4. *Xanthomonas*: the worldwide, infamous plant-associated genus

To contextualize, the genus *Xanthomonas* (two Greek words; *xanthos*, meaning “yellow”, and *monas*, meaning “entity”) comprises plant-associated Gram-negative rod-shaped bacteria, that form yellow colonies, and have a mobility-conferring polar flagellum [22,40]. This genus comprises 34 validly described species [22,40,41].

Most *Xanthomonas* spp. synthesize xanthan gum, an exopolysaccharide thought to be essential for its pathogenetic role, as mutations in *xpsI* or gum 12 genes cluster, essential to the xanthan synthesis pathway, make up for attenuated plant virulence [42]. In addition, xanthan gum has many applications because of its rheological properties making it of economic interest [42].

Xanthomonas have historically been described as pathogens since they cause disease to 124 monocots and 268 dicots [22,40]. Hosts like banana, bean, cabbage,

cassava, citrus, pepper, rice, tomato, and wheat are all victims of *Xanthomonas* infectious diseases [22]. Symptoms can include water-soaked spots evolving to necrosis on leaves, wilting, hyperplasia, rotting, hypertrophy, canker, dieback, and blights [22]. These negatively impacted cultivations compromise the global trade of seeds, plants, and food; resulting in economic-environmental losses with real-life consequences for farmers and consumers [22]. Recently *Xanthomonas* have been dodging this status, being rather considered a plant-associated bacteria [22]. Indeed, some strains of *Xanthomonas* were isolated from plant material (of various organs and species) free of disease symptoms, and equally no symptoms were seen after artificial inoculation [22]. Specifically, the *Xanthomonas arboricola* species encompass, at an infraspecific level, non-pathogenic and pathogenic strains, and, curiously, both archetypes have been found to co-colonize the same diseased walnut host, this paradigm renders useful the genotyping and/or phenotyping of strains so that no harmful socio-economic consequences ensue (when unnecessary) [22].

5. The way in: the infectious mechanisms of *Xanthomonas*

To understand plant infection by *Xanthomonas,* one must start with its infectious cycle. If the bacteria perceive the environment as favorable, the epiphytic stage occurs, where xanthomonads make use of bacterial surface polysaccharides, adhesion proteins, and the type IV pilus (T4P) to attach to the surface of aerial plant tissues and proceed to grow; then because these pathogens are equipped with a flagellum that confers motility, the cells begin to cluster in microcolonies (that are single-species aggregates) and form an epiphytic biofilm (Figure I.5) [15,19,35,40]. A biofilm is a bacteria-originated solid surface-associated three-dimensional matrix constituted of exopolysaccharides (EPSs), xanthan gum, extracellular DNA, proteins, and lipids, that connect congregated bacteria cells to each other and plays an important role in bacteria survival and persistence on plant surface [19,40,43]. Followed by the endophytic stage, when said bacteria through motility and directional chemotactic movement reach a wound or natural opening (e.g. stomata) and enter the plant colonizing the mesophyll parenchyma (Figure I.5) [15,19,35,40]. Once it has reached a high interior population density, bacteria return to the plant surface where it is carried (e.g., through wind or rain), and possibly land on another host plant, thus reinitiating the infectious cycle [15,19,35,40]. Equally to meteorology factors, agricultural practices are also considered ways of pathogen transmission [15,19,35,40].

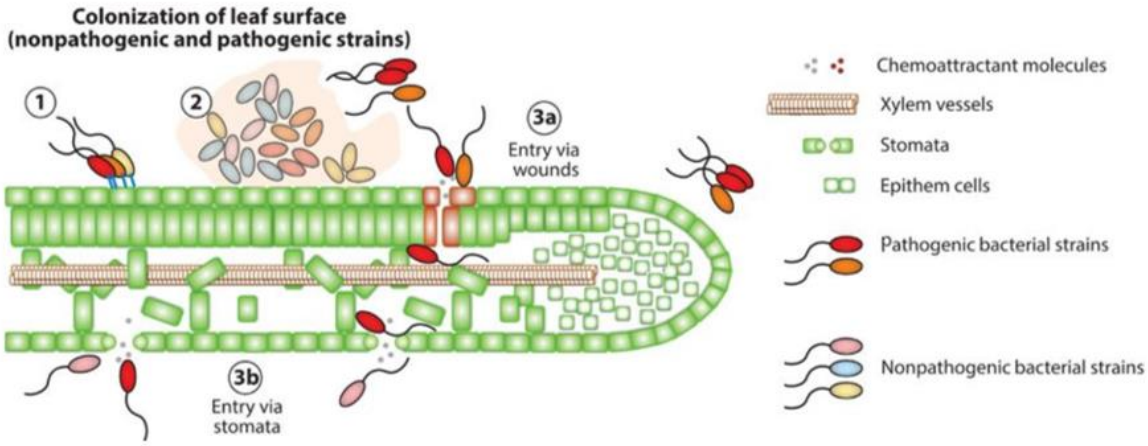


Figure I.5. (1) Bacteria are suggested to exhibit epiphytic growth and attach via their adhesins; (2) bacterial aggregation in microcolonies and biofilm synthesis; (3) bacterial cells reach leaf tissues through (3a) wounds or (3b) stomata [35]. This figure has been adapted from Jacques et al. [35].

Because some studies have isolated non-pathogenic *Xanthomonas* spp. strains from walnut trees, the epiphytic stage has been hypothesized in scientific literature to occur in these isolates, however, the endophytic stage remains undescribed [16,17]. Conjectures about the role of these non-pathogenic bacteria at the epiphytic stage arise, as well as the putative part that they play if perhaps capable of breaching into the plant via a speculated transporter [16,17]**.**

Plant-pathogenic bacteria of the *Xanthomonas* genus are extremely adapted to their hosts as a consequence of their coevolution [35]. Interestingly, it has been suggested that Xanthomonas spp. host specialization, translated into distinct pathovars, emerged as a consequence of intense agricultural practices [35].

6. Phytocontrol of *Xanthomonas*

With the ongoing climate changes, it is foreseeable that not only the geographical area subjected to plant pathogens but also the epidemic severity, number of etiological agents, and frequency of bacterial disease outbreaks will arise [22]. All in all, without adequate phytosanitary control, these plant diseases spread unchecked to many parts of the globe, and many unpleasant surprises (i.e., the emergence of novel illnesses) await the agroecosystems as the virulence of pathogens (i.e., the fitness to cause disease) increases through recombination-mediated evolution [18,21,29,35–37]. Furthermore, the diseases for which *Xanthomonas* are responsible are particularly difficult to control and may involve cultural practices, bactericides, plant defense inducers, and even, if available, plant resistance and biocontrol strategies [22]. On that account, effective Integrated Pest Management (IPM) strategies are more important than

ever [22,44]. Naturally, the success of IPM strategies relies on specific, sensitive, and reproducible diagnostic techniques, as precocious detection is critical for early-on sanitary intervention aimed at eradicating the pathogen and at reducing the inoculum spread [14,16,19,22,35,44,45,46].

The National Plant Protection Organization (NPPO) proposes guidelines for diagnostic protocols, that involve the identification and detection of regulated *Xanthomonas* [22]. Description of symptoms, sampling procedures of plants and plant products, isolation and culturing of bacteria are usually required components of the laboratory testing of these plants and products of plants during official controls (mandatory phytosanitary practices) [22,45].

Over the past decade, with the exceeding improvements in genomic information, molecular methods for the detection of phytopathogenic bacteria taxa and pathovars have been developed, for *X. arboricola* pv. *juglandis* inclusively, and it is broadly acknowledged that these are more specific, sensitive, reliable, and faster (than biochemical methods) [18,22,45,47]. To be precise, molecular methods can be hybridization-based methods, PCR-based methods, LAMP and other isothermal methods, and viability PCR [22,47]. PCR techniques, because they do not require pathogen isolation and cultivation are less time-consuming and have more analytical sensitivity, thus being considered the golden standard for the diagnosis of plant bacterial diseases [22,47]. PCR assays are also interesting as they allow us to detect and identify different species of *Xanthomonas* that are responsible for the same disease, and more remarkably to identify multiple bacteria within the same sample (multiplex PCR) [22]. A disadvantage of the PCR is that it does not assess bacterial viability, hence understudying the risk of the sample at hand [22]. The mentioned innovations in the field of genomics have enhanced the suitability of new DNA markers as PCR tools, a cornerstone for the development of more specific and sensitive taxa-specific identification assays [22]. DNA markers are a tool that is first selected through a plethora of available pipelines (usually reliant on comparative genomics), that then can be adapted to several detection methods, being that a specific probe is designed according to the method selected [22].

With how molecular tools are progressing (e.g. with the design of DNA markers) the future promises that phytopathologists will be paying more attention to public genomic databases; and also that portable molecular detection tools for in-field diagnosis will become more of a commodity [22].

7. Objectives of this dissertation

Early detection is crucial to eradicate pathogens and to reduce the inoculum spread to other plantations. As a specific detection method for *Xanthomonas euroxanthea* is not yet available, the present dissertation aims to provide a reliable method for its detection and isolate identification and, eventually, for the genotyping of its strains. In addition, the detection method would be rendered useful in distinguishing *X. euroxanthea* from *X. arboricola* pv. *juglandis*, for risk assessment analysis, as these bacteria can cause diseases with the same symptomatology.

Furthermore, this project aimed to unravel if within the novel species *X. euroxanthea*, encompassing both pathogenic and non-pathogenic strains, the type III secretion system and its effectors also play a major role as pathogenicity determinants (relevant in the plant-infection process) as described for its closely related species *X. arboricola*.

Ultimately, the main goals of this dissertation were to:

- ✓ Develop an *in silico* workflow heavily based on comparative genomics for the selection and validation of taxa-specific DNA markers (unique and discriminative genomic regions gifted with high species-specificity and genomic stability);
- ✓ Select and validate the specificity, stability, and consistency of *X. euroxanthea*-specific DNA markers;
- ✓ Provide a reliable method namely, a multiplex PCR targeting DNA markers, for the detection and isolate identification of *X. euroxanthea*;
- ✓ Ascertain the genotyping potential of *X. euroxanthea*-specific DNA markers;
- ✓ Attain *X. euroxanthea* mutants in genes of the type three secretion system - *hrcT*, *hrpG*, and *hrpX*;
- ✓ Experimentally validate *X. euroxanthea* mutants depleted of *hrcT*, *hrpG*, and *hrpX* genes, through hypersensitive response (HR) assays;
- ✓ Assess the fitness growth of *X. euroxanthea* mutants depleted of *hrcT*, *hrpG*, and *hrpX* genes;
- ✓ Understand the role of type III secretion system genes, specifically, *hrcT*, *hrpG*, and *hrpX* in the pathogenicity of *X. euroxanthea*.

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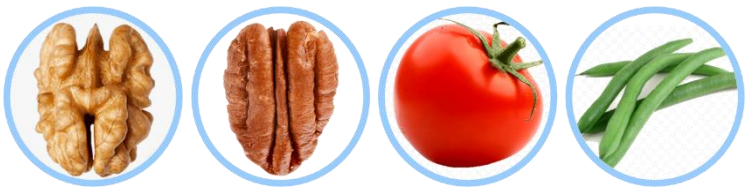
CHAPTER II

DNA Markers for Detection and Genotyping of *Xanthomonas euroxanthea*



Chapter II: DNA Markers for Detection and Genotyping of Xanthomonas euroxanthea

Chapter II of this dissertation is composed of the following original publication:

- ✓ Silva, K.G., Martins, L., Teixeira, M.; Pothier, J.F., Tavares, F. DNA Markers for Detection and Genotyping of *Xanthomonas euroxanthea*. *Microorganisms* **2022**, *10*, 1078, doi:https://doi.org/10.3390/microorganisms10061078.

Chapter II of the current dissertation includes results presented at the following events:

- ✓ Silva, K.G.; Martins, L.; Teixeira, M.; Pothier, J.F.; Tavares, F. 2022. Highlighting the genome of *Xanthomonas euroxanthea*: DNA Markers for its Detection and Genotyping. Presented at the 15th Encontro de Investigação Jovem da U.Porto (IJUP 2022), Porto, Portugal, May 4-6, 2022.
- ✓ Martins, L.; Silva, K.G.; Teixeira, M.; Pothier, J.F.; Tavares, F. *Xanthomonas euroxanthea*-specific DNA markers for genotyping and multiplex PCR-based detection. Presented at the 14th International Conference on Plant Pathogenic Bacteria (ICPPB), Assisi, Italy, July 3-8, 2022.
- ✓ Silva, K.G.; Martins, L.; Tavares, F. 2022. DNA markers profiling reveals host adaptation of *Xanthomonas euroxanthea*. Presented at the III Plant Pests and Diseases Forum: Exploiting Sustainability Through Biotechnology, Porto, Portugal, November 2-3, 2022.

DNA Markers for Detection and Genotyping of *Xanthomonas euroxanthea*

Abstract

Xanthomonas euroxanthea is a bacterial species encompassing both pathogenic and nonpathogenic strains and is frequently found colonizing the same host plants as *X. arboricola*. This presents the need to develop a detection and genotyping assay able to track these bacteria in microbial consortia with other xanthomonads. Eight *X. euroxanthea*-specific DNA markers (XEA1—XEA8) were selected by comparative genomics and validated *in silico* regarding their specificity and consistency using BLASTn, synteny analysis, CG content, codon usage (CAI/eCAI values) and genomic proximity to plasticity determinants. *In silico*, the selected eight DNA markers were found to be specific and conserved across the genomes of 11 *X. euroxanthea* strains, and in particular, five DNA markers (XEA4, XEA5, XEA6, XEA7, and XEA8) were unfailingly found in these genomes. A multiplex of PCR targeting markers XEA1 (819 bp), XEA8 (648 bp), and XEA5 (295 bp) was shown to successfully detect *X. euroxanthea* down to 1 ng of DNA (per PCR reaction). The topology of trees generated with the concatenated sequences of three markers (XEA5, XEA6 and XEA8) and four housekeeping genes (*gyrB*, *rpoD*, *fyuA* and *acnB*) underlined the equal discriminatory power of these features and thus the suitability of the DNA markers to discriminate *X. euroxanthea* lineages. Overall, this study displays a DNA-marker-based method for the detection and genotyping of *X. euroxanthea* strains, contributing to monitoring for its presence in *X. arboricola*-colonizing habitats. The present study proposes a workflow for the selection of species-specific detection markers. Prospectively, this assay could contribute to unveil alternative host species of *Xanthomonas euroxanthea*; and improve the control of phytopathogenic strains.

Keywords: *Xanthomonas euroxanthea*; taxa-specific DNA markers; multiplex PCR; comparative genomics; genotyping

1. Introduction

Following extensive genotyping and comparative genomics studies performed on walnut-associated bacterial isolates, it has been shown that not all the isolates could be identified as *Xanthomonas arboricola* pv. *juglandis*, the phytopathogen commonly acknowledged as causing walnut bacterial blight (WBB) [1–3]. Further studies showed that some walnut-associated *Xanthomonas* isolates were taxonomically distinct from any of the other described *Xanthomonas* species.

These were proposed as members of the new species *Xanthomonas euroxanthea* [4]. Pathogenicity assays indicated that *X. euroxanthea* encompasses non-pathogenic and pathogenic strains that can cause WBB-like symptoms, being in a privileged position to investigate genetic determinants of pathogenesis and its evolution [4–6].

Interestingly, apart from its occurrence in walnuts (*Juglans regia*), recent evidence was gathered reporting the isolation of *X. euroxanthea* from distinct plant host species, such as *Carya illinoensis* (pecan; strains CPBF 761 and CPBF 766), that together with walnut (*Juglans regia*; strains CPBF 367, CPBF 424^{T}, CPBF 426 and CFBP 7653) belong to the Juglandaceae family; *Solanum lycopersicum* (tomato plants; strains BRIP 62409, BRIP 62411, BRIP 62415, and BRIP 62418) a member of the Solanaceae family [7–9]; and *Phaseolus vulgaris* (common bean; strain CFBP 7622, previously misclassified as *X. arboricola* [10,11]), a Fabaceae plant species.

More strikingly is that *X. arboricola* strains were also isolated from all the mentioned plant species, suggesting that both *X. euroxanthea* and *X. arboricola* share the same host plants, including the same plant specimen, which raises questions regarding co-colonization and niche-specific adaptations [7,8,12]. Still, taking into consideration the recent taxonomic refinement of *X. arboricola* species and the unearthing of six new *X. euroxanthea* strains (2949, 2955, 2957, 2974, 3640, and F2) [11], it is foreseeable the identification of additional *X. euroxanthea* isolates from plant species that have passed unnoticed so far.

Altogether, the common ecological niche of these two closely related species, plus their cosmopolitan distribution and co-occurrence in different host plant species resulted in misclassifying some *X. euroxanthea* isolates as *X. arboricola* [8,10,11], which calls for the need to develop methods for the detection and genotyping of this bacterial species. Over the years, different diagnostic and molecular typing tools have been developed for a variety of xanthomonads [13], namely for *X. arboricola* including their most studied pathovars *juglandis* [14,15] and *pruni* [16–19], aiming to address their diversity within a geographic or epidemiological context.

Generally, these approaches consist of culture-based detection of the phytopathogen by PCR, followed by multilocus sequence analysis (MLSA) of several housekeeping genes to define haplotypes. In fact, multiplex-PCR and dot-blot hybridization assays showed that three *X. arboricola* pv. *juglandis* specific DNA markers (XAJ1, XAJ6, and XAJ8) were absent from *X. euroxanthea* strains CPBF 367, CPBF 424^{T}, and CPBF 426, which formed a distinct MLSA cluster [12,14].

A robust method to detect *X. euroxanthea* while undoubtedly distinguishing it from *X. arboricola* has not been described so far. Furthermore, while several genotyping techniques have been used to assess the diversity of phytosanitary regulated *Xanthomonas*, as recently reviewed [13], the data currently available regarding genotyping of *X. euroxanthea* is scarce and limited to MLSA studies performed on *Xanthomonas* isolated from walnut trees [12].

In this study, comparative genomics and *in silico* validation tools were combined as previously described [20] to select eight *X. euroxanthea*-specific DNA markers located in conserved genomic regions. While XEA1, XEA5, and XEA8 DNA markers were chosen to optimize a multiplex PCR detection method for the reliable detection of the target bacteria, altogether the number of SNPs recorded for DNA markers XEA5, XEA6, and XEA8 within the studied *X. euroxanthea* strains revealed an allelic variation capable to discriminate *X. euroxanthea* strains as efficiently as the housekeeping genes used in MLSA. Ultimately, this work may unlock the possibility to conciliate bacterial detection and diversity assessment using the same DNA markers, which may be particularly useful to survey *X. euroxanthea* populations in environmental samples.

2. Materials and Methods

2.1. *In silico* selection and validation of *X. euroxanthea*-specific DNA markers

A synteny analysis of 11 *X. euroxanthea* strains and other 24 representative xanthomonads, downloaded from the NCBI database (Table II.1), was performed using MaGe v3.15.3 [21]. This led to the identification of *X. euroxanthea*-specific coding DNA sequences (CDSs) that are concomitantly present in *X. euroxanthea* genomes and absent from non-*X. euroxanthea* strains.

Table II.1. Bacterial strains used for MaGe synteny analysis to retrieve *Xanthomonas euroxanthea*-specific coding sequences (CDSs).

***Xanthomonas* Species and Pathovars**	**Strains**	**GenBank, NCBI Accession/WGS Prefix**
X. euroxanthea	CPBF 367	LR861803.1
X. euroxanthea	CPBF 424^{T}	LR994544.1
X. euroxanthea	CPBF 426	LR861805.1
X. euroxanthea	CPBF 761	HG999363.1
X. euroxanthea	CPBF 766	HG999364.1
X. euroxanthea	CFBP 7622	MIGF01.1
X. euroxanthea	CFBP 7653	MIGK01.1
X. euroxanthea	BRIP 62409	QEZJ01.1
X. euroxanthea	BRIP 624011	QEZI01.1
X. euroxanthea	BRIP 62415	QEZH01.1
X. euroxanthea	BRIP 62418	QEZG01.1
X. arboricola	CPBF 1494	HG999362.1
X. arboricola	CPBF 765	HG999365.1
X. arboricola pv. *juglandis*	CPBF 427	LR861807.1
X. campestris pv. *campestris*	LMG 568^{PT}	NC_003902
X. campestris pv. *campestris*	8004	NC_007086
X. campestris pv. *campestris*	B100	NC_010688
X. citri pv. *citri*	306	NC_003919
X. citri pv. *bilvae*	NCPPB 3213^{PT}	CDHI01
X. phaseoli pv. *phaseoli*	CFBP 412	NZ_CP020964.2
X. citri subsp. *aurantifolli*	ICPB 10535	ACPY01
X. vasicola pv. *musacearum*	BCC282	RRCQ01
X. vasicola pv. *musacearum*	NCPPB 4381	ACHT01
X. oryzae pv. *oryzae*	PX099^{A}	NC_010717.1
X. oryzae pv. *oryzae*	MAFF 311018	NC_007705.1
X. oryzae pv. *oryzae*	KACC 10331	NC_006834.1
X. oryzae pv. *oryzicola*	BLS256	NZ_AAQN
X. translucens pv. *translucens*	569	VIWM01.1
X. translucens pv. *translucens*	LMG 876^{PT}	NZ_CAPJ.1
X. sacchari	NCPPB 4393	AGDB01.1
X. hortorum pv. *gardneri*	ICMP 7383	CP018731.1
X. hortorum pv. *gardneri*	LMG 962^{T}	NZ_AEQX.1
X. vesicatoria	LMG 911^{T}	NZ_AEQV
X. euvesicatoria pv. *perforans*	91-118	NZ_AEQW
X. albilineans	GPE PC73	NC_013722

Then, a BLAST was performed in NCBI (accessed 22 November 2021 at https://www.ncbi.nlm.nih.gov/) using as a query the putative *X. euroxanthea*-specific CDSs and the database nr/nt to further confirm their specificity. Sequences with hits pertaining solely to *X. euroxanthea* were considered putative *X. euroxanthea*-specific genomic regions (Table II. S1) and were used for primer and specific DNA marker design

using Geneious® 9.1.8 [22]. The affinity and complementarity of these primers to target *X. euroxanthea* CDSs were checked using NCBI's Primer-BLAST tool [22,23]. The obtained eight DNA markers were then evaluated for specificity by a BLASTn analysis in Geneious® 9.1.8 and NCBI (using the database nr/nt).

The genomic context of the markers was assessed to ensure its consistency across the diversity of *X. euroxanthea* strains. Particularly, a comparative genomics analysis was performed by local alignment of CDSs in MaGe (Figure II. S1a–d), to evaluate the syntenic context. Additionally, features, such as the CAI/eCAI values [24]; GC content (deducted from MaGe); chromosomal location; and proximity to determinants of genomic plasticity, namely transposons, integrases, recombinases and phage-related ORFs (Geneious® 9.1.8), were annotated. The number of Single Nucleotide Polymorphisms (SNPs) included in the eight DNA markers and housekeeping genes of the 11 *X. euroxanthea* genomes considered in this study were summed up recurring to Geneious® 9.1.8 and normalized using the formula $\left(\frac{\text{SNPs number}}{\text{total nucleotide sequence length}} \times 100\right)$. The described procedure followed for specific *X. euroxanthea* DNA markers design is systematized in Figure II.1.

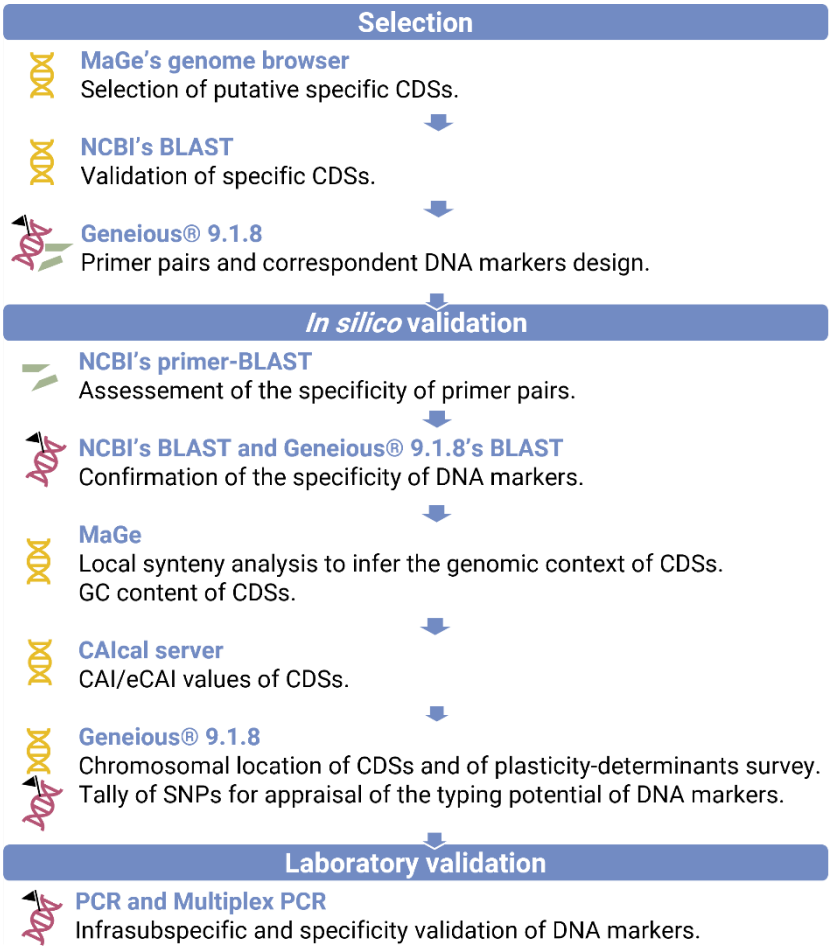


Figure II.1. Flowchart for the selection and validation of *Xanthomonas euroxanthea*-specific DNA markers.

2.2. Bacterial strains, culture conditions and DNA extraction

The bacterial strains used for the validation of the eight *X. euroxanthea*-specific markers are listed in Table II.2 and include seven *X. euroxanthea* strains; and other closely related and niche-sharing strains, namely 10 strains of *X. arboricola*, seven strains representing six pathovars of *X. arboricola* and 11 strains belonging to non-*arboricola Xanthomonas* species. Bacterial strains were cultured as previously described [14] or in peptone-sucrose-agar (PSA) medium (10 g peptone; 10 g sucrose; 1 g glutamic acid; 15 g agar and distilled water up to 1.0 L) at 28 °C. DNA was extracted from pure cultures using the EZNA Bacterial DNA Purification kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions and quantified using a DS-11 microvolume spectrophotometer (DeNovix, Wilmington, DE, USA).

Table II.2. List of bacterial strains used for validation of the *Xanthomonas euroxanthea*-specific DNA markers.

***Xanthomonas* Species and Pathovars**	**Strains [1]**	**Geographic Origin**	**Year of Isolation**
X. euroxanthea	CPBF 367	Portugal (Loures)	2016
X. euroxanthea	CPBF 424 ^{T}	Portugal (Loures)	2016
X. euroxanthea	CPBF 426	Portugal (Loures)	2016
X. euroxanthea	CPBF 761	Portugal (Alcobaça)	2016
X. euroxanthea	CPBF 766	Portugal (Alcobaça)	2016
X. euroxanthea	CFBP 7622	USA	1985
X. euroxanthea	CFBP 7653	France	2008
X. arboricola	CPBF 122	Portugal (Ponte da Barca)	2015
X. arboricola	CPBF 237	Portugal (Ponte de Lima)	2015
X. arboricola	CPBF 554	Portugal (Carrazeda de Ansiães)	2016
X. arboricola	CPBF 765	Portugal (Alcobaça)	2016
X. arboricola	CPBF 796	Portugal (Alcobaça)	2016
X. arboricola	CPBF 1494	Portugal (Alcobaça)	2014
X. arboricola	CPBF 1483	Portugal (Alcobaça)	2014
X. arboricola	CPBF 1514	Portugal (Estremoz)	2014
X. arboricola	CPBF 1567	Portugal (Bombarral)	2015
X. arboricola	CPBF 1586	Portugal (Loures)	2015
X. arboricola pv. *juglandis*	CPBF 427	Portugal (Loures)	2016
X. arboricola pv. *juglandis*	CPBF 1521	Portugal (Loures)	2014
X. arboricola pv. *celebensis*	LMG 677 ^{PT}	New Zealand	1960
X. arboricola pv. *corylina*	LMG 689 ^{PT}	USA	1939
X. arboricola pv. *fragariae*	LMG 19145 ^{PT}	Italy	1993
X. arboricola pv. *populi*	CFBP 3123 ^{PT}	Netherlands	1979
X. arboricola pv. *pruni*	LMG 852 ^{PT}	New Zealand	1953
X. citri pv. *citri*	LMG 9322 ^{T}	USA	1989
X. campestris pv. *campestris*	LMG 568 ^{PT}	United Kingdom	1957
X. axonopodis pv. *dieffenbachiae*	LMG 695 ^{PT}	Brazil	1965
X. fragariae	LMG 708 ^{T}	USA	1960
X. oryzae pv. *oryzicola*	LMG 797 ^{PT}	Malaysia	1964
X. translucens pv. *translucens*	LMG 876 ^{PT}	USA	1933
X. vesicatoria	LMG 911 ^{T}	New Zealand	1955
X. euvesicatoria pv. *euvesicatoria*	LMG 922	USA	1939
X. hortorum pv. *gardneri*	LMG 962 ^{T}	Yugoslavia	1953
X. euvesicatoria pv. *perforans*	NCPPB 4321 ^{T}	USA	1933
X. oryzae pv. *oryzae*	LMG 5047 ^{PT}	India	1965

[1] CPBF: Portuguese Collection of Phytopathogenic Bacteria, Instituto Nacional de Investigação Agrária e Veterinária, I.P. Oeiras, Portugal. CFBP: French Collection for Plant-associated Bacteria, Institut National de la Recherche Agronomique, Angers, France. LMG: Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection, Universiteit Gent—Laboratorium voor Microbiologie, Gent, Belgium. NCPPB: National Collection of Plant Pathogenic Bacteria, Fera Science Ltd., York, UK. Superscript following strain names indicate ^{T} the type strain of a species and PT the pathotype strain for a pathovar.

2.3. Experimental validation of putative *X. euroxanthea*-specific DNA markers by multiplex PCR

A multiplex PCR targeting the most promising DNA markers was optimized to validate a method to rapidly identify *X. euroxanthea* isolates. XEA1, XEA5, and XEA8 were the chosen markers with distinct amplicon lengths of 819, 295, and 648 bp, respectively; and their broad occurrence in the tested *X. euroxanthea* strains, apart from XEA1 (absent in CFBP 7622). A 20 µL PCR reaction mix consisted of 1 × DreamTaq Buffer (ThermoFisher Scientific, Waltham, MA, USA), 0.2 mM of each deoxynucleotide triphosphate (dNTP) (Grisp, Porto, Portugal), 0.2 mM of each forward and reverse

primers (Table II.3), 1.5 U of DreamTaq DNA Polymerase (ThermoFisher Scientific, Waltham, MA, USA) and 25 ng of DNA template.

Sterile distilled water was used as the negative control. PCR cycling parameters consisted of a first amplification cycle of 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 61 °C for 15 s, and 72 °C for 30 s as well as a final DNA extension at 72 °C for 10 min. The same DNA samples were used as templates in PCR reactions using each of the markers individually (XEA1, XEA5, and XEA8) and 1.0 U of DreamTaq DNA polymerase per PCR reaction.

PCR products were separated by electrophoresis on a 0.8% agarose gel (1 × TAE buffer) and visualized using Xpert Green DNA stain (Grisp, Porto, Portugal) with a Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA). The obtained PCR products for each marker and each strain were purified using the Illustra GFX GEL Band Purification kit (GE Healthcare, Buckingham-shire, UK), following the reference protocol available and sequenced on both strands (STAB Vida, Caparica, Portugal) to confirm their identity and determine the number of SNPs.

Table II.3. Selected *Xanthomonas euroxanthea*-specific DNA markers (XEA1—XEA8), corresponding primer pair sequences, expected amplicon sizes and best BLASTn hits of amplicons with non-*X. euroxanthea* genomes.

DNA Markers	CDS (MaGe)[1]	Locus Tag (NCBI)	Gene Annotation (MaGe)	Primers	Sequences (5′→3′)	Length (bp)	Best BLASTn Hit with Non-*X. euroxanthea* (E Value/Query Coverage)
XEA1	XE424_v1_a0582	XTG_000508	Conserved protein of unknown function	XEA1F	CTGCCGAGCGTGAAATCCAG	819	-
				XEA1R	CCTTCAGTTGCACCGAACGC		
XEA2	XE424_v1_a2605	XTG_002379	Conserved protein of unknown function	XEA2F	AGTCCACCAATGCCATCGCC	425	-
				XEA2R	AGTCCACCAATGCCATCGCC		
XEA3	XE424_v1_a2606	XTG_002380	Conserved protein of unknown function	XEA3F	CGGATCGGACAATGACGCTG	612	-
				XEA3R	GCTCTACATCGCCGCTGGAG		
XEA4	XE424_v1_a1415	XTG_001287	TetR/AcrR family transcriptional regulator	XEA4F	GACGCATCCGCCCACGACC	341	*Dickeya zeae* A586-S18-A17 (2×10^{-50}/95%)
				XEA4R	TAGGCGGCAGACCCCTTCC		
XEA5	XE424_v1_a0462	XTG_000401	MarR family transcriptional regulator	XEA5F	AACGACGCTGACCTGGACC	295	*Sphingomonas* sp. AP4-R1 (5×10^{-13}/73%)
				XEA5R	CGACACCGCACGACCCCG		
XEA6	XE424_v1_a0617	XTG_000542	Conserved protein of unknown function	XEA6F	GCGGCTGCAGCGTCGTTG	237	*Xanthomonas* sp. GW (2×10^{-4}/19%)
				XEA6R	TCACCTGATGATCGAAGCCTGG		
XEA7	XE424_v1_a1414	n/a	Protein of unknown function	XEA7F	GGACGCGCCATGATCTGCC	212	-
				XEA7R	GGTGTCCGAGGMTCAGGTGC		
XEA8	[2]	[2]	[2]	XEA8F	ATCGCCTCTGGATGACGGC	648	*Dickeya zeae* A586-S18-A17 (1×10^{-95}/73%)
				XEA8R	GGTGATGTCGGCAAGCTCG		

n/a: not available. [1]: no significant hit has been found. [2]: XEA8 DNA marker was designed within the genomic subsequent and partially overlapping CDSs for markers XEA7 and XEA4.

2.4. PCR detection limit

The detection limit of the multiplex PCR was determined using 10 µL from each of the 10-fold dilutions of *X. euroxanthea* CPBF 424^T chromosomal DNA prepared in distilled sterile water, ranging from 100 ng to 1 pg per PCR reaction. Multiplex PCR conditions were kept as described above.

2.5. Typing potential of *X. euroxanthea*-specific DNA markers

Unrooted trees using XEA5, XEA6, and XEA8 markers (transversal to all strains of *X. euroxanthea*) (Table II. S2) and partial sequence analysis of the housekeeping genes *acnB*, *fyuA*, *gyrB,* and *rpoD* (Table II. S3) of 11 *X. euroxanthea* strains were built to infer the typing potential of these DNA markers. Markers XEA5 (295 bp), XEA6 (237 bp), and XEA8 (648 bp) sequences were concatenated using the Geneious ® v. 9.1.7 and used to build a maximum-likelihood tree based on Tamura-Nei model on MEGA X [25], as previously described [14].

The nucleotide sequences of housekeeping genes *acnB*, *fyuA*, *gyrB,* and *rpoD* were retrieved from the 11 *X. euroxanthea* genomes, aligned and trimmed to 513, 640, 828, and 793 bp, respectively, and subsequently concatenated using the Geneious ® v. 9.1.7 to build a maximum-likelihood tree as described for the markers. Since these three markers are *X. euroxanthea* specific, and no homologous marker could be found to define a coherent outgroup, the relatedness of the *X. euroxanthea* strains was inferred by an unrooted tree.

3. Results

3.1. *In silico* selection of DNA markers for *X. euroxanthea*

The selection of *X. euroxanthea*-specific DNA markers was conducted according to the workflow detailed in Figure II.1, which consisted on the use of a platform of comparative genomics (Mage) to screen for unique *X. euroxanthea* CDSs, further validated by BLASTn.

A list of CDSs exclusively present in the genomes of 11 *X. euroxanthea* strains and absent from other 12 *Xanthomonas* species, including the closely related *X. arboricola*, was deducted from synteny analysis between the 24 genomes (Table II.1) along the full length of *X. euroxanthea* CPBF 424^{T} genome used as reference (Figure II. S1). The candidate CDSs (from the reference genome of *X. euroxanthea* CPBF 424^{T}) were submitted to a BLASTn to confirm their exclusivity in *X. euroxanthea* considering high stringency values of E-value, percentage of identity and query coverage and their suitability as *X. euroxanthea*-specific DNA markers appraised by the design of robust primers.

The data showed that four CDSs had no significant BLAST hits; three CDSs showed no hits with other *Xanthomonas* sp. and solely one CDS coding for a putative conserved protein of unknown function matched with *Xanthomonas* sp. GW (E-value of 2×10^{-4} and query coverage of 19%) (Table II.3). These seven CDSs were selected for the design of eight DNA markers, designated XEA1–XEA8; being that two consecutive and partly overlapping CDSs (XE424_v1_a1415 and XE424_v1_a1414) were used to design marker XEA8 (Table II.3). Five of these CDSs are predicted to be proteins of unknown function and two to be transcriptional regulators (Table II.3). Ultimately, five DNA markers, XEA4–XEA8, are unfailingly present in the genome of the 11 *X. euroxanthea* strains analyzed; XEA1 is present in eight genomes; and XEA2 and XEA3 are present in five strains (Figure II.2).

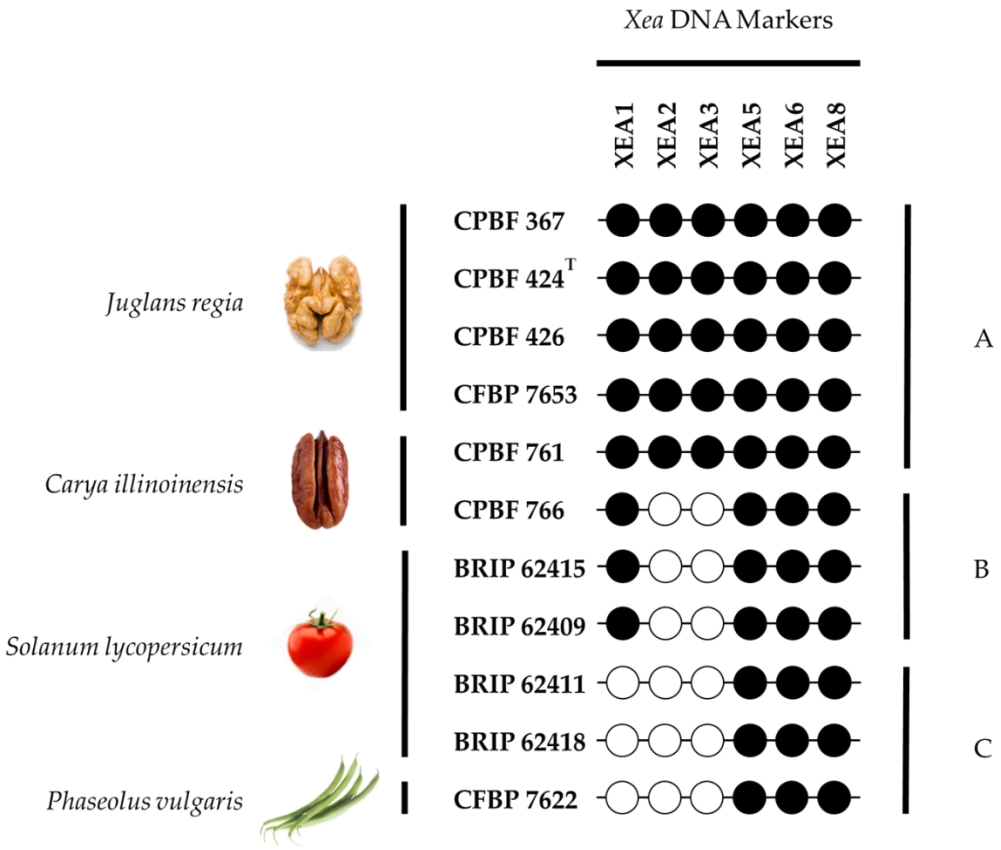


Figure II.2. Distribution of six *Xanthomonas euroxanthea* (*Xea*)-specific DNA markers (XEA1, XEA2, XEA3, XEA5, XEA6, and XEA8) in 11 X. euroxanthea genomes. The presence/absence of six XEA DNA markers was assessed by BLASTn analysis in Geneious, allowing to disclose three patterns, A to C, that do not translate strain-host affinities.

3.2. Genomic analysis unearths the stability of XEA DNA markers

To further assess the uniqueness of these *X. euroxanthea*-specific CDSs used for DNA marker design, several features, including SNPs number, CAI/eCAI values, GC content, chromosomal location, and chromosomal proximity to genomic plasticity determinants were surveyed (Figure II.3).

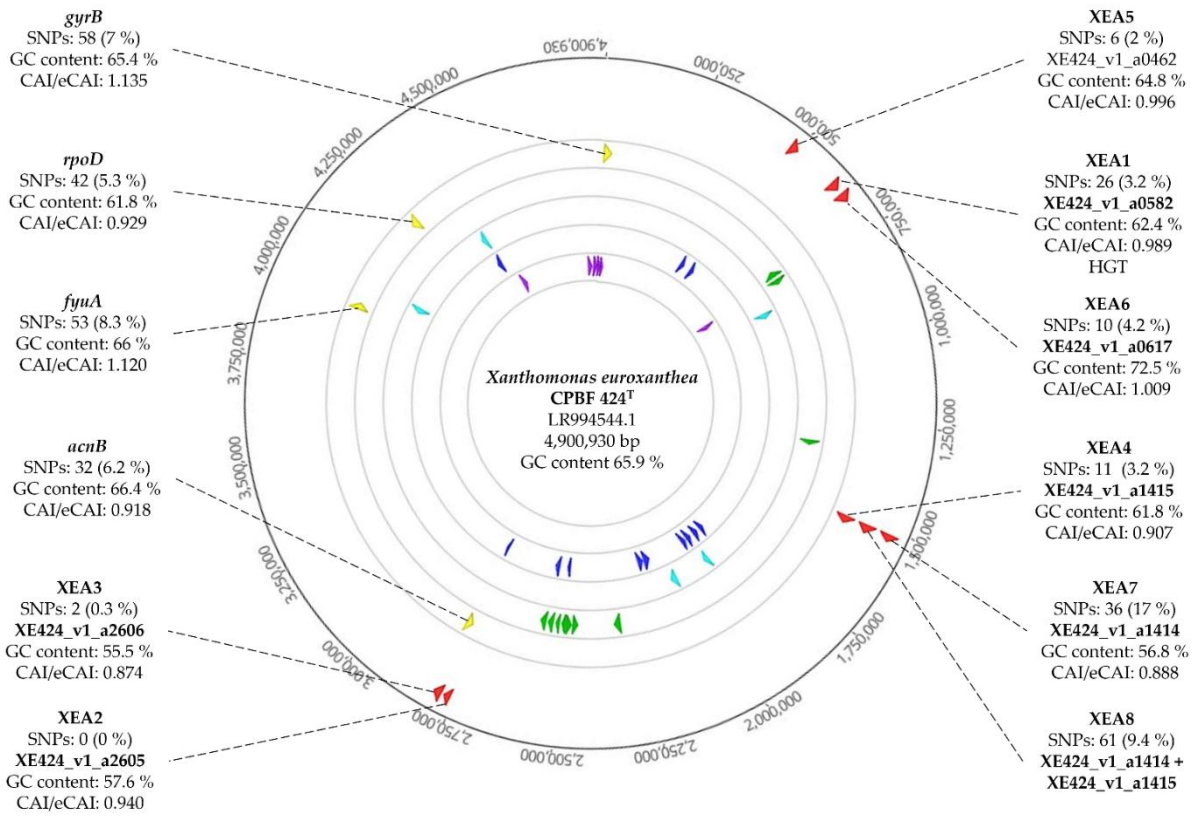


Figure II.3. Circular map of *Xanthomonas euroxanthea* strain CPBF 424^T chromosome. Outside to inner circles are showing genome coordinates (bp); *X. euroxanthea*-specific DNA markers XEA1- to XEA8 (red); housekeeping genes *gyrB*, *rpoD*, *fyuA,* and *acnB* (yellow); transposases (green); recombinases (light blue); integrases (dark blue) and phage-related ORFs (purple). For each XEA DNA marker and housekeeping gene the number of SNPs (calculated based on 11 genomes of X. euroxanthea), GC content, and CAI/eCAI values are shown.

Within the 11 *X. euroxanthea* genomes considered in this study, the number of SNPs for each marker and housekeeping gene was determined and normalized to the full length of the sequence (i.e., DNA marker or housekeeping gene) as a percentage indicative of the allelic diversity of the sequences (Figure II.3).

CAI/eCAI values of CDSs range between 0.874 (for XE424_v1_a2606 corresponding to DNA marker XEA3) to 1.009 (for XE424_v1_a50617 corresponding to DNA marker XEA6); which are values similar to the range attained with housekeeping genes, namely, 0.918 (*acnB*) to 1.135 (*gyrB*) (Figure II.3). GC content values for *X. euroxanthea*-specific CDSs varied between 55.5% (XE424_v1_a2606, XEA3) to 72.5% (XE424_v1_a0617, XEA6), which parallels with *X. euroxanthea* CPBF 424^T genome GC content value of 65.9% (Figure II.3).

Moreover, chromosomal location studies revealed that the XEA markers are scattered throughout the first half of the chromosome and not in the vicinity of genomic plasticity-determinants, including transposases, recombinases, integrases, and phage-related ORFs (Figure II.3).

The synteny analysis performed with MaGe allowed to investigate the genomic context of the most representative DNA markers, i.e., XEA1, XEA5, XEA6, and XEA8, across 11 *X. euroxanthea* genomes and comparatively to *Xanthomonas arboricola* strains CPBF 427 (*X. arboricola* pv. *juglandis*) and CPBF 765 (Figure II.4) and other *Xanthomonas* spp. representative strains (Figure II. S1).

The data showed that XEA1, XEA5, XEA6, and XEA8 are located in highly syntenic regions across all the *X. euroxanthea* genomes studied, underlining the absence of genomic rearrangements (Figure II.4). Furthermore, CDSs used for the design of markers XEA5, XEA6 and XEA8 are exclusive of *X. euroxanthea*, being either absent (CDSs encompassing XEA5 and XEA8) or truncated (CDS encompassing XEA6) in *X. arboricola* strains (Figure II.4).

Particularly, the region upstream and including marker XEA1 appears to have suffered erosion in the *X. arboricola* strains analyzed (CPBF 427 and CPBF 765) and in three of the X. euroxanthea strains studied (BRIP 62411, BRIP 62418, and CFBP 7622). In parallel, this scenario is observed for the region upstream of XEA5 for all strains, except for CPBF 424^T; and downstream of XEA8 for the non-*X. euroxanthea* strains.

Within the flanking regions of the XEA markers, the CDSs annotated as unknown proteins are the ones where the presence/absence across strains is inconsistent, suggesting that they have been decaying. While, for XEA6 and XEA8 markers, these events are limited to a single CDS immediately flanking the markers, for XEA5 this paradigm is particularly clear as several CDSs annotated as unknown proteins are present in CPBF 424^T, have been lost by the other strains.

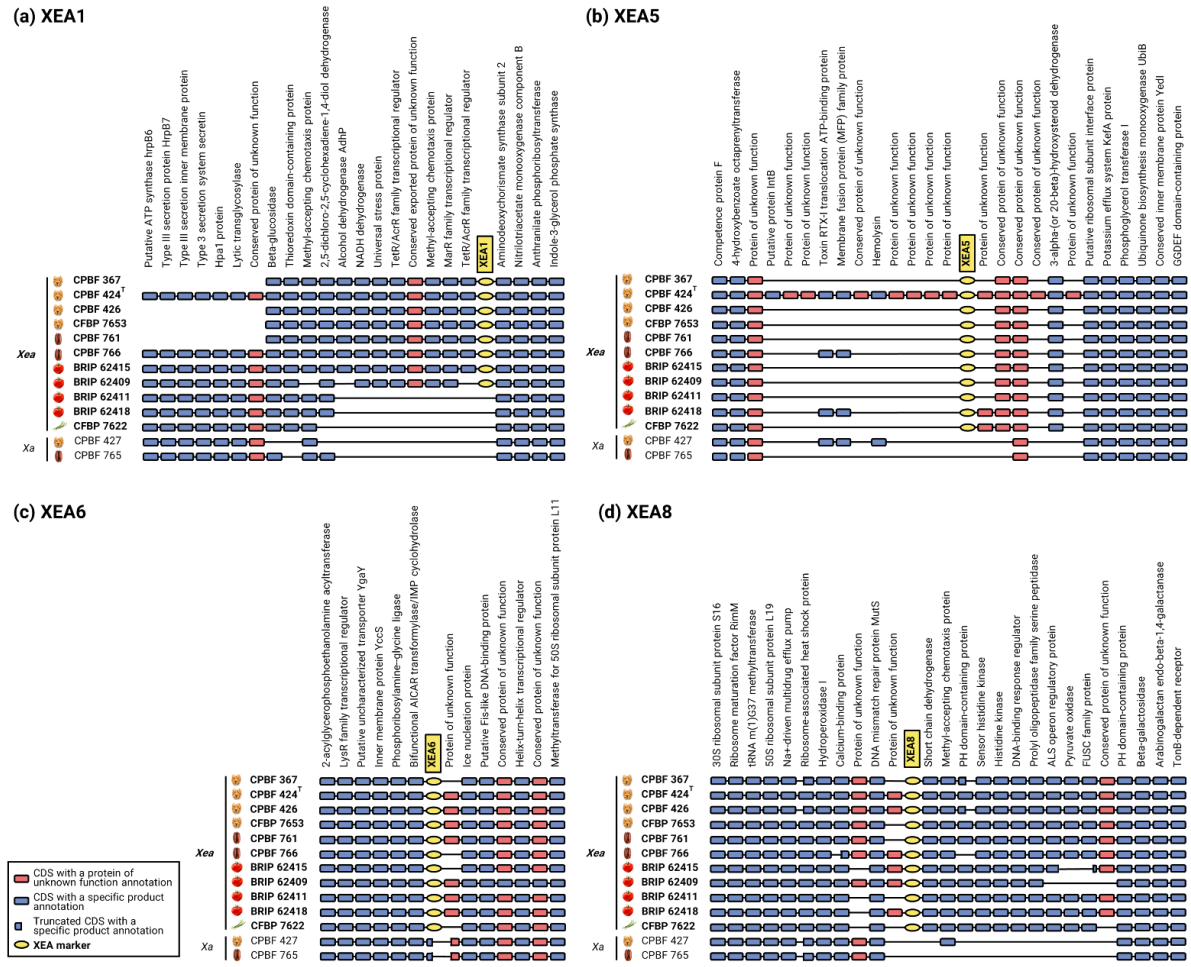


Figure II.4. Comparative syntenic maps of four *Xanthomonas euroxanthea*-specific DNA marker-harboring-regions (a) XEA1 (designed from a conserved protein of unknown function sequence), (b) XEA5 (design from a MarR family transcriptional regulator), (c) XEA6 (designed from a conserved protein of unknown function sequence) and (d) XEA8 (designed from a protein of unknown function and a TetR/AcrR family transcriptional regulator sequences) DNA markers across 11 *X. euroxanthea* (*Xea*) and two *Xanthomonas arboricola* (*Xa*) strains.

3.3. Multiplex PCR allows for the confident identification of *X. euroxanthea* strains

Reliable identification of *X. euroxanthea* isolates by multiplex PCR was optimized for DNA markers XEA1, XEA5, and XEA8, as these originate amplicons of distinguishable size (819, 295 and 648 bp, respectively) and are located in three independent genomic regions of *X. euroxanthea*. The three markers were successfully amplified in all *X. euroxanthea* strains analyzed, with the exception of XEA1 in *X. euroxanthea* CFBP 7622 strain (Figure II.5) as expected by *in silico* studies. In addition, no amplification was observed for any of the other 28 xanthomonads, namely 17 strains of *X. arboricola*, including different pathovars and 9 non-*arboricola* *Xanthomonas* species.

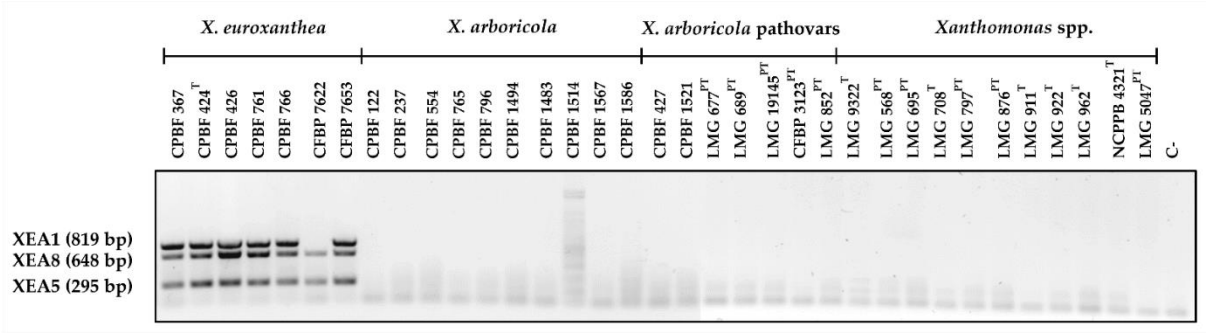


Figure II.5. Multiplex PCR using *Xanthomonas euroxanthea*-specific DNA markers XEA1 (819 bp), XEA8 (648 bp), and XEA5 (295 bp) on 7 *X. euroxanthea* strains, 10 *Xanthomonas arboricola* strains, 6 pathovars of *Xanthomonas arboricola* and 9 non-*arboricola Xanthomonas* species. Markers XEA5 and XEA8 were successful in detecting X. euroxanthea strains, while XEA1 identified all *X. euroxanthea* strains, except for CFBP 7622. No amplification was observed for any of the other xanthomonads tested, namely *X. arboricola* and other *Xanthomonas* species. C-: negative control.

3.4. Detection limit of multiplex PCR with XEA DNA markers

The detection limit of the multiplex PCR targeting the *X. euroxanthea*-specific markers XEA1, XEA5, and XEA8, determined through serial dilution of chromosomal DNA, was 1 ng for PCR reaction (Figure II. S2). When assessing the PCR detection limit of each marker individually, while for XEA1 and XEA8 the detection limit was identical to the multiplex PCR, i.e., 1 ng/PCR reaction, it was ascertained that the limit of detection lowered to 100 pg/PCR reaction for XEA5.

3.5. Typing potential of informative XEA DNA markers

The concatenated sequences of XEA5, XEA6, and XEA8 (1180 bp) for each *X. euroxanthea* strain studied were aligned and used to generate a maximum-likelihood tree to investigate the discriminatory potential of these *X. euroxanthea*-specific markers comparatively to four housekeeping genes, corresponding to a concatenated sequence length of 2774 bp, (*acnB*, *fyuA*, *gyrB*, and *rpoD* genes) commonly used for MLSA (Figure II.6).

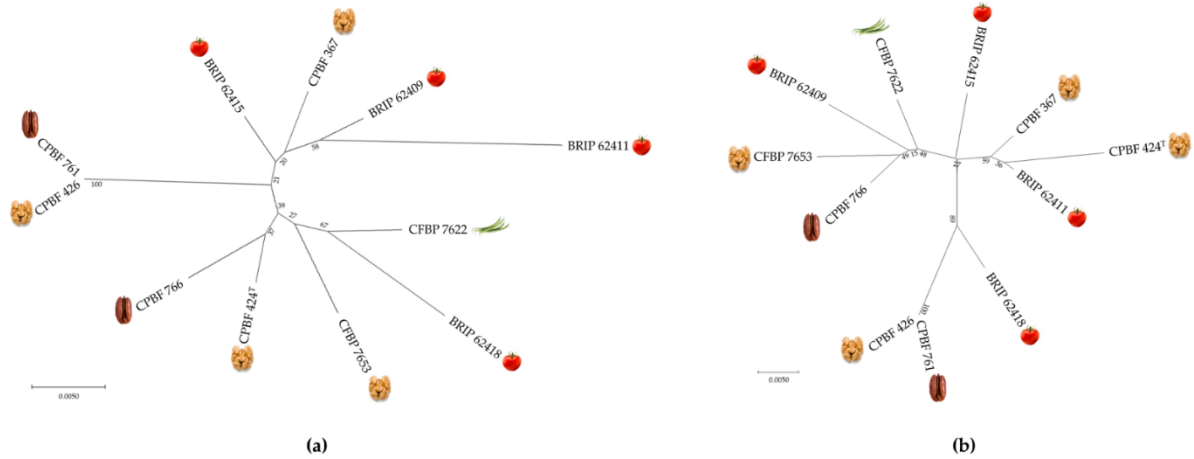


Figure II.6. Maximum-likelihood phylogenetic tree based on concatenated sequences of (a) DNA markers XEA5, XEA6, and XEA8 (1180 bp); and (b) partial housekeeping gene sequences for *acnB*, *fyuA*, *gyrB* and *rpoD* (2774 bp) extracted from 11 *X. euroxanthea* genomes. The tree was constructed using the Tamura-Nei model using MEGA X (Kumar et al., 2018). Supporting values from 1000 bootstrap replicates are indicated near nodes.

Attending that these XEA detection markers are highly specific to *X. euroxanthea*, no homologous sequences have been found in other bacterial taxa, and therefore the allelic diversity determined by the number of SNP (6 SNP/295nt for XEA5, 10 SNP/237nt for XEA6, and 61 SNP/648nt for XEA8; Figure II.3) is represented by an unrooted maximum-likelihood tree. For both trees, each *X. euroxanthea* strain is represented by an independent tree branch, with exceptions for strains CPBF 426 and CPBF 761, which clustered together in a single branch. Furthermore, the topology of both trees does not reflect any clustering according to plant–host of isolation nor by pathogenicity or non-pathogenicity phenotypes (Figure II.6).

4. Discussion

It is important to investigate the distribution and role played by *X. euroxanthea* and the closely related *X. arboricola* within the plant hosts that they frequently co-colonize [12]. Therefore, it is essential to develop a reliable and efficient method for the accurate detection and identification of *X. euroxanthea* strains and its differentiation from *X. arboricola* [12].

Additionally, early detection is a critical first step towards timely sanitary intervention aimed at eradicating the pathogen and at reducing the inoculum spread to other plants; thus lessening the disease-induced damage in crops from growth to postharvest processing of products and ensuring agricultural sustainability [26]. Likewise, discriminating one bacterial species from the other would be useful in the application of suitable management procedures.

Recommended standard diagnostic protocols from OEPP/EPPO for other plant-diseases caused by *Xanthomona*s spp. (namely, *X. arboricola* pv. *corylina*, *X. axonopodis* pv. *dieffenbachiae*, *X. arboricola* pv. *pruni*, *X. axonopodis* pv. *citri*, *X. fragariae*, *X. oryzae*, *X. axonopodis* pv. *allii*, *X. euvesicatoria*, *X. hortorum* pv. *gardneri*, *X. perforans*, and *X. vesicatoria*) still require several steps-observation of disease symptoms, microscopic examination, pathogen isolation, pathogenicity tests, and molecular tests [27–34]. Having in mind that only recently *X. euroxanthea* was proposed as a new species [4], isolated from different plant hosts [7–10], for which the nature of the bacteria–plant interaction is still unknown, and no distinct symptoms have been described [4], the herein proposed multiplex PCR is the tool available for accurate detection and identification of *X. euroxanthea*.

While multiplex PCRs have been proposed to detect *X. arboricola* pv. *juglandis* [14] or *X. arboricola* pv. *pruni* [35], the present work describes eight *X. euroxanthea*-specific DNA markers (XEA1–XEA8) and proposes a methodology to detect and genotype *X. euroxanthea* strains. In view of that, using a comparative genomics strategy and by assessing a pool of numerous xanthomonads genomes, including several *X. euroxanthea* and *X. arboricola*, it was possible to narrow down *X. euroxanthea*-specific genomic regions to only seven CDSs. Genomes alignment is emerging as a fast and convenient solution to rapidly identify species-specific DNA markers to implement PCR-based detection methods, as recently evidenced for *Xanthomonas campestris* pv. *raphani* [36].

The specificity of these putative *X. euroxathea*-specific DNA markers was further validated by BLASTn analysis using *X. euroxanthea* type strain CPBF 424^{T} as a query sequence, followed by an in silico workflow essentially as described in previous studies

[20,37], to determine the genomic context of each *X. euroxanthea*-specific loci and particularly to ensure that these putative DNA markers are within conserved and stable genomic regions.

Specifically, four CDSs showed no significant BLASTn hits outside of *X. euroxanthea*, three CDSs showed some similarity with non-*Xanthomonas* species, and only one CDS matched with a *Xanthomonas* spp. but with a poor similarity as shown by an E-value of 2×10^{-2} and query coverage of 19%. Based on these data, eight primer pairs corresponding to eight markers (XEA1–XEA8), were designed to nest in these seven CDSs. Marker XEA8 was designed from two consecutive and partly overlapping CDSs.

Furthermore, the chromosomal distance between each of the *X. euroxanthea* DNA markers (XEA1–XEA8) and genomic plasticity-determinants, such as transposases, recombinases, integrases, and phage-related ORFs attests to the low genomic plasticity and high stability of the DNA marker-harboring-regions, as previously suggested [20]. All seven specific CDSs on which XEA markers are founded appear to be well adapted to the codon usage and CG content, thereby, suggesting that these CDSs were not recently acquired by Horizontal Gene Transfer (HGT) [20,38].

Such findings are consistent with the hypothesis that these CDSs are conserved in the *X. euroxanthea* genomes and likely present across all the *X. euroxanthea* strains regardless of infrasubspecific variability. A BLAST analysis of each DNA-marker (XEA1–XEA8) against the genomes of 11 *X. euroxanthea* showed that five DNA markers (XEA4–XEA8) are unfailingly present in the genome of the 11 *X. euroxanthea* strains analyzed.

One marker (XEA1) is absent in three out of the 11 *X. euroxanthea* genomes analyzed, and markers XEA2 and XEA3 are present in the four *X. euroxanthea* strains isolated from walnut (CPBF 367, CPBF 424^{T}, CPBF 426, and CFBP 7653) and in one strain isolated from pecan (CPBF 766). These results suggest that the CDSs used for the design of markers XEA4–XEA8 are within the core genome of the 11 *X. euroxanthea* studied, whereas the CDSs corresponding to XEA1–XEA3 markers, although *X. euroxanthea*-specific, are part of the accessory genome [39].

An interesting attribute of *X. euroxanthea* is the encompassment of pathogenic (CPBF 424^{T}) and non-pathogenic strains (CPBF 367) by the same host [4]. Considering the pathogenicity and non-pathogenicity phenotypes; different plant hosts of isolation; and the occurrence of markers XEA1–XEA8 across the 11 studied *X. euroxanthea*, one may conclude that these *X. euroxanthea*-specific markers are not biased by pathogenicity phenotype or plant host species.

To determine the allelic variation of the markers and infer their potential to discriminate *X. euroxanthea* strains, the number of SNPs of markers shown to be present in the 11 *X. euroxanthea* strains (i.e., XEA5, XEA6, and XEA8) was determined. The

data indicate that XEA8 with a 9.4% of SNPs 61 SNP/648 bp) stands as the most informative *X. euroxanthea*-specific DNA marker for typing purposes, as it surpasses the 2% and 4.2% of SNPs recorded for XEA5 and XEA6 markers, respectively (6 SNP/295 bp for XEA5; and 10 SNP/237 bp for XEA6).

Although the allelic variation of XEA5 and XEA6 may be inferior to the allelic variation observed for the housekeeping genes commonly used for MLSA (*acnB* (6.2%), *fyuA* (8.3%), *gyrB* (7%) and *rpoD* (5.3%)), when combined with XEA8, it is nevertheless sufficient to separate the 11 *X. euroxanthea* strains as efficiently as the housekeeping genes. These results suggest that these three *X. euroxanthea*-specific DNA markers (XEA5, XEA6, and XEA8) may be used both for the detection and genotyping of *X. euroxanthea*. Since the allelic variation was conducted considering 11 genomes of *X. euroxanthea*, the number of SNPs may be underestimated, i.e., novel *X. euroxanthea* strains may reveal novel single nucleotide substitutions in the sequence of the mentioned markers.

The synteny analyses conducted to investigate the genomic context of the XEA markers across 11 *X. euroxanthea* and two strains of the closely related species *X. arboricola*, revealed that XEA markers and their flanking CDSs are syntenic for *X. euroxanthea*. It is worth mentioning that, within the flanking regions of XEA markers, CDSs annotated as unknown proteins are intermittently absent when compared to CDSs of proteins of known function, suggesting genomic erosion.

This paradigm is further supported by the genomic context of the XEA5 marker where two CDSs, namely, the toxin RTX-I translocation ATP-binding protein and the membrane fusion protein (MFP) family protein remain present in CPBF 424^{T}, CPBF 766, BRIP 62418, and CPBF 427 despite the overall loss of the flanking unknown proteins. The fact that the CDS of the XEA6 marker is present in *X. euroxanthea* strains and truncated in *X. arboricola* strains suggests a common and recent ancestrality of these two species.

Distinctively, markers designed from CDSs annotated as a family transcriptional regulator, namely XEA5 and XEA8, are likely to portray an essential role in cell life and consequently remain conserved in *X. euroxanthea*, which is further supported by their inclusion as part of the core genome.

Overall, the data gathered regarding synteny, CAI/eCAI values, and the GC content of *X. euroxanthea*-specific CDSs concerning markers XEA1, XEA5, XEA6, and XEA8, suggest that these markers and their flanking regions are not the result of recent HGT acquisitions events by *X. euroxanthea*, which increases its reliability for *X. euroxanthea* detection and identification, as has been hypothesized for other bacteria [20,37].

A multiplex PCR detection assay targeting XEA1, XEA5 and XEA8 markers was successfully developed for the identification of *X. euroxanthea*. The annealing temperature and extension times were optimized to steer clear of non-specific amplifications of DNA, which was also reportedly done in previous studies [40].

This multiplex PCR was shown to be both specific and efficient given that neither non-specific amplifications (bands of unexpected sizes) in *X. euroxanthea* nor amplification in non-target *Xanthomonas* spp. tested were observed. As predicted by the in silico assessments, the amplicons corresponding to XEA1, XEA5, and XEA8 markers were obtained for all X. euroxanthea strains, with the exception of XEA1 for *X. euroxanthea* CFBP 7622. These results emphasize the robustness of the multiplex PCR for the detection and identification of *X. euroxanthea*.

When assessing the PCR detection limit of each marker individually, it was observed that XEA5 lowered the limit of detection value to 10 pg/µL, indicating that the sensitivity of the simplex PCR targeting XEA5 marker is ten-fold higher than the multiplex PCR. The detection limit of 0.1 ng/µL attained for the multiplex PCR is similar to what has been reported in other studies (0.02 and 0.5 ng/µL) [40,41].

To investigate the genotyping potential of the chosen markers, the allelic variation of the three markers present in all studied *X. euroxanthea* strains (XEA5, XEA6, and XEA8) was compared with four housekeeping genes commonly used for MLSA (*acnB*, *fyuA*, *gyrB,* and *rpoD*) and represented as unrooted trees. The trees obtained had a similar topology, as each strain was allocated to an independent tree branch, except for strains CPBF 426 and CPBF 761, which clustered together in a single branch, and no SNPs were observed within the concatenated sequences of the XEA markers (1180 bp) nor within the partial housekeeping genes (2774 bp).

Such data reveals that markers and housekeeping genes are similarly informative in discriminating *X. euroxanthea* strains; and that the studied *X. euroxanthea* strains are not clustered according to plant–host isolation, pathogenicity or non-pathogenicity phenotypes.

By comparing marker-presence profiles to plant host species from which the *X. euroxanthea* strains were isolated, three patterns identified as A, B, and C (Figure II.2) are observed. Attending that all XEA markers are in syntenic genomic regions that overlap with homologs regions in the closely related *X. arboricola* strains, we may hypothesize that the *X. euroxanthea* strains isolated from two Juglandaceae species (*Juglans regia* and *Carya illinoinensis*) with pattern A, i.e., possessing all XEA markers (XEA1–XEA8), are ancestors of strains included in pattern B (i.e., which lost XEA2 and XEA3) and isolated from *Carya illinoinensis* and Solanaceaceae (*Solanum*

lycopersicum) and pattern C (i.e., which lost XEA1, XEA2 and XEA3), isolated from *Solanum lycopersicum* and a Fabaceae (*Phaseolus vulgaris*).

Thus, rather than an acquisition of XEA2 and XEA3 markers in five of the eleven studied *X. euroxanthea* strains (pattern A), these markers were likely lost in the other *X. euroxanthea* strains, followed by XEA1 marker shown to be absent in 3 out of the 11 *X. euroxanthea* strains, leading to the emergence of recent *X. euroxanthea* lineage characterized by marker-profile C (BRIP 62411, BRIP 62418) and *Phaseolus vulgaris* (CFBP 7622). These data suggest that the loss of markers XEA2, XEA3, and XEA1 i.e., patterns A1–C, occurred progressively as *X. euroxanthea* lineages extended to a new host, specifically, from walnut to pecan, to tomato, to the common bean. These results are aligned with studies describing events of genome erosion as a consequence of bacterial adaptation to a new plant–host [42,43].

To summarize, we may infer that the evolutionary trend of XEA marker loss follows progressive host-jump events from walnut to pecan, to tomato, and finally to the common bean.

5. Conclusions

The present study proposes eight specific, efficient and reliable DNA markers for the detection and identification of *X. euroxanthea* isolates. The allelic variation of some of these markers allows to conciliating the detection and genotyping of *X. euroxanthea* strains, contributing to survey these bacteria in ecological niches colonized by the closely related *X. arboricola*. The multiplex PCR was shown to be highly specific, as solely the target DNA (i.e., *X. euroxanthea*) was amplified; and efficient, as an amplicon was observed with all tested X. euroxanthea strains.

The present study also provided a successful workflow for the selection of molecular markers, which is able to be implemented in the selection of species-specific genomic regions for any other taxa.

By analyzing a marker's presence across strains of different colonizing plant host species, we may infer that *X. euroxanthea* colonization of different plant host species occurred at different points in time.

6. References

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7. Supplementary material

Table II. S1. MaGe labels of the seven selected *Xanthomonas euroxanthea*-specific CDSs (for DNA markers design) of 11 *X. euroxanthea* genomes.

***X. euroxanthea* strains**	**MaGe label for CDS used in DNA marker design**						
	XEA1	**XEA2**	**XEA3**	**XEA4**	**XEA5**	**XEA6**	**XEA7**
CPBF 367	XSP_000481	XSP_002020	XSP_002019	XSP_001326	XSP_000402	XSP_000515	[1]
CPBF 424^T	XE424_v1_a0582	XE424_v1_a2605	XE424_v1_a2606	XE424_v1_a1415	XE424_v1_a0462	XE424_v1_b0617	XE424_v1_b1414
CPBF 426	XSP_000491	XSP_001973	XSP_001972	XSP_001326	XSP_000410	XSP_000525	[1]
CPBF 761	XE761_v1_b0564	XE761_v1_b2153	XE761_v1_b2152	XE761_v1_b1437	XE761_v1_b0475	XE761_v1_b0602	[1]
CPBF 766	XE766_v1_a0570	-	-	XE766_v1_b1398	XE766_v1_b0459	XE766_v1_b0604	[1]
CFBP 7622	-	-	-	MIGF01_270014	MIGF01_300018	MIGF01_80115	[1]
CFBP 7653	MIGK01_60160	MIGK01_30158	MIGK01_30157	MIGK01_260023	MIGK01_60023	MIGK01_60196	[1]
BRIP 62409	QEZJ01_150401	-	-	QEZJ01_340018	QEZJ01_150518	QEZJ01_150362	QEZJ01_340017
BRIP 62411	-	-	-	QEZI01_160016	QEZI01_420165	QEZI01_420020	[1]
BRIP 62415	QEZH01_490082	-	-	QEZH01_30015	QEZH01_270036	QEZH01_490118	[1]
BRIP 62418	-	-	-	QEZG01_250061	QEZG01_340025	QEZG01_33023	QEZG01_250062

-, CDS absent from this particular genome.

[1] CDS not annotated in MaGe (Geneious® 9.1.8 confirmed the presence of the coding sequences).

Marker XEA8 was designed for the two partly overlapping CDSs used to design markers XEA4 and XEA7.

Table II. S2. Chromosomal coordinates of the eight *Xanthomonas euroxanthea*-specific DNA markers of 11 *X. euroxanthe*a genomes.

-, DNA marker is absent from this particular genome

***X. euroxanthea* strains**	**Genomic coordinates of DNA markers (bp)**							
	XEA1 (819 bp)	**XEA2 (425 bp)**	**XEA3 (612 bp)**	**XEA4 (341 bp)**	**XEA5 (295 bp)**	**XEA6 (237 bp)**	**XEA7 (212 bp)**	**XEA8 (648 bp)**
CPBF 367	570,244-571,062	2,366,790-2,367,214	2,365,643-2,366,254	1,531,285-1,531,625	468,013-467,719	605,286-605,522	1,531,067-1,530,854[3]	1,530,890-1,531,539
CPBF 424^{T}	611,841-612,659	2,762,517-2,762,093	2,763,664-2,763,053	1,513,728-1,514,068	478,064-477,770	646,704-646,940	1,513,510-1,513,299	1,513,335-1,513,982
CPBF 426	579,870-580,688	2,294,375-2,294,799	2,293,228-2,293,839	1,544,553-1,544,893	476,032-475,738	615,051-615,287	1,544,335-1,544,124	1,544,160-1,544,807
CPBF 761	579,877-580,695	2,264,312-2,264,736	2,263,165-2,263,776	1,514,531-1,514,871	476,039-475,745	615,058-615,294	1,514,313-1,514,102	1,514,138-1,514,785
CPBF 766	605,555-606,373	-	-	1,507,857-1,508,197	471,200-470,906	640,559-640,795	1,507,639-1,507,426	1,507,462-1,508,111
CFBP 7622	-	-	-	16,055-16,395	19,751-19,457	121,664-121,900	15,837-15,624	15,660-16,309
CFBP 7653	175,010-175,828	178,250-178,674	177,103-177,714	28,344-28,004	20,944-20,650	209,888-210,124	28,562-28,775	28,739-28,090
BRIP 62409	444,851-444,042[1]	-	-	15,994-16,334	575,504-575,798	409,763-409,527	15,776-15,562	15,598-16,248
BRIP 62411	-	-	-	17,152-17,492	184,923-185,217	19,788-19,552	16,934-16,721	16,757-17,406
BRIP 62415	84,958-85,776	-	-	16,009-16,349	52,386-52,680	119,886-120,122	15,791-15,578	15,614-16,263
BRIP 62418	-	-	-	67,088-66,748	20,888-20,594	22,667-22,428[2]	67,306-67,517	67,481-66,834

[1] XEA1 in the genome of BRIP 62409 has 810 bp
[2] XEA6 in the genome of BRIP 62418 has 240 bp
[3] XEA7 in the genome of CPBF 367 has 214 bp
[4] XEA7 in the genome of CPBF 766 has

Table II. S3. MaGe labels of four housekeeping genes from 11 *Xanthomonas euroxanthea* genomes used in the construction of an unrooted tree.

***X. euroxanthea* strains**	**Genomic coordinates of housekeeping genes (bp)**			
	***acnB* (513 bp)**	***fyuA* (640 bp)**	***gyrB* (828 bp)**	***rpoD* (793 bp)**
CPBF 367	2,309,810- 2,310,322	3,978,234-3,978,873	6,525-7,352	4,291,063-4,290,271
CPBF 424^T	2,819,809-2,819,297	3,941,493-3,942,132	6,524-7,351	4,277,647-4,276,855
CPBF 426	2,238,318-2,238,830	3,807,436-3808,075	6,523-7,350	4,281,395-4,280,603
CPBF 761	2,208,262-2,208,774	3,777,389-3778,028	6,523-7,350	4,251,279-4,250,487
CPBF 766	2,190,263-2,190,775	3,825,810-3826,449	6,525-7,352	4,200,512-4,199,720
CFBP 7622	18,414-18,926	23,387-22,748	4,421-3,594	18,005-18,797
CFBP 7653	117,059-117,571	23,271-22,632	74,161-74,988	108,162-108,954
BRIP 62409	189,818-190,330	37,662-37,023	87,916-88,743	108,320-109,112
BRIP 62411	27,980-28,492	730,566-731,205	104,086-103,259	103,346-104,138
BRIP 62415	51,710-51,198	38,083-37,444	125,771-126,598	108,315-109,107
BRIP 62418	251,033-250,521	182,168-182,807	197,832-197,005	89,064-88,272

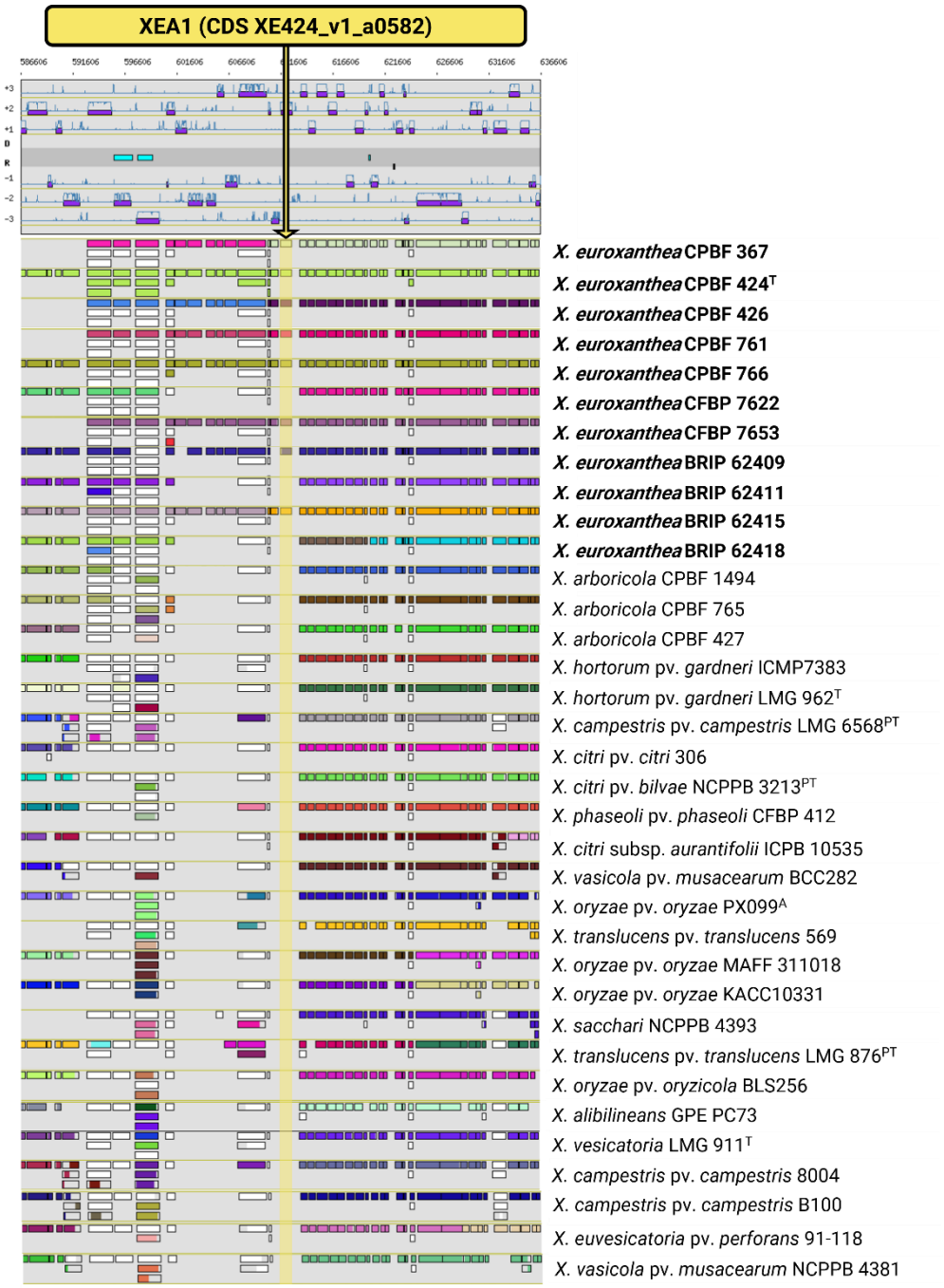


Figure II. S1(a). Synteny map of XEA1 (highlighted in yellow and designed from a conserved protein of unknown function and its flanking regions) across 11 *Xanthomonas euroxanthea* strains (CPBF 367, CPBF 424^{T}, CPBF 426, CPBF 761, CPBF 766, CFBP 7622, CFBP 7653, BRIP 62409, BRIP 62411, BRIP 62415, and BRIP 62418) and 24 other *Xanthomonas* spp. strains.

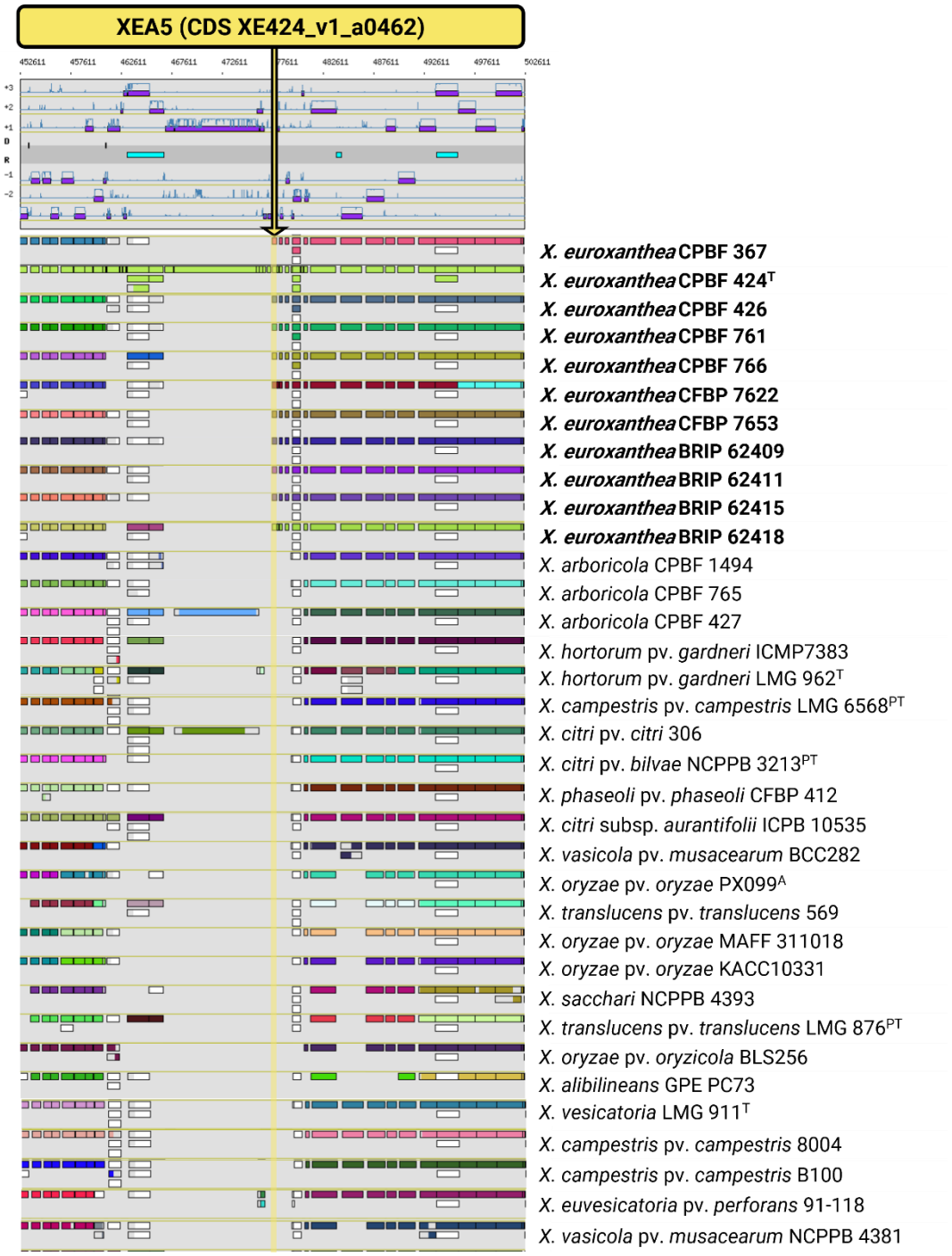


Figure II. S1(b). Synteny map of XEA5 (highlighted in yellow and designed from a MarR family transcriptional regulator) across 11 *Xanthomonas euroxanthea* strains (CPBF 367, CPBF 424^{T}, CPBF 426, CPBF 761, CPBF 766, CFBP 7622, CFBP 7653, BRIP 62409, BRIP 62411, BRIP 62415, and BRIP 62418) and 24 other *Xanthomonas* spp. strains.

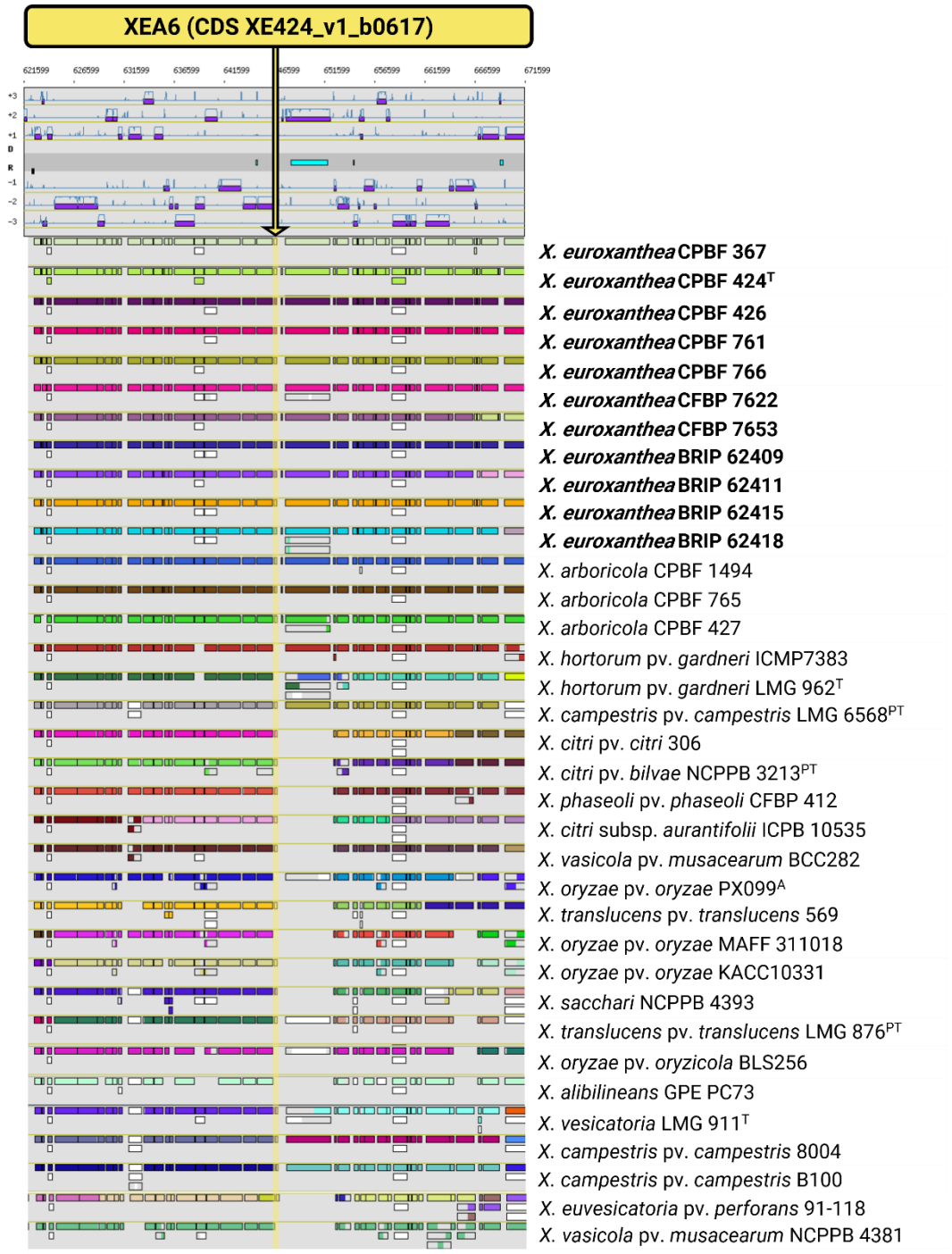


Figure II. S1(c). Synteny map of XEA6 (highlighted in yellow and designed from a conserved protein of unknown function sequence) across 11 *Xanthomonas euroxanthea* strains (CPBF 367, CPBF 424^{T}, CPBF 426, CPBF 761, CPBF 766, CFBP 7622, CFBP 7653, BRIP 62409, BRIP 62411, BRIP 62415, and BRIP 62418) and 24 other *Xanthomonas* spp. strains.

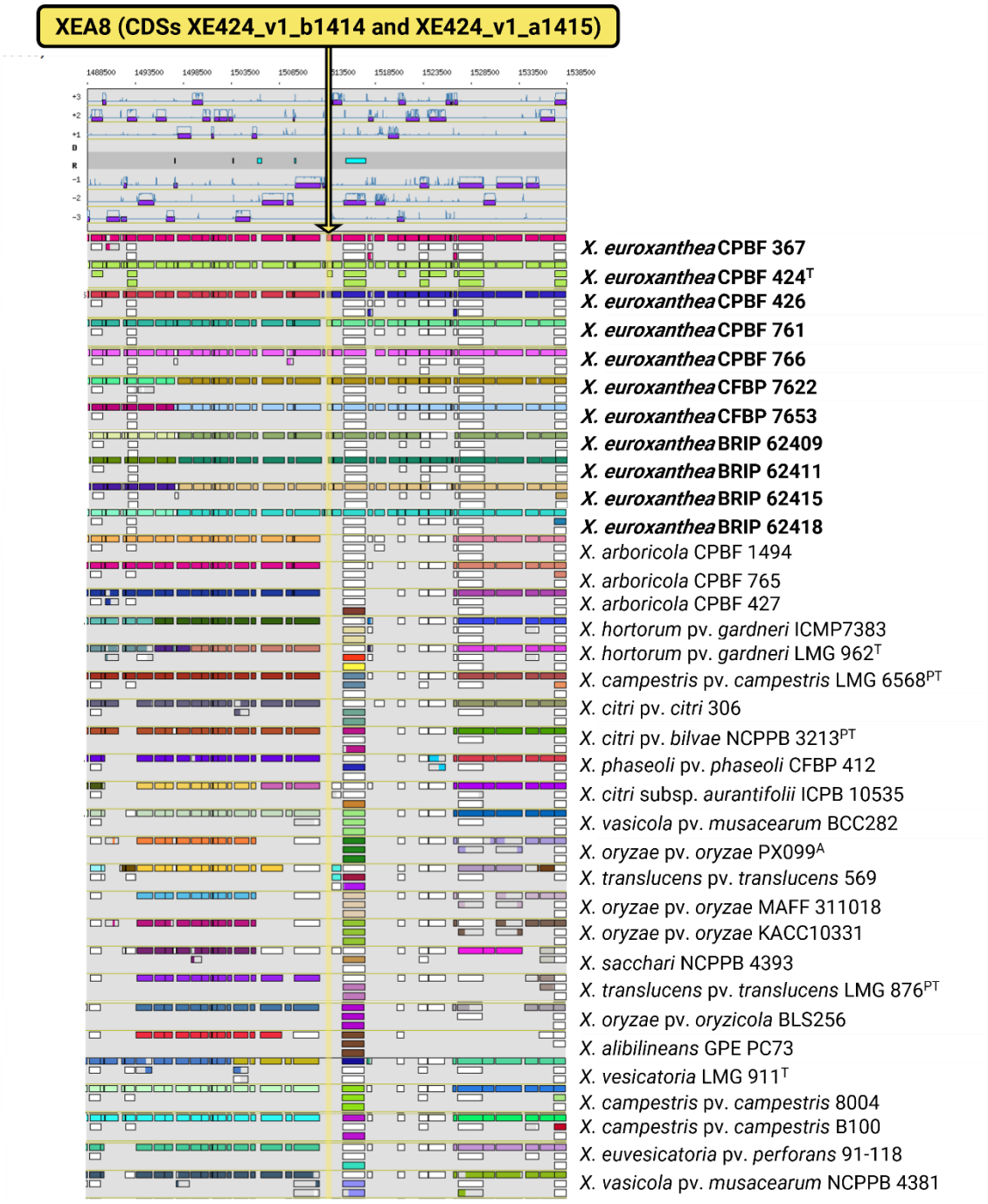


Figure II. S1(d). Synteny map of XEA8 (highlighted in yellow and designed from a conserved protein of unknown function, and a TetR/AcrR family transcriptional regulator sequences) across 11 *Xanthomonas euroxanthea* strains (CPBF 367, CPBF 424^{T}, CPBF 426, CPBF 761, CPBF 766, CFBP 7622, CFBP 7653, BRIP 62409, BRIP 62411, BRIP 62415, and BRIP 62418) and 24 other *Xanthomonas* spp. strains.

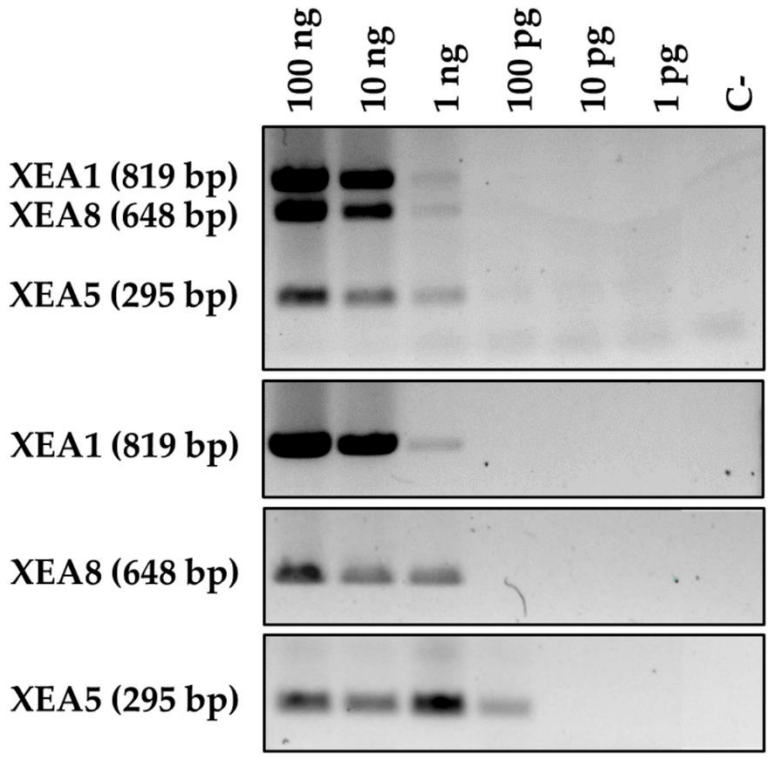
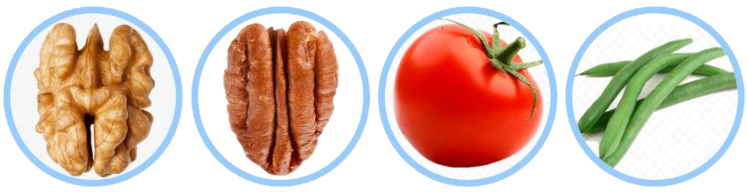


Figure II. S2. PCR detection limits assessed using purified DNA from CPBF 424^{T}. C-: negative control (sterile distilled water).

CHAPTER III

HrcT is a key module of the type III secretion system as seen in *Xanthomonas euroxanthea* mutants



Chapter III: HrcT is a key module of the type III secretion system as seen in *Xanthomonas euroxanthea* mutants

1. Introduction

The *Xanthomonas* genus comprises 34 species with valid and correct names [1], some of which are infamously known to cause worldwide disease to economically interesting plants, such as banana, bean, cabbage, cassava, citrus, pepper, rice, soy, sugarcane, tomato, and wheat [2,3]. Part of what makes these bacterial pathogens so successful in surviving, adapting, and colonizing host and non-host environments are secretion systems [2,3].

A recent study from our research group isolated from the same *Juglans regia* (walnut) three strains, CPBF 367, CPBF 424^T, and CPBF 426, and further classified these as a new species, *Xanthomonas euroxanthea* [4,5]. While no symptoms of the disease were observed upon walnut inoculation with CPBF 367, strain CPBF 424^T was shown to be pathogenic, making this species particularly appealing to address pathoadaptation to walnut [6–8]. Additionally, strain CPBF 766 isolated from *Carya illinoinensis* in Portugal (2016), and CFBP 7622 isolated from *Phaseolus vulgaris* in the USA (1985) were also found to pertain to the *X. euroxanthea* species [9].

X. euroxanthea is very closely related to the infamous *X. arboricola* pv. *juglandis* pathogen. When comparing the genome of strains of these two species (CPBF 367, CPBF 424^T, CPBF 426, and CPBF 427) our group found major differences regarding the type III secretion system (T3SS) and type III effector (T3E) gene repertoire [4]. The T3SS is a well-studied multi-protein machine acknowledged to sponsor bacteria with pathogenicity, in both plant and animal hosts; and it has also been associated with symbiotic interactions between bacteria and plants [3,10]. The structure of the T3SS resembles a protruding needle with a channel through which the T3Es are translocated into host cells and, once there, are free to trigger a host response [3,4,8,10,11].

Studies point out that the T3SS evolved by exaptation from a primal flagellar-like apparatus mandatory for bacterial swimming motility [3]. As these features diversified throughout time, 13 families of non-flagellar T3SS formed [3]. *Xanthomonas* spp. harbors T3SS genes from the Hrp2 family, known to be present in the majority of phytopathogenic bacteria [3,12]. In *Xanthomonas* spp. the production and assembly of this nanomachine come at a high energetic cost, as the T3SS is composed of one to more than a hundred copies of the structural proteins HrcQ, HrcL, HrcN, HrpB2, HrcD, HrcJ, HrcC, HrcV,

HrcR, HrcS, hrcT, HrcU, HrpB7, HrpE, and HrpF, whose genes are clustered in pathogenicity islands (PAIs) usually within the chromosomal DNA [3,13,14]. The *hrp* designation is due to the T3SS genes having been historically named “hypersensitive response and pathogenicity” because of their role in the development of response in resistant plants and disease in host plants [3]. This term if further divided into *hrc*, meaning *hrp*-conserved, and *hpa* meaning *hrp*-associated [3].

HrpG and HrpX are master regulators of the T3SS and T3Es in *Xanthomonas* [12]. HrpG controls the expression of *hrpX*, which consequently controls the expression of T3SS structural genes and effectors [12]. The activation of the HrpG/HrpX regulon occurs *in planta* under strict metabolic and genetic regulation, that is influenced by the bacterial environment and metabolic state [12]. It is the accumulation of these active proteins that majorly controls the expression of the T3SS [12].

The T3Es are proteins that interfere with plant basal immunity or manipulate cellular processes of the host cell to promote optimal physiological conditions for pathogen multiplication in the intercellular spaces of the plant tissue, which inevitably triggers the development of disease symptoms [4,8,15,16]. While T3Es are candidate determinants of host specificity of pathogenic bacteria and thus reflect the adaptation of strains to the walnut tree, some T3Es are host-restricting as they induce an immunity response [8,17]. Fascinatingly, pathogenic strains of *Xanthomonas* spp. encode on average 25–35 T3Es; being that the T3SS can export hundreds to thousands of T3Es per second [12,13].

For both T3SS and T3E proteins, the non-pathogenic *X. euroxanthea* strains CPBF 367, CPBF 426, and even, CFBP 7653 (recently reclassified as *X. euroxanthea* [4]) have a reduced number of these features (*hrpG*, *hrpX*, *hrcN*, x*opAZ,* and *xopR*), when compared to the pathogenic strain CPBF 424^{T} of the same species [4].

Conversely, the pathogenic CPBF 424^{T} and CPBF 766 *X. euroxanthea* strains demonstrated a more complete T3SS-proteins profile (*hrpG, hrpX, hrcN, hrcJ, hrcQ, hrcR, hrcS, hrcT, hrcV, hrpB1, hrcC, and hrcU*), which is similar to the pathogenic *X. arboricola* strain CPBF 427 [4]. Regardless, CPBF 424^{T} strain also included fewer type III effector proteins (*xopAZ, xopR, hpaA, xopM, xopF1, xopA,* and *xopZ2*), similarly to the non-pathogenic *X. arboricola* strains CFBP 7652, CFBP 7651, and CITA14, isolated from *Juglans regia* and *Prunus persica* [4]. Although CPBF 424^{T} has a less complete TE3s arsenal, *in vivo* walnut assays suggest that it performs as pathogenic, though with attenuated virulence, when compared to other strains of *X. arboricola* pv. *juglandis* (LMG 747^{T} and CPBF 1480) [4]. Since *X. euroxanthea* strains CPBF 424^{T} [5] and CPBF 766 [9] are pathogenic in walnut, it would be interesting to assess if, similarly to *X. arboricola*

pv. *julgandis,* the T3SS and T3Es also play a significant role in its ability to cause disease [6].

Therefore, it is the ambition of this study to obtain *X. euroxanthea* mutants deficient in *hrcT*, *hrpG*, and *hrpX* genes to identify the key genetic determinants of pathogenicity and infection by *X. euroxanthea*.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table III.1. Strains were cultivated at 28 °C in PSA (peptone-sucrose agar) medium (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, and 16 g/L agar). The *Escherichia coli* DH10B strain containing the constructs used in this study was grown at 37°C in LB (lysogeny broth) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl).

Electro-competent cells of *Xanthomonas euroxanthea* CPBF 766 and CFBP 7622 strains were prepared as follows. First, a single colony of each *X. euroxanthea* strain was inoculated into 100 mL PSA medium and incubated overnight at 28 °C with 225 rpm shaking. The overnight cultures (with OD_{600nm}s around 0.3–0.6) were equally distributed into two 50 mL sterile Falcon tubes and placed on ice. All subsequent steps were performed on ice. Cell suspensions were centrifuged at 4300 rpm, 4 °C for 10 minutes. The supernatant was discarded, and the cell pellets were carefully resuspended in sterile milliQ water and centrifuged at 4300 rpm, 4 °C for 10 minutes. These washes with sterile milliQ water were repeated two times. Then all the water was discarded, and the cells were resuspended in 50 mL of 15% glycerol and centrifuged at 4300 rpm, 4 °C for 10 minutes. The resulting supernatant was discarded, and the cells were resuspended in the glycerol remaining in the falcon (around 400 µl), then 1 mL of 15% glycerol was added. Finally, 100 µL aliquots of the cells (in Eppendorf tubes) were immediately placed in liquid nitrogen and subsequently stored at -80 °C.

Additionally, to evaluate the viability and natural antibiotic resistance of electro-competent *X. euroxanthea* cells these were, respectively, plated on PSA medium and PSA medium with 20 µg/mL gentamycin (Gm), and kept at 28 °C.

Electro-transformation was used to introduce the plasmids into the electro-competent *X. euroxanthea*. Kanamycin and gentamycin-resistant *X. euroxanthea* mutants were selected upon plating, respectively, on 20 µg/mL kanamycin and 20 µg/mL gentamycin-containing PSA.

Table III.1. Bacterial strains and plasmids used and produced.

Bacterial strains and plasmids	Description	Source
CPBF 424^T	*Xanthomonas euroxanthea* CPBF 424^T wild type	[7]
CPBF 766	*Xanthomonas euroxanthea* CPBF 766 wild type	[9]
CFBP 7622	*Xanthomonas euroxanthea* CFBP 7622 wild type	[9]
DC3000	*Pseudomonas syringae* pv. *tomato* DC3000	[18]
DH10B	*Escherichia coli* DH10B	[19]
DH10B_ pUC57::hrcT	*Escherichia coli* DH10B containing pUC57::hrcT construct	[20]
DH10B_ pUC57::hrpG	*Escherichia coli* DH10B containing pUC57::hrpG construct	[20]
DH10B_ pUC57::hrpX	*Escherichia coli* DH10B containing pUC57::hrpX construct	[20]
pBBR1MCS-5	Broad-host-range vector, Gm^R	[21]
DH10B_ pBBR1MCS-5	*Escherichia coli* DH10B containing pBBR1MCS-5 construct	[20]
766_ pBBR1MCS-5	Strain CPBF 766 containing pBBR1MCS-5 construct (by electroporation with pBBR1MCS-5), Gm^R	This study
7622_ pBBR1MCS-5	Strain CFBP 7622 containing pBBR1MCS-5 construct (by electroporation with pBBR1MCS-5), Gm^R	This study
pUC57::hrcT	pUC57 vector containing a 614 bp internal *hrcT* fragment consensus for *X. euroxanthea* strains	[20]
pUC57::hrpG	pUC57 vector containing a 587 bp internal *hrpG* fragment consensus for *X. euroxanthea* strains	[20]
pUC57::hrpX	pUC57 vector containing a 570 bp internal *hrpX* fragment consensus for *X. euroxanthea* strains	[20]
424ΔhrcT	Strain CPBF 424^T with the *hrcT* gene mutated (by electroporation with pUC57::hrcT), Km^R	[20]
424ΔhrpG	Strain CPBF 424^T with the *hrpG* gene mutated (by electroporation with pUC57::hrpG), Km^R	[20]
424ΔhrpX	Strain CPBF 424^T with the *hrpX* gene mutated (by electroporation with pUC57::hrpX), Km^R	[20]
766ΔhrcT	Strain CPBF 766 with the *hrcT* gene mutated (by electroporation with pUC57::hrcT), Km^R	[20]
7622ΔhrcT	Strain CFBP 7622 with the *hrcT* gene mutated (by electroporation with pUC57::hrcT), Km^R	This study
7622ΔhrpX	Strain CFBP 7622 with the *hrpX* gene mutated (by electroporation with pUC57::hrpX), Km^R	[20]

Gm^R - resistance to gentamicin (Gm)
Km^R - resistance to kanamycin (Km)

2.2. *Xanthomonas euroxanthea hrcT*, *hrpG*, and *hrpX* mutants

Single cross-over knock-out mutants in *X. euroxanthea* were obtained by introducing the construct of the non-replicative plasmid pUC57 containing an internal fragment of the target gene, relying upon the single cross-over event by homologous recombination to disrupt the target gene [20]. The constructs targeting *hrcT* (pUC57::hrcT), *hrpX* (pUC57::hrpX), and *hrpG* (pUC57::hrpG), were already available at the lab [20]. Briefly, the nucleotide sequences for *hrcT* (*X. euroxanthea* CPBF 424^T, CPBF 766, and CFBP 7622), *hrpX* and *hrpG* (*X. euroxanthea* CPBF 367, CPBF 424^T, CPBF 426, CPBF 766, CFBP 7622, and CFBP 7653) were retrieved from *X. euroxanthea* genomes using the Artemis software and aligned using Clustal Omega platform [20]. Consensus sequences with 3-13 mismatches, depending on the gene, were built and the corresponding DNA fragments were synthesized and cloned into pUC57-Kan by the company GenScript [20]. The pBBR1-MCS5 plasmid resistant to gentamycin was used as the positive control. The constructs were recovered from *E. coli* DH10b strain with Monarch® Plasmid DNA Miniprep Kit.

The integrity and correct size of the plasmid were evaluated in 0.8% agarose gel electrophoresis with uncut and enzyme-cut plasmids, respectively. Constructs using pUC57 were digested with EcoRI and pBBR1-MCS5 with AgeI, at 37 °C for 4 h, with enzyme inactivation at, respectively, 65 °C and 80 °C. 10 µL of the digestion mix and 2 µL of the plasmid were separated in a 0.8% agarose gel (1 × TAE buffer), and results were visualized using the Xpert Green DNA stain (Grisp, Porto, Portugal) with a Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA). These restriction enzymes were selected as they cut the plasmid at a single specific site (as verified by the tool available at http://heimanlab.com/cut2.html).

Cells of *X. euroxanthea* were transformed with the appropriate plasmid DNA by electroporation to attain mutants 766ΔhrpG, 766ΔhrpX, 7622ΔhrcT, and 7622ΔhrpG (Table III.2). CPBF 766 and CFBP 7622 were electroporated with pBBR1MCS-5 to obtain the positive controls 766_pBBR1MCS-5 and 7622_pBBR1MCS-5. Electroporation was carried out with 50 µL and 100 µL of electro-competent cells and 1, 1.5, and 3 µL of plasmid DNA (corresponding to a concentration between 26.6–100 ng/µL). Electroporation was carried out in a 0.1 cm ice-cold Gene Pulser®/MicroPulser™ Cuvette (Bio-Rad) on a MicroPulser™ Electroporator (Bio-Rad). The electric field strength tested was 1.8 and 2.8 kV/cm, and the pulse was 1.2, 2.7, and 4 ms. Plasmid-free cells were used as the negative control, and cells transformed with the pBBR1-MCS5 were used as the positive control. After the pulse, 900 µL of liquid PSA medium at 28 °C was added to the electroporation cuvette for cell resuspension, and 1 mL of

these contents were transferred to 1.5 mL and 2 mL sterile Eppendorf tubes. These electroporated cells were incubated at 28 °C for 1–5 hours. Following an incubation period at 250 rpm, 28 °C for 1–5 hours, 50 µL of the transformation mixture was directly spread on the PSA medium containing the appropriate antibiotic at 20 µg/mL. The remaining transformation mixture was concentrated by centrifugation at 4000 rpm for 5 minutes, and supernatant resuspension in a smaller volume, which was spread on a second PSA plate with the antibiotic. The plates were incubated at 28 °C until colonies were obtained.

Table III.2. Mutants obtained and in-progress for the T3SS genes *hrcT*, *hrpG*, and *hrpX* of *Xanthomonas euroxanthea* strains CPBF 424^T, CPBF 766, and CFBP 7622.

Strain	hrcT in WT	ΔhrcT mutant	hrpG in WT	ΔhrpG mutant	hrpX in WT	ΔhrpX mutant
CPBF 424^T	🧬	🟢	🧬	🟢	🧬	🟢
CPBF 766	🧬	🟢	🧬	🚧	🧬	🚧
CFBP 7622	🧬	✓	🧬	🚧	🧬	🟢

🧬: gene present in the genome of the respective WT (wild-type) strain
🟢: mutant constructed in a previous study [20]
✓: mutant constructed in this study
🚧: mutant under construction

The candidates recovered from the electroporation were screened by colony PCR to confirm the mutation status, i.e. both the insertion and orientation of the construct. A sterile micropipette tip was used to transfer a few cells from each colony to 100 µL of sterile distilled water (SDW), followed by cell lysis by incubation at 100 °C for 10 minutes. After, centrifugation at 14500 rpm for 5 minutes, 10 µL of the supernatant was added to the PCR reaction mix consisting of 1 × DreamTaq Buffer (ThermoFisher Scientific, Waltham, MA, USA), 0.2 mM of each deoxynucleotide triphosphate (dNTP) (Grisp, Porto, Portugal), 0.2 mM of each forward and reverse primers (Table III.3), 1 U of DreamTaq DNA Polymerase (ThermoFisher Scientific, Waltham, MA, USA). The primer pair, pUC57_Kan1Fw, and pUC57_Kan1Rv, was previously designed to validate the presence of the construct; whilst the primer pairs (hrcT_5prime/ hrpG_5prime/ hrpX_5 prime and M13F; hrcT_3prime/ hrpG_3prime/ hrpX_3prime and M13R) were designed to validate the orientation of the construct (Table III.3). Sterile distilled water was used as the negative control. PCR cycling parameters consisted of a first amplification step of 3 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, as well as a final DNA extension at 72 °C for 5 min. PCR products were separated by electrophoresis on a 0.8% agarose gel (1 × TAE buffer) stained with

Xpert Green DNA stain (Grisp, Porto, Portugal) and visualized with a Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA).

Table III.3. Primers for monitoring the presence and orientation of pUC57::hrcT, pUC57::hrpG, and pUC57::hrpX constructs

Primer name	Nucleotide sequence (5'→3')	Expected amplicon size (bp)
pBBR1MCS5_Rep_Fwd	ATGGCCACGCAGTCCAGAGAAATC	663
pBBR1MCS5_Rep_Rev	CTACCGGCGCGGCAGCGTG	
pUC57_Kan_fwd-1	ATGCCTCTTCCGACCATCAA	429
pUC57_Kan_rev-1	TCCGACTCGTCCAACATCAA	
hrcT_5prime	TCGTGATGTTCTTCGTGCTGC	772
hrcT_3prime	GAT-GTT-GGC-GGC-ATC-GTG	712
hrpG_5prime	AAGGATCGGCTTTCCTGTTG	858
hrpG_3prime	GCGAATACACGGTTCTGATGC	715
hrpX_5prime	GGTCTGCAACATCTTCAACAA	1420
hrpX_3prime	GCATCGAAACCGAAGGTG	669
M13Fshort	CGCCAGGGTTTTCCCAGTC	
M13R	AGCGGATAACAATTTCACACAGGA	

2.3. Plant material and hypersensitive response assays

Tobacco plants (*Nicotiana tabacum*) were grown in growth chambers for 6 weeks with cycles of 16 hours of light per day at 23 ºC and 50% relative humidity. Bacterial suspensions at an OD_{600} of 0.4, corresponding to 3.2 x 10^8 CFU/mL, were infiltrated into the lower epidermis of the tobacco leaves using a needleless syringe, and symptom development was assessed four days after. Three replicates were done for each leaf situation. SDW was used as negative control; *Pseudomonas syringae* pv. *tomato* DC3000 and CFBP 7622 strains were used as positive controls of hypersensitive response.

2.4. Growth assessment of wild-type and mutant *Xanthomonas euroxanthea*

The growth curves of mutants obtained in the current (candidates E and G of 7622ΔhrcT) and a previous study (424ΔhrcT, 424ΔhrpG, 424ΔhrpX, 766ΔhrcT, and 7622ΔhrpX) were carried out to verify if the mutants show the same growth fitness as their corresponding wild-type strains or if the depletion of these T3SS components interferes with their growth in standard culture conditions. A 96-well microplate was filled with 200 µL of the pre-cultures, previously adjusted to 10^6 CFU/mL, and incubated at 28

°C, under shaking, for 2 days. Bacterial growth was monitored by reading optical density values at 600 nm utilizing a Thermo Scientific™ Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific) at 1 h intervals. Each well was replicated three times, and the overall assay was repeated once. For data analysis, the average of each three replicates was calculated, and standard deviation error-bars were added.

3. Results and Discussion

3.1. Colony PCR confirms *Xanthomonas euroxanthea hrcT* mutants

Electrophoresis was performed to learn the integrity and correct size of the plasmid. Results show that the uncut plasmids pUC57::hrcT, pUC57::hrpG, pUC57::hrpX, and pBBR1MCS-5 have three plasmid conformations – nicked open-circular, relaxed circular, and supercoiled, being that the band corresponding to the latter is more intense in the gel indicating that this conformation is the most represented within working samples, meaning that these plasmids are intact and ready for transformation into electro-competent cells of *X. euroxanthea* (Figure III.1) [22].

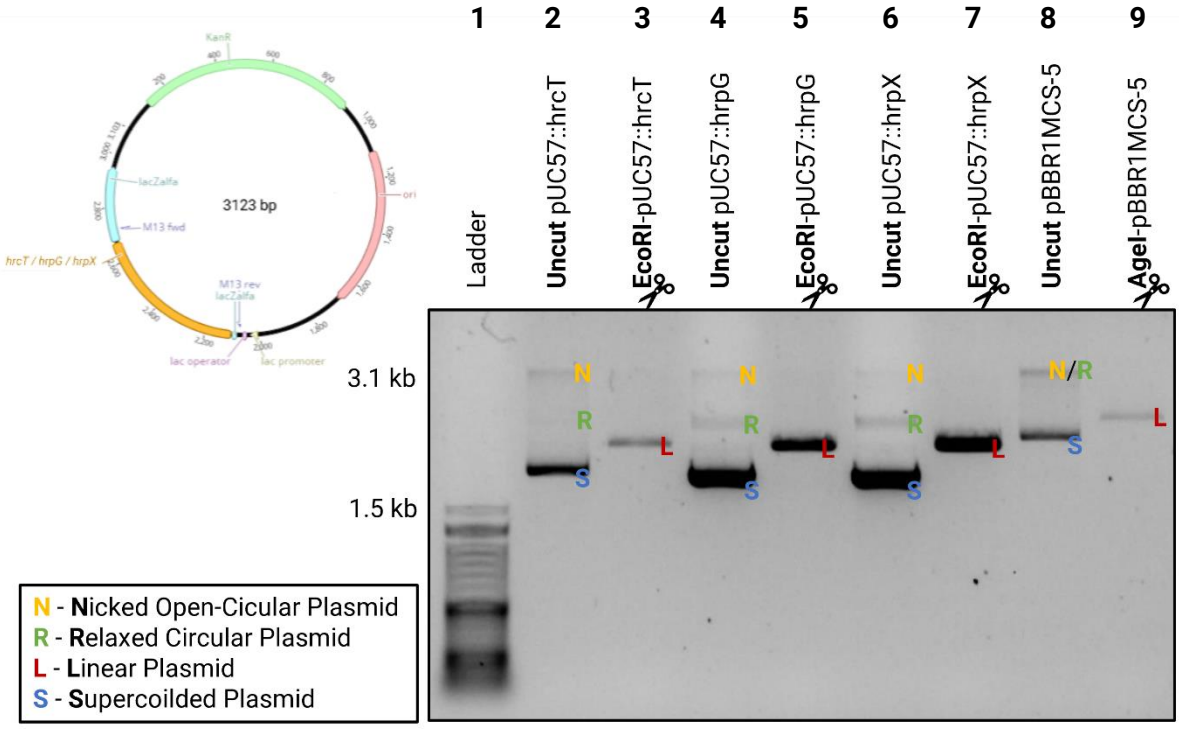


Figure III.1. Agarose gel 0.8% electrophoresis of plasmids pUC57::hrcT, pUC57::hrpG, pUC57::hrpX before and after digestion with EcoRI; and pBBR1MCS-5 before and after digestion with AgeI. Lane 1: NZYDNA Ladder VI (NZYTech, Lisbon, Portugal). Lanes 2, 4, and 6 show that plasmids pUC57::hrcT, pUC57::hrpG, and pUC57::hrpX have three conformations nicked open-circular, relaxed circular, and supercoiled, being that the latter is the most representative and ideal for bacteria transformation assays. In lane 8, plasmid pBBR1MCS-5 formed two bands in the gel, and thus, has 2 conformations – the more abundant supercoiled, and a nicked open-circular or relaxed circular.

X. euroxanthea electro-competent cells grew on PSA plates free of antibiotics (Table III. S1), indicating that the cells are still culturable and viable, and so that absence of colonies (in other plates) is not due to the inability of cells in dividing. No growth was observed in the PSA plates with 20 µg/mL gentamycin inoculated with *X. euroxanthea* electro-competent cells (Figure III. S1), hinting that these are not naturally gentamycin-

resistant; hence, positive control growth corresponds to bacteria that have successfully acquired the plasmid (of gentamycin-resistance) by electroporation.

Several parameters of the electroporation protocol were altered to attain strains CPBF 766 and CFBP 7622 of *X. euroxanthea* deficient in *hrcT*, *hrpG*, and *hrpX* genes (see Table III. 2). Finally, putative 7622ΔhrcT mutant colonies A, C, D, E, F, G, H, and I; and a putative 7622ΔhrpG mutant colony B were detected.

When constructs pUC57::hrcT, pUC57::hrpG, and pUC57::hrpX recombine with the bacterial chromosome, they disrupt the target T3SS gene and introduce the kanamycin-resistance gene. Colonies E and G were confirmed as 7622ΔhrcT mutants, as an amplicon of 500 bp was obtained from a PCR using primers targeting the kanamycin-resistance gene, pUC57_Kan_fwd-1 and pUC57_Kan_rev-1 (Figure III.2a). For candidates A, B, C, D, F, H, and I a 500 bp amplicon was not observed, hinting that these are not mutants (Figure III.2a).

The primers hrcT_5prime and M13Fshort target 772 nucleotide bases where the chromosomal DNA fuses with the plasmid DNA, as a result of the disruption of the *hrcT* gene by plasmid cross-over recombination. In a PCR with the mentioned primers, candidate E formed an amplicon of approximately 800 bp, therefore tested positive as a 7622ΔhrcT mutant (Figure III.2b).

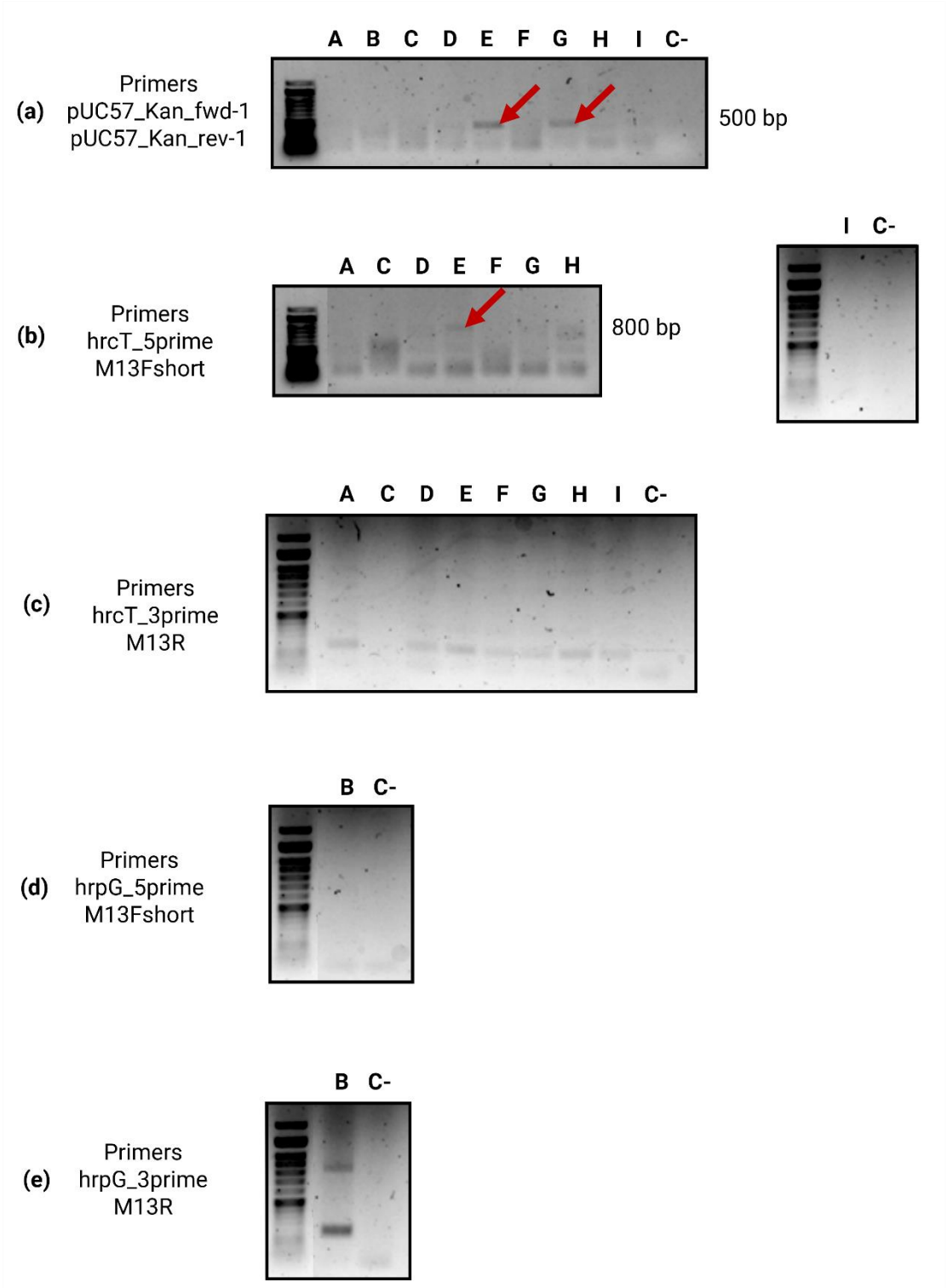


Figure III.2. Electrophoresis gel of PCR using primers targeting (a) the kanamycin resistance gene; the 5' chromosomal-plasmid DNA fusion site of *hrcT* mutants (b); the 3' chromosomal-plasmid DNA fusion site of *hrcT* mutants (c); the 5' chromosomal-plasmid DNA fusion site of *hrpG* mutants (d); and the 3' chromosomal-plasmid DNA fusion site of *hrpG* mutants (e). Clones E and G tested positive as putative 7622ΔhrcT mutants due to specific amplicons indicated with an arrow.

3.2. *Xanthomonas euroxanthea* CFBP 7622 strain without functional *hrcT* is unable to cause a hypersensitive response in tobacco

The results showed that 96 h post-inoculation clones E and G of the 7622ΔhrcT mutant were unable to induce an HR in tobacco when compared to the CFBP 7622 wild-type strain that induced HR, as seen by the necrosis symptoms (Figure III.3); which suggests that the mutants were successfully obtained.

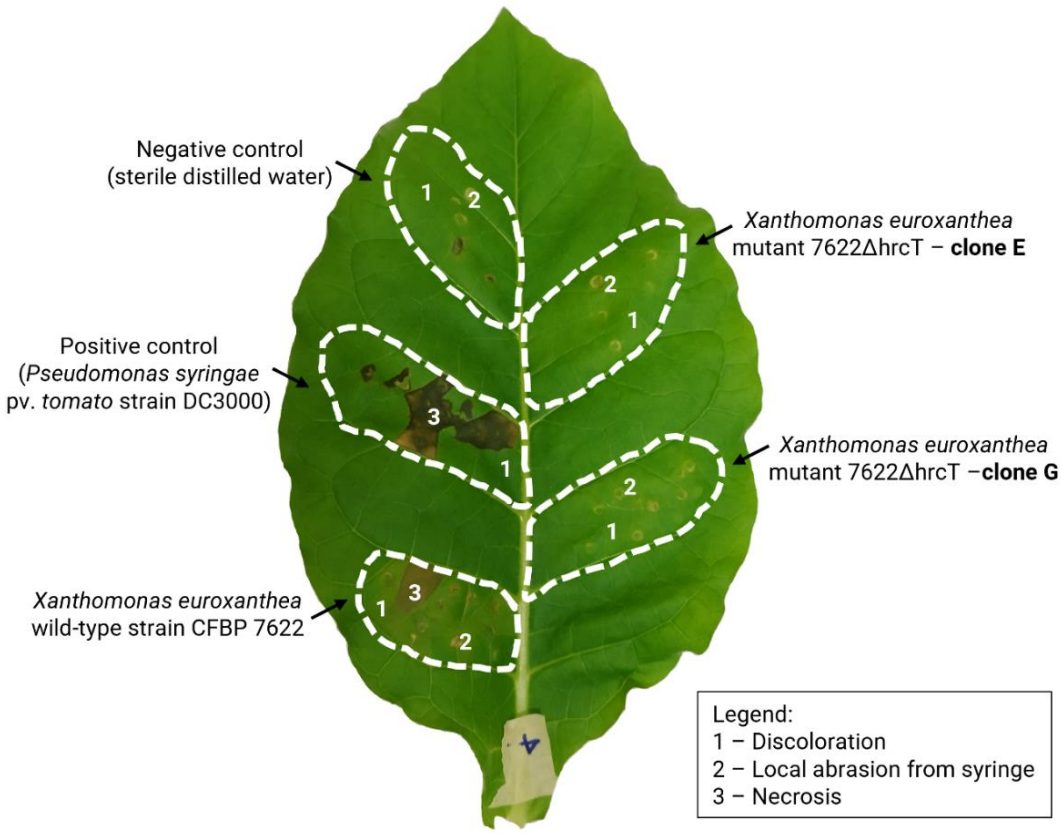


Figure III.3. Tobacco leaf 96 h after infiltration with SDW (negative control), the *Pseudomonas syringae* pv. *tomato* DC3000 (positive control), *Xanthomonas euroxanthea* CFBP 7622 wild-type, and two clones, E and G, for 7622ΔhrcT. No hypersensitive response symptomology was observed for the 7622ΔhrcT clones "E" and "G", when compared to the wild-type CFBP 7622 strain that caused visible necrosis of the leaf.

Effector-Triggered Immunity is an immune response from plants in which plant resistance proteins recognize pathogen effector molecules, which amidst other consequences, triggers HR characterized by a local plant cell death that stops the progressive spreading and multiplication of bacteria [3,8]. The HrcT is a structural protein of the T3SS, that is characterized by a rigorous assembly hierarchy in which the HrcT protein is an early intervenient; attending that these *X. euroxanthea* mutants are missing *hrcT*, it is possible that the assembly of the T3SS may not occur or if it does the T3SS may have its secretion role impaired [13,23,24]. Hence, It can be hypothesized that

clones E and G missing the HrcT are unable to cause HR in tobacco because the secretion function of its T3SS was abolished and, consequently, no T3Es were translocated into the plant cells, where these did not interact with plant defenses, and subsequently did not trigger HR [12]. These results suggest that HrcT is a crucial structural component of the T3SS and, consequently, key for the translocation of T3Es into the cells of *X. euroxanthea* hosts (thus triggering infection) [12,23]. This has been reported in other studies where *hrcT* mutants of the pathogen *Xanthomonas campestris* pv. *vesicatoria* had the secretion of several T3Es abolished [23], and where *X. oryzae* pv. *oryzicola* was unable to secret the T3E AvrXa27 [24].

3.3. *Xanthomonas euroxanthea hrcT*, *hrpG*, and *hrpX* deficient mutants get a head start in bacterial proliferation

To assess the physiological fitness of the *Xanthomonas euroxanthea* mutants in comparison to their wild-type counterparts, in standard culture conditions, the growth of these mutants was evaluated during 40 h (as similarly done in another study [25]).

In general, the typical sigmoidal growth curve was obtained, where the lag, exponential, and stationary phases are observed (Figure III.4) [26–28]. Interestingly, results show that all mutant variants entered the logarithmic phase earlier when compared to wild-type strains (Figure III.4). These results indicate that the depletion of the *hrcT*, *hrpG*, and *hrpX* genes did not impair bacterial growth; on the contrary, this suggests that these mutants may benefit from a quicker adaptation to the culture conditions or environment when compared to the wild-type strains.

The ΔhrcT mutants share the same growth kinetics as ΔhrpG and ΔhrpX mutants, i.e. the early entering of the exponential phase. This study suggests, due to the non-existence of HR response, that the absence of HrcT impairs the assembly of the T3SS and the successful translocation of T3Es [29,30]. Likewise, a previously conducted study [20] shows that ΔhrpG and ΔhrpX mutants are not able to trigger HR in tobacco; thus that the inexistence of HrpG and HrpX, as master transcriptional regulators of the T3SS, results in the non-expression of structural and effector genes of the T3SS [12]. With this in mind, the present growth curve assays suggest that *X. euroxanthea* bacteria mutated in the studied T3SS genes have the expression of the T3SS hindered, resulting in an early entering of the exponential phase.

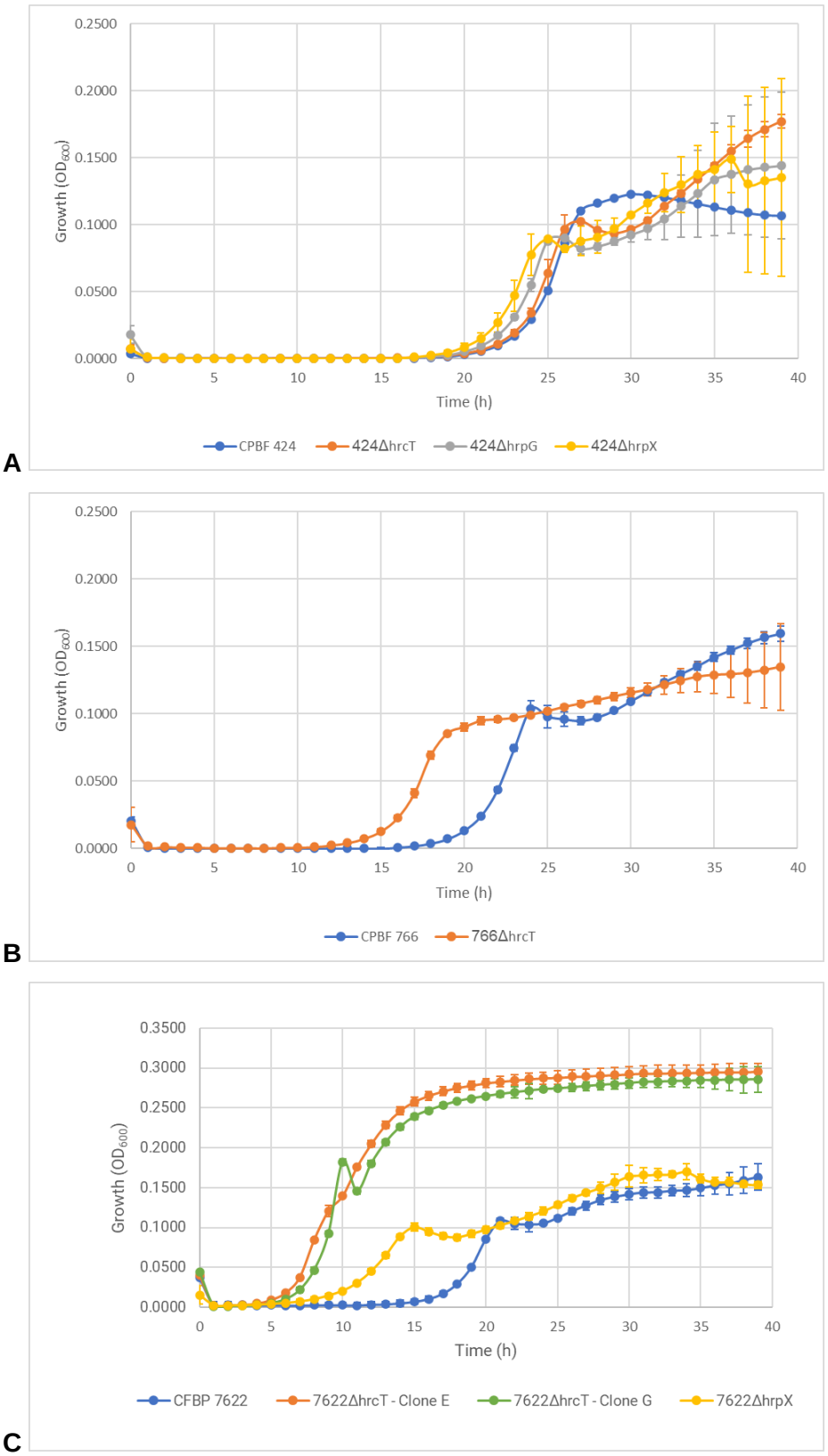


Figure III.4. Optical density (600_{nm})-incubation time plot for the growth of initial inoculum of 10^6 CFU/mL of *Xanthomonas euroxanthea* wild-type and T3SS-mutants at 28 °C. Optical density at 600 nm was read by a Thermo Scientific™ Multiskan™ FC Microplate Photometer at 1 h intervals. The points in the graph correspond to the average of three experimental replicas of optical density readings at 600 nm. **A.** Growth curve of CPBF 424^T, 424ΔhrcT, 424ΔhrpG, and 424ΔhrpX. **B.** Growth curve of CPBF 766 and 766ΔhrcT. **C.** Growth curve of CFBP 7622, 7622ΔhrcT candidates E and G, and 7622ΔhrpX. *X. euroxanthea* wild-type strains show a growth delay in comparison to strains mutated in the *hrcT*, *hrpX*, and *hrpG* genes.

A tendency for previously pathogenic *Xanthomonas* to undergo T3SS and T3Es loss events has been reported [31]. Indeed, this is a paradigm theorized for *X. euroxanthea*, thought to be initially pathogenic, though some of its strains lost their pathogenic ability by genomic degeneracy of several T3SS and T3Es genes [4]. In fact, it may be hypothesized that by giving up energetically costly machinery such as the T3SS, *X. euroxanthea* can re-allocate where to invest the energy, otherwise directed to the regulation, maintenance, and assembly of the T3SS, and production and translocation of T3Es [13,32]. This hypothesis may explain why T3SS-deficient strains enter the exponential growth phase earlier and thus may colonize plant surfaces in initially bigger numbers than T3SS-carrying wild-type strains; then as these latter strains rise in number they also provide the tools for infection and proceed to protrude inside the host-plant, and, once inside, deliver molecules that favor bacterial growth or survival [14]. This further suggests that in hosts where T3SS-positive and T3SS-negative *X. euroxanthea* coexist, strains of *X. euroxanthea* T3SS-negative are not necessarily less fit because of bacterial cheating [14]. Indeed, non-pathogenic strains of *Pseudomonas aeruginosa* have been found to do the same - the presence of T3SS-negative strains is a reoccurrence in the population where T3SS-positive strains exist, being that the earlier ones have been found to take advantage of the T3SS existing without having to support its energy costs themselves [13,14].

4. Conclusions

In this study is provided the first evidence for two clones of *Xanthomonas euroxanthea* CFBP 7622 deficient in the *hrcT* gene (7622ΔhrcT).

These preliminary findings suggest that HrcT acts as a key module of the T3SS of *Xanthomonas euroxanthea*, and is crucial for the assembly of the T3SS machinery and secretion of T3Es into plant cells.

X. euroxanthea whether depleted of HrcT or HrpG or HrpX may enter the exponential growth phase earlier likely due to a faster metabolic adaptation to the environment; when compared to wild-type strains.

5. References

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6. Supplementary material

Table III. S1. Culturability and gentamycin resistance assessment of electro-competent *Xanthomonas euroxanthea* CPBF 766 and CFBP 7622 strains.

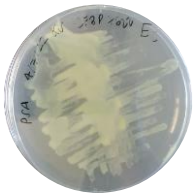
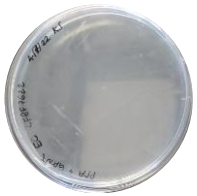
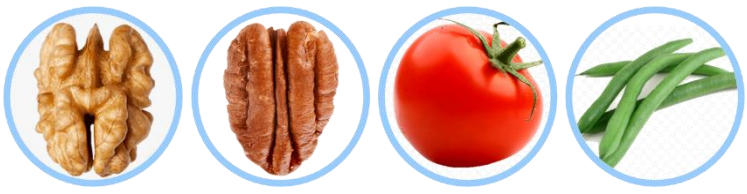
Strain	PSA medium	PSA medium with 20 µg/mL gentamycin
Electro-competent CPBF 766 cells	Not applicable	No growth 
Electro-competent CFBP 7622 cells	Growth 	No growth 
Overall conclusion	Culturable electro-competent cells	No natural gentamycin resistance of electro-competent cells

Table III. S2. Tested conditions in the electroporation protocol of *Xanthomonas euroxanthea***.**

Electrocompetent cells (µL)	Plasmid (µL)	Voltage (kV)	Pulse (ms)	Temperature of the 1 mL PSA medium	Incubation time, temperature, shaking and container	PSA medium + antibiotic plate (°C)	Candidate mutants
100	3	1.8	1.2	Room temperature	2 h, 28 °C, 250 rpm, 1.5 mL Eppendorf	28 °C	No growth
100	3	1.8	1.2	Room temperature	2 h, 28 °C, 250 rpm, 1.5 mL Eppendorf	28 °C	Growth
100	1.5	1.8	1.2	Room temperature	2 h, 28 °C, 250 rpm, 1.5 mL Eppendorf	28 °C	No growth
100	1	1.8	1.2	Room temperature	2 h, 28 °C, 250 rpm, 1.5 mL Eppendorf	28 °C	No growth
100	1	2.8	2.7	28 °C	4 h, 28 °C, 250 rpm, 1.5 mL Eppendorf	28 °C	No growth
50	1	2.8	4	28 °C	5 h, 30 °C, 250 rpm, 15 mL Falcon	32 °C	No growth
50	1	1.8	4	28 °C	1 h, 28 °C, 0 rpm, 2 mL Eppendorf	28 °C	No growth
100	1	1.8	4	28 °C	1 h, 28 °C, 250 rpm, 2 mL Eppendorf	28 °C	No growth
100	1	1.8	4	28 °C	1 h, 28 °C, 250 rpm, 2 mL Eppendorf	28 °C	No growth
100	1	1.8	4	28 °C	1 h, 28 °C, 250 rpm, 2 mL Eppendorf	28 °C	Growth

CHAPTER IV

General discussion



Chapter IV: General discussion

1. Discussion

Xanthomonas euroxanthea surely raises some interesting discussions within the scientific world – from the “how”s of pathogenesis (as it includes pathogenic and non-pathogenic strains, pushing it as an interesting model to study pathogenicity evolution in *Xanthomonas*) to the role of non-pathogenic strains within an infection consortium [1,2]. Certainly, the interest in studying this species is also very much attributed to the threat to food security and economic losses that it represents [3—6]. Bacterial disease problems are extremely important when trying to maintain food quality and quantity available for the foreseeable future [6]. By managing plantations and the related pests and diseases, adequate decisions and actions can be put in place if the species of concern is detected early [6]. This work aimed at filling in the gaps between food safety regulations and hands-on management; by proposing an inexpensive, specific, and applicable method for the detection of *Xanthomoas euroxanthea*, a recently described species [7]. Moreover, this dissertation intended to attain, validate and assess the fitness of *X. euroxanthea* mutants in genes of the T3SS - *hrcT*, *hrpG*, and *hrpX*; to understand the role of the T3SS genes in the pathogenicity of this species.

This study developed and validated eight specific, efficient and reliable DNA markers for the detection and identification of *X. euroxanthea* isolates. The allelic variation of some of these markers allowed conciliating the detection and genotyping of *X. euroxanthea* strains, which could contribute to the survey of these bacteria in ecological niches colonized by the closely related *X. arboricola*. After the publication of the scientific article, that corresponds to Chapter II [7], the genomes of seven strains deposited in the NCBI database, and confirmed by us as *X. euroxanthea* (data not shown) were scanned for the presence of XEA markers by BLAST analysis using Geneious; from which Figure IV.1 ensued. These results indicate that there are five *X. euroxanthea*-universal markers, XEA4–XEA8, which corroborates the specificity of the XEA markers (Figure IV.1). These findings also unveiled DNA marker pattern D, corresponding to the presence in the genome of DNA markers XEA2–XEA8 (Figure IV.1). While doing this analysis it was verified that *Physalis peruviana*, the ground berry, is also a possible plant host of *X. euroxanthea*.

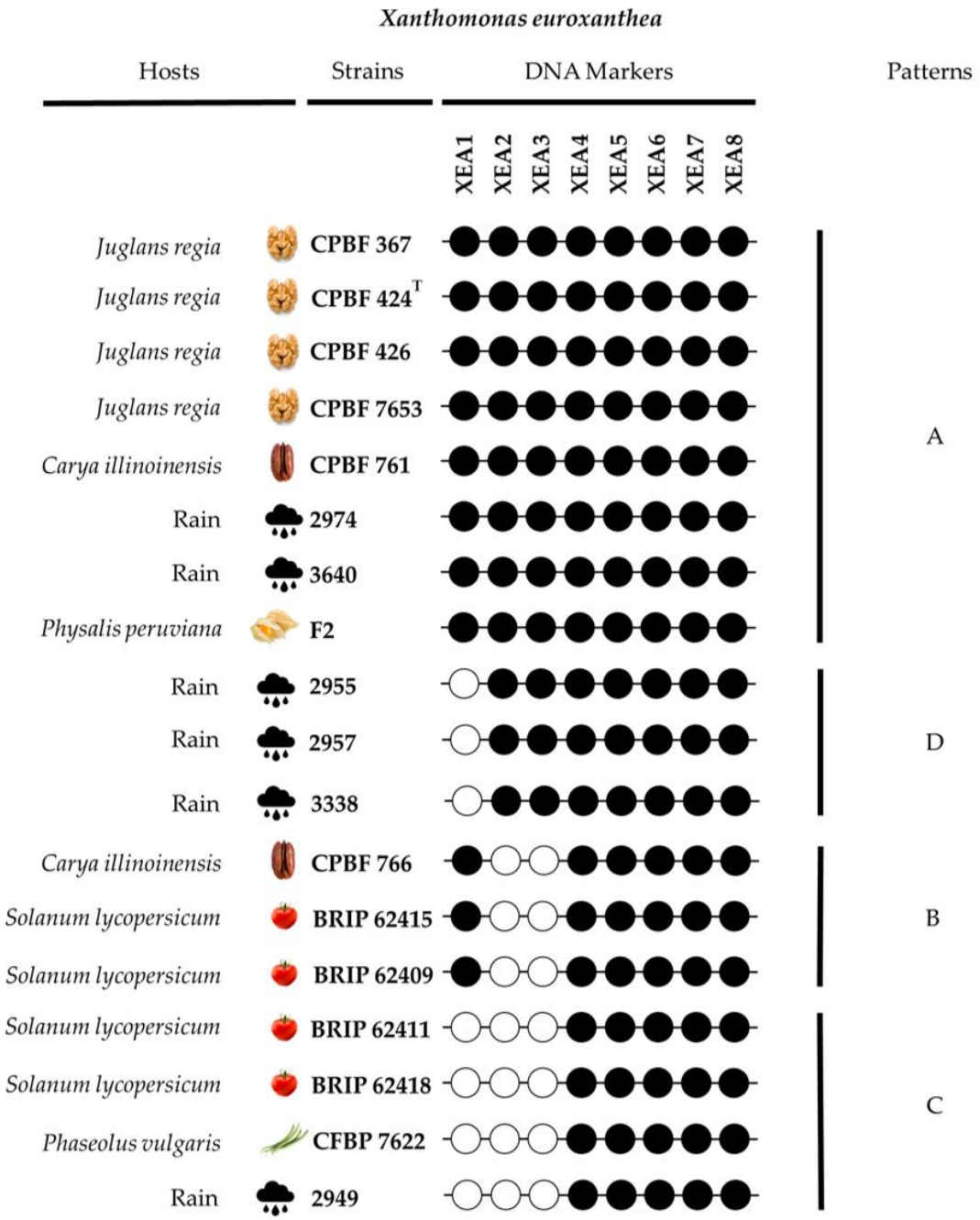


Figure IV.1. Distribution of eight *Xanthomonas euroxanthea* species-specific DNA markers (XEA1, XEA2, XEA3, XEA4, XEA5, XEA6, XEA7, and XEA8) in 18 *X. euroxanthea* genomes. The presence and absence of the eight DNA markers were assessed by BLASTn analysis in Geneious, allowing the disclosure of four patterns, A, B, C, and D, that do not translate strain-host affinities.

In Chapter II, a pattern of plant-host-adaptive-erosion was suggested as both strains with a more complete DNA marker pattern and with a less complete one (likely due to genome erosion) were isolated from the same host species, namely, *Carya illinoinensis* (patterns A and B) and *Solanum lycopersicum* (patterns B and C) [8,9]. Here it can be proposed that strains with pattern D diverged from the strains with pattern A, at some point, attested by the erosion of marker XEA1 (Figure IV.2). Evidence suggests

that strains with pattern D may have been isolated from a fourth different plant host, as a new marker pattern befalls host transition, as seen in patterns B and C.

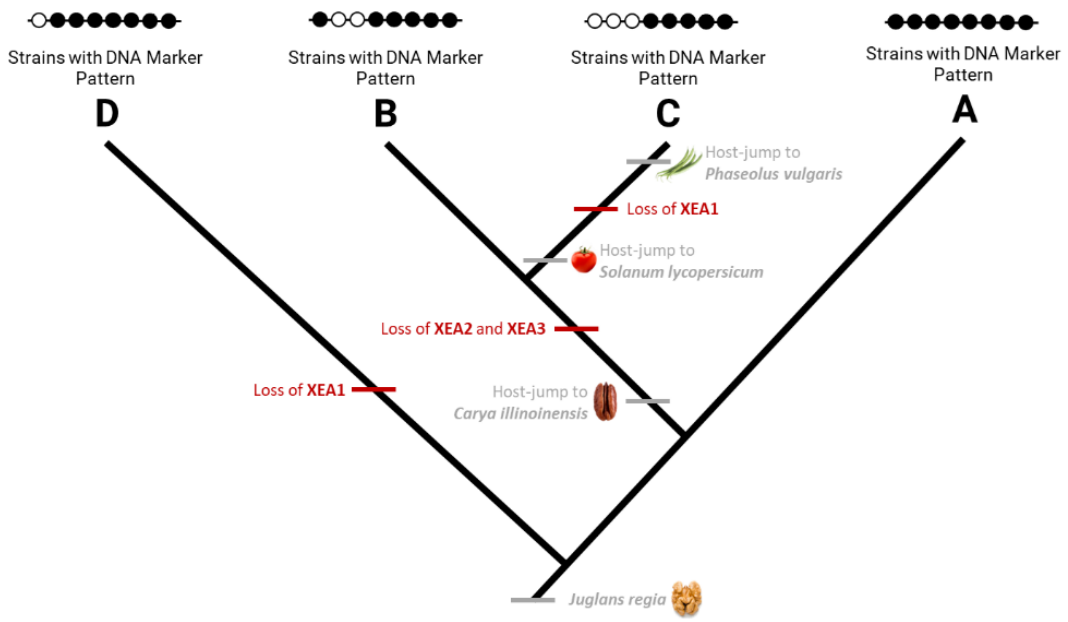


Figure IV.2. Cladogram depicting the evolutionary trend of DNA markers loss by *Xanthomonas euroxanthea* strains as part of the adaptation that follows new host colonization, from walnut to pecan, to tomato, and finally to the common bean. Four DNA marker patterns are identified – pattern A (XEA1–XEA8, the more ancestral pattern), pattern B (XEA1, XEA4–XEA8), pattern C (XEA4–XEA8), and pattern D (XEA2–XEA8).

Chapter III reveals that pattern A (XEA1—XEA8) is present in both the walnut-pathogenic strain CPBF 424^{T} and the walnut-non-pathogenic strain CPBF 367, dismissing these XEA patterns as hints to ascertain the phenotype of *X. euroxanthea* strains. These pathogenicity issues are far from being understood, and may hardly be tackled solely from a genomics perspective. Understanding pathoadaptation and pathogenesis demands functional studies capable of clarifying the role played by genetic determinants of pathogenicity and virulence [10]. Unavoidably, T3SS and T3Es are acknowledged as key players in this process [11]. In this regard, in chapter III, mutants in genes of the T3SS were constructed. The T3SS is a molecular structure present in Gram-negative phytopathogenic bacteria that allows the delivery of T3Es into plant cells, where these promote pathogenicity in susceptible host plants or trigger the hypersensitive response in resistant or non-host plants [12,13]. This study suggests that the depletion of the HrcT, HrpG, and HrpX is correlated to bacteria having a faster metabolic adaptation to the environment. This finding allowed us to rethink the way we perceived this consortium of pathogenic and non-pathogenic *X. euroxanthea* strains. One may hypothesize that these strains with different phenotypes are under co-infection, that is, joint efforts towards plant-surface colonization and within-plant bacterial multiplication [14,15]; or rather that bacteria deficient in T3SS are cheating and finding

refuge and nutrients inside the plant by taking advantage of the T3SS-carrying strains colonizing the same host, but without actually having to endure its energetic costs [16,17].

2. Final remarks and future perspectives

This work “The two-faced *Xanthomonas euroxanthea*: from DNA markers to genomic determinants of pathogenicity” provides useful tools to identify and genotype both pathogenic and non-pathogenic strains of *Xanthomonas euroxanthea*, i.e. the XEA DNA markers; and two clones of the *X. euroxanthea* CFBP 7622 strain mutated in the *hrcT* gene.

The herein presented PCR assay, in the future, can be validated in laboratories all over the globe, thus testifying to its accuracy and robustness. This may lead to the technique being adopted by regulatory agents, such as EPPO (European and Mediterranean Plant Protection Organization) and further stakeholders involved in walnut production.

This work lays out the ground for sensitive and accurate identification of *Xanthomonas euroxanthea* isolates; meanwhile, it is important to optimize the limit of resolution of this assay by making it compatible with the bacterial load of other phytopathogens found in infected plants.

Future work is directed towards performing detection in environmental samples or *in planta*, in hopes of optimizing an unwavering culture-independent method for the identification of *X. euroxanthea*.

Prospectively, this assay can contribute to unveiling alternative host species of *X. euroxanthea*; and improve the control of phytopathogenic strains.

Epidemiological studies are an interesting future hypothesis to learn about the prevalence of some *X. euroxanthea* strains within the overall diversity of this species.

Additionally, co-infection assays with pathogenic and non-pathogenic strains are rendered useful to determine if bacterial cheating is taking place.

In the future, by complementing the *X. euroxanthea* mutants with the deficient T3SS protein and assessing if the bacteria regain their pathogenicity it would further highlight the crucial role of these components in the infection ability of *X. euroxanthea*.

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