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Zucchini vein clearing disease is caused by several lineages within *Pseudomonas syringae* species complex.

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Abstract

Zucchini (Cucurbita pepo) is worldwide affected by Pseudomonas syringae inducing vein clearing, stunting and necroses during plantlet development. A collection of 58 P. syringae strains isolated from diseased zucchini plantlets was characterized by multilocus sequence analysis (MLSA). A subset of 23 strains responsible for vein clearing of zucchini (VCZ) was evaluated for pathogenicity on zucchini and their genomes were sequenced. Host range of six VCZ strains was evaluated on 11 cucurbit species. Most VCZ strains belong to clades 2a and 2b-a within phylogroup 2 of P. syringae species complex and are closely related to other strains previously isolated from cucurbits. Genome analyses revealed diversity among VCZ strains within each clade. One main cluster, once referred to by the invalid pathovar name [peponis], gathers VCZ strains presenting a narrow host range including zucchini and squashes. Other VCZ strains present a large host range including zucchini, squashes, cucumber, melons and in some cases watermelon. The VCZ strain pathogenic features are strongly associated with type III effector repertoires. Presence of avrRpt2 and absence of hopZ5 are associated with a narrow host range, while presence of hopZ5 and absence of avrRpt2 are most generally associated with a large host range. In order to better detect the different clusters identified with whole genome sequence and pathogenicity analyses, we used a specific-k-mers approach to refine the MLSA scheme. Using this novel MLSA scheme to type P. syringae isolates from diseased cucurbits would give insight into distribution of worldwide strains and origin of epidemics.

Additional key-words: seedborne disease, bacteria

Key-words: Pseudomonas syringae, Cucurbita pepo, cucurbits, bacterial seedborne disease
Introduction

In 2004 diseased plantlets of zucchini (*Cucurbita pepo* subsp. *pepo*) were observed in plant grower’s facilities in Europe. Diseased plants show cotyledon and leaf necroses, vein clearing and stunting. This disease is observed on plantlets and impacts plant development at early stages but to date no symptoms are observed on fruits. One causal agent has been identified as belonging to the *Pseudomonas syringae* species complex and was provisionally named *P. syringae* pv. *peponis* (Manceau et al. 2011). This pathogenic bacterium was shown to be seed-transmitted (Manceau et al. 2011). Climatic conditions drastically influence symptom expression at early stage development, for example low temperatures are associated with increased disease severity. Because most generally efficient chemical options are not available toward bacterial threats, gain of knowledge about *P. syringae* responsible for vein clearing on zucchini (VCZ) is essential to implement appropriate control strategies.

The *P. syringae* species complex comprises 19 phylogenomic species based on Average Nucleotide Identity (ANI) (Gomila et al. 2017), some of which are formally described (*P. amygdali*, *P. asturiensis*, *P. avellanae*, *P. cannabina*, *P. caricapapayae*, *P. caspiana*, *P. cerasi*, *P. cichorii*, *P. congelans*, *P. syringae*, and *P. viridiflava*). Moreover, *P. coronafaciens* has been proposed recently by Dutta and colleagues (2018). However, some of the crop pathogenic species are not easy to refer to as recombination between lineages is sufficiently high to blur the taxonomic boundaries (Baltrus et al. 2017; Dillon et al. 2019a). Many studies devoted to classify pathogenic strains of *P. syringae sensu lato* refer to phylogroups. Thirteen phylogroups (i.e some phylogroups cluster several phylogenomic species) generally subdivided into clades were defined in the *P. syringae*
species complex based on Multi Locus Sequence Analysis (MLSA) (Berge et al. 2014) and over 60 pathovars were described based on pathogenicity (Young 2010). Indeed, a pathovar is defined as a group of strains showing the same or similar characteristics at an infra-subspecific level but presenting a distinctive pathogenicity, which means either a unique host range and/or unique symptomatology (Dye et al. 1980).

Various pathogenic *P. syringae sensu lato* have been reported on cucurbits. *P. amygdali pv. lachrymans* causes angular leaf spots on cucumber (Bradburry 1986) and other cucurbits (Hopkins and Schenck 1972; Shila et al. 2013). Other strains of *P. syringae* that were named pathovar *lachrymans* cause different symptoms on cucumber and belong to the phylogenomic species *P. tomato* within the phylogroup 1 (Gomila et al. 2017; Słomnicka et al. 2018). Diverse strains isolated from melons and squashes within phylogroup 2 belong to *P. syringae pv. aptata* that was initially described as pathogenic on sugar beet (Morris et al. 2000; Sedighian et al. 2014). Other strains of *P. syringae sensu lato* genetically different from the two previously mentioned pathovars were shown to induce bacterial leaf spots on adult watermelon, melon and squash (Newberry et al. 2016, 2017, 2018, and 2019; Tian et al. 2017) and bacterial warts on pumpkin fruits (Tymon and Inglis 2017). Most of these cucurbit pathogenic strains are phylogenetically diverse (Newberry et al. 2016, 2017, 2018, and 2019). These strains belong to different species including *P. cerasi* (Kaluzna et al. 2016) that corresponds to phylogroup 2a, (Berge et al. 2014), *P. syringae* (corresponding to phylogroup 2b, Berge et al. 2014), the unnamed group A *sensu* Gomila et al. (2017; also named phylogroup 2d, Berge et al. 2014) and the phylogroup 2b-a issued from the recombination of *P. syringae* and *P. cerasi* (Newberry et al. 2019). In other words, these strains belong to different clades, i.e. 2a, 2b,
2b-a and 2d, within the phylogroup 2 of the *P. syringae* species complex. The phylogroup 2 appears to be the most ubiquitous group and comprises numbers of pathogenic strains with narrow or wide host range but also strains recovered from the environment pathogenic or not (Baltrus et al. 2017; Berge et al. 2014; Monteil et al. 2013; Morris et al. 2000).

Cucurbitaceae is a family of plants including economically important crops such as cucumbers (*Cucumis sativus* L.), melons (*Cucumis melo* L.), watermelons (*Citrullus lanatus*) and squashes. Squash is a generic name for various plant species including *C. pepo*, *C. maxima* and *C. moschata*. Zucchini together with pumpkin, vegetable marrow and cocozelle form *C. pepo* subsp. *pepo*. Acorn, scallop, straightneck and crookneck form *C. pepo* subsp. *texana* (Paris 1989; Paris et al. 2015). Genetic relationships between many *C. pepo* were inferred from the calculation of average dissimilarity values using DNA markers (Paris et al. 2015). These results indicate that zucchini and pumpkin from the same subspecies are genetically close while zucchini and straightneck are more distant.

Pathogenicity assays are essential to determine epidemic potential of phytopathogenic bacteria. Such assays constitute a prerequisite to designate pathovars distinguished by distinct host range and symptoms (Bull et al. 2008). Host range and virulence of *P. syringae sensu lato* are shaped by virulence factors such as Type III effectors (T3Es) and phytotoxins (Silby et al. 2011; Xin et al. 2018). T3Es are secreted via the Type III secretion system (T3SS) to specifically target host proteins or DNA sequences inside plant cells and alter physiological processes to the benefit of the pathogens (Feng and Zhou 2012). The *P. syringae* content of T3Es evolves and T3Es can be acquired via horizontal transfer as response to host-mediated selection (Baltrus et al. 2011; Dillon et al. 2019a, 2019b).
Several methods based on genomic DNA sequences are used to study organism phylogeny and update taxonomy. MLSA is very useful to assign strains to previously described taxa and reveal phylogenetic relationships among strains (Jacques et al. 2012, Jacques et al. 2016, Young et al. 2010). This portable and easy to use method is based on four or more housekeeping genes. Within the *P. syringae* species complex, a classical MLSA scheme initially proposed by Sarkar and Guttman (2004) and refined by Hwang et al. (2005) is frequently used to describe genetic structure of strain groups and to assign unknown strains to the correct genetic group and pathovar within the *P. syringae* species complex (Bull et al. 2011; Cunty et al. 2015; Ferrante and Scortichini 2015; Martin-Sanz et al. 2013; Newberry et al. 2016; Słomnicka et al. 2015). Furthermore, several methods based on whole genome sequence comparisons are currently used to infer closely related bacteria relationships, such as the average nucleotide identity based on BLAST (ANIb) (Konstantinidis and Tiedje 2005). Other methods, based on the number of words of length k (k-mers) shared between genome sequences, could be used as proxy for species delimitation (Briand et al. 2019). In addition, distribution of *k*-mers in specific genomic group could provide a list of markers for the identification of bacterial strains (Denancé et al. 2019).

In order to gain knowledge on bacteria causing VCZ and to define appropriate field management strategies, we developed a three-step approach. Our first aim was to correctly allocate the VCZ strains in the *P. syringae* species complex and infer how these strains are related to other *P. syringae* isolated from cucurbits with a MLSA and a whole-genome studies. Second, different pathogenicity assays were designed to finely phenotype strains on their host of origin (zucchini) but also on diverse cucurbit genotypes.
Third, based on draft genome sequences, some specific T3Es were highlighted for their potential link with host range specialization and a new MLSA scheme dedicated to this group of pathogens was developed. This work allows for better evaluation of the epidemic potential of VCZ strains.

**Materials and methods**

**Bacterial strains collection.** Fifty-eight strains were isolated from zucchini seedlings (*C. pepo* subsp. *pepo*) with symptoms of vein clearing issued from seed lots harvested in different geographic locations between 2002 and 2015 (Table 1). Bacterial strains were stored in a -80°C freezer in 40% glycerol solution and were routinely cultivated at 28°C on TSA$_{10\%}$ media (3 g/L Tryptone Soya broth, 15 g/L Agar). All these strains induced typical VCZ symptoms (necrosis, vein clearing and stunting) on zucchini following inoculation by rubbing the cotyledons (see the description below in pathogenicity test section).

**Housekeeping gene sequencing.** Amplicons were obtained from fresh pure cultures (overnight growth at 28°C on TSA$_{10\%}$) using primers developed by Hwang and collaborators (2005) for the four following housekeeping genes, *gapA* (encoding the glyceraldehyde-3-phosphate dehydrogenase A), *gltA* (encoding the citrate synthase), *gyrB* (encoding the B subunit of DNA gyrase) and *rpoD* (encoding the RNA polymerase sigma70 factor RpoD). Briefly, PCRs were performed in a 50 µL reaction mixture containing 0.2 µL 5X GoTaq reaction buffer (Promega), 0.2 µM dNTP, 0.5 µM of each primer and 10 µL of boiled bacterial cells. The amplification program consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 55 °C (*gapA*) or 64°C (*gltA, gyrB, rpoD*) for 30 s, extension at 72 °C for 1 min 30 s and a final extension for 7 min at 72°C. Sanger sequencing (Genoscreen, Lille, France) was performed on forward and reverse strands.
Sequence acquisition and alignment. Forward and reverse nucleotide sequences were assembled and aligned according to the reading frame of a complete gene sequence and trimmed with Bionumerics, version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) and Geneious, version 9.1.7 (https://www.geneious.com). Sequences were trimmed to obtain fragments of the following size: 474 bp (\textit{gapA}), 528 bp (\textit{gltA}), 507 bp (\textit{gyrB}) and 498 pb (\textit{rpoD}) and eventually concatenated according to the alphabetical order in a 2007 bp long sequence.

MLSA. Phylogenetic analyses were first performed on individual partial gene sequences of \textit{gapA}, \textit{gltA}, \textit{gyrB} and \textit{rpoD} and then on the concatenated dataset to obtain one single fragment of 2007 bp. These sequences were analyzed together with publicly available sequences from 22 strains representing the main phylogroups and clades (Berge et al. 2014) of the \textit{P. syringae} species complex and from 17 \textit{P. syringae} strains (13-509A, Ps711, 13-140A, 13-C2, 14-32, 14-410, 14-Gil, Zum3584, 200-1, Bs2121, Zum3984, 03-19A, Bs13-139B, 13-429, Ps03-A13, PsBS3058 and Ps02-B3) isolated from various cucurbits (Newberry et al. 2016, 2019) (Supplementary table 1). Sequences from strain CFBP 2107 (\textit{Pseudomonas viridiflava}) were used to root the trees.

For the novel MLSA scheme proposed in this study, the analysis of the four gene sequences previously mentioned (\textit{gapA}, \textit{gltA}, \textit{gyrB}, \textit{rpoD}) and genes identified in this study \textit{Psyr3420}, \textit{Psyr4880} and \textit{Psyr3208} (see results section) lead to the acquisition of one unique fragment of 4431 bp. Sequences were recovered from the genome sequences of a strain subset from our collection (23 VCZ strains), a subset of 14 strains isolated from cucurbits by Newberry et al. (2019) and 22 diverse strains from phylogroup 2 (Supplementary table 1).
The Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa 1999) test implemented in
the DnaML program from PHYLIP, Phylogeny Inference Package, version 3.69
(Felsenstein 1989) was used to test whether tree topologies fell within the same
confidence limits. Maximum-likelihood (ML) trees were generated with MEGA 7 (Kumar
et al. 2016) using the Tamura-Nei model with gamma distributed with invariant sites (G+I),
and 1,000 bootstrap replicates.

**Genome sequencing.** A subset of 23 VCZ strains were chosen to maximize their genetic
diversity and their geographical origin (P99, P121, P123, P12855, P84, P12831, P139,
P12857, P127, P79, P22, P12854, P12832, P113, P5, P118, P66, P77, P78, P87, P89,
P73 and P108). The pathotype strain of *P. syringae* pv. *dysoxyli* (strain CFBP 2356) was
also included in this study. Genomic DNAs of these strains were obtained using the
Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer’s
instructions. The genomic DNA quality was checked with NanoDrop ND-1000
spectrophotometer (the NanoDrop Technologies, Wilmington, DE) and quantified using a
Qubit fluorometer (Invitrogen). Sequencing libraries were prepared using TruSeq DNA
PCR-Free kits (Illumina). DNAs of P66 and P99 strains were paired-end sequenced (2 x
300 bp) using the Illumina MiSeq v3 (ANAN plateform, SFR QuaSav, Angers, France).
DNAs of the other VCZ strains and strain CFBP 2356 were paired-end sequenced (2 x
150 bp) using the Illumina HiSeq X-Ten (Beijing Genomics Institute, China).

**Genome sequence analysis.** Genome assembly was performed with SOAPdenovo
version 2.04, SOAPGapCloser version 1.12 (Luo et al. 2012) and Velvet version 1.2.02.
(Zerbino and Birney 2008) Genome sequences were annotated using EuGene-PP (Sallet,
Gouzy, and Schiex 2014). A dataset of 116 genome sequences (Supplementary table 1)
composed of 23 genome sequences from strains responsible for VCZ, a collection of 14
genome sequences (13-509A, Ps711, 13-140A, 13-C2, 14-32, 14-410, 14-Gil, Zum3584,
200-1, Bs2121, Zum3984, 03-19A, Bs13-139B and 13-429) of *P. syringae* isolated from
cucurbits (Newberry et al. 2019), the genome sequence of CFBP 2356 (*P. syringae* pv.
dysoxyli) and 78 genome sequences available in NCBI (to represent *P. syringae* diversity
from phylogroup 2) was used for phylogenetic analyses. To assess relationships between
genome sequences, the average nucleotide identity using blast (ANIb; Konstantinidis and
Tiedje 2005) and percentage of shared 15-mers (Briand et al. 2019) were employed. ANIb
values were obtained with the online version of pyani
(https://github.com/widdowquinn/pyana) while percentage of shared *k*-mers and distance
matrices were calculated with Ki-S as were dendrograms
(https://iris.angers.inra.fr/galaxypub-cfbp) (Briand et al. 2019). Specific *k*-mers in
predicted CDSs of each genomic group were identified with SkIf (Denancé et al. 2019).
SkIf was also used to identify sequences shared by all VCZ strains and absent in the
predicted proteins of other closely-related pathovars. Homology groups were identified in
predicted proteomes by an OrthoMCL analysis (80% identity on 80% length) using Family
companion tool (http://family-companion.toulouse.inra.fr) (Cottret et al. 2018). Finally, the
T3E repertoire of each strain was predicted through tBLASTN search (identity higher than
80% on at least 80% of CDS length) on 138 amino acid sequences of T3Es (Hops
database at http://www.pseudomonas-syringae.org/). Putative frameshift mutations were
checked by aligning each T3E sequence with the corresponding curated reference
Pathogenicity tests on zucchini. To characterize the pathogenicity of strains on their host of origin (zucchini), a cotyledon rubbing test and two seed soaking tests were run on *C. pepo* subsp. *pepo* cvs. Tosca and Opale. For each test, 23 strains responsible for VCZ (P99, P121, P123, P12855, P84, P12831, P139, P12857, P127, P79, P22, P12854, P12832, P113, P5, P118, P66, P77, P78, P87, P89, P73 and P108) and four controls were inoculated. Water was used as negative control, CFBP 5427 (*P. syringae* pv. *aptata*) and CFBP 6463 (*P. amygdali* pv. *lachrymans*) were used as pathogenic control on zucchini and CFBP 2356 (*P. syringae* pv. *dysoxyli*), which is a woody plant pathogen was used as negative control.

In the cotyledon rubbing test, five plantlets at the cotyledon stage were inoculated per strain and cultivar by rubbing three times the cotyledon surface with a finger soaked in bacterial suspensions calibrated at $1 \times 10^8$ cfu/mL. Plantlets were produced in a greenhouse from healthy seeds during seven days in pots containing Klassman substrate and watered as required for optimal growth. Inoculated plantlets were incubated in a growth chamber (18°C night, 25°C day, 14h day-light, relative humidity near 100 %) and the health status of each plantlet was recorded eight-days post-inoculation (dpi). Plantlets were qualitatively rated and the number of plantlets with necrosis on leaves, vein clearing on leaves and stunting was counted.

For the two seed soaking tests, 10 seeds per strain and per zucchini cultivar (*C. pepo* subsp. *pepo* cvs. Tosca, Opale) were inoculated by a 30 min soaking in a fresh bacterial suspension calibrated at $1 \times 10^6$ cfu/mL or $1 \times 10^7$ cfu/mL. Then, seeds were dried under a laminar flow in individual Petri dishes containing filter paper during 2 hours. Each dried seed was sown in an individual small pot containing Klassman substrate and was watered.
Pots were incubated in a climatic chamber (18°C night, 25°C day, 14h day-light, relative humidity near 100 %). Health status of each plantlet was evaluated eight days after sowing for both inoculum doses and up to 14 days for test with a 1 × 10⁷ cfu/mL inoculum concentration. Plantlets were considered healthy when no macroscopic symptom was visible and diseased when plantlets presented at least one of the following symptoms: necrosis on cotyledons, vein clearing of the first leaf or stunting.

**Pathogenicity tests on different cucurbit species.** To evaluate the host range of the VCZ strains, pathogenicity tests were run on a collection of 11 cucurbit genotypes. Six squashes including zucchini (*Cucurbita pepo* subsp. *pepo* cvs. Opale and Tosca), a straightneck squash (*C. pepo* subsp. *texana* cv. Cheetah), a pumpkin (*C. pepo* subsp. *pepo* cv. *Mischief*), a French pumpkin (*Cucurbita maxima* cv. Rouge Vif d’Etampes) and a butternut (*Cucurbita moschata* cv. Sibelle) were tested. Three melon varieties including a cantaloupe (*Cucumis. melo* var. *cantalupsis* cv. Perseus), a Galia melon (*C. melo* var. *galia* cv. Citrex) and a Canary melon (*C. melo* var. *inodorus* cv. Helios), a cucumber (*Cucumis sativus* cv. Dahab) and a watermelon (*Cucumis lanatus* cv. Livia) were tested. Seeds were sown individually in pots containing Klassman substrate and germinated in a greenhouse until the first true leaf stage. The day before inoculation, plantlets were moved for acclimation to inoculation cages in growth chamber (18°C night, 25°C day during 14h). Each inoculation cage contained five plantlets of each cucurbit genotype. Six strains responsible for VCZ (P99, P79, P66, P73, P77, P108) and three controls including water, CFBP 5427 (*P. syringae* pv. *aptata*) and CFBP 6463 (*P. amygdali* pv. *lachrymans*) were evaluated. The assay was independently done twice for each genotype (except for *C. pepo* subsp. *texana* cv. Cheetah). Bacterial suspensions were prepared from fresh
cultures and calibrated at $1 \times 10^7$ cfu/mL. Each strain was sprayed until runoff on each plantlet. Plantlets were incubated seven days (18°C night, 25°C day during 14 h and near 100 % relative humidity). Pathogenicity was qualitatively rated as (0) when a plantlet was asymptomatic and (1) when plantlets presented necrosis. First leaves were collected for photography on a light table.

**Test of hypersensitive response (HR).** Ability of strains P99, P66, P108 and P12857 to elicit a HR was tested on melons (*C. melo* var. *galia* cv. Citrex, *C. melo* var. *cantalupsis* cv. Perseus, *C. melo* var. *inodorus* cv. Helios), cucumber (*C. sativus* cv. Dahab) and watermelon (*C. lanatus* cv. Livia). Additionally, strains P79, P77 and P73 were tested on watermelon. These strains were chosen based on phylogenetic position and to represent different pathogenicity groups. Pathogenicity controls were CFBP 5427 (*P. syringae* pv. *aptata*) on melons, CFBP 6463 (*P. amygdali* pv. *lachrymans*) on cucumber and CFBP 4459 (*Acidovorax citrulli*) on watermelon. HR control was given by strain CFBP 2356 (*P. syringae* pv. *dysoxyli*) as this strain is non-pathogenic on cucurbits. Water was used as a negative control. At the time of inoculation, fresh inocula calibrated at $1 \times 10^8$ cfu/mL were infiltrated with a needle in the limb of the second leaf of 3-weeks old plantlets produced in Klassman substrate in a greenhouse. The HR characteristics (rapid death of cells in the infiltration area) were visually evaluated at 48 and 72 h after inoculation on each inoculated leaf. Dried brown necrosis limited to the infiltrated area corresponded to HR-like while spreading lesions that eventually collapsed with a water-soaked aspect corresponded to disease.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the partial sequences used in this study are MN176747 to MN176804 for *gapA*; MN176805...
to MN176862 for gltA; MN176863 to MN176920 for gyrB and MN176921 to MN176978 for rpoD. Genomic sequences of VCZ strains sequenced in this study were deposited in NCBI, accession numbers are listed in table 1. The genomic sequence of *P. syringae* pv. *dysoxyli* CFBP 2356 sequenced in this study was deposited in NCBI, accession number is VLHW00000000.

**Results**

**Phylogenetic analyses of VCZ strains.** All strains responsible for VCZ belonged to phylogroup 2 of the *P. syringae* species complex (Fig. 1A). The Maximum Likelihood tree clearly showed that all VCZ strains grouped in the same branch as did type strains and pathotype strains of phylogroup 2 (Fig. 1A). Other strains of *P. syringae sensu lato* isolated from various cucurbits (Newberry et al. 2016, 2019) also grouped in the same cluster. VCZ strains were genetically different from strain NM002 of *P. amygdali* pv. *lachrymans*, which is a pathovar previously reported as pathogenic on various cucurbits (Bradbury 1986) and belonging to phylogroups 3 (Słomnicka et al. 2018).

Within phylogroup 2, VCZ strains were mainly assigned to clade 2b-a and to clade 2a of the *P. syringae* species complex (Fig. 1B). Thirty-nine VCZ strains grouped in one highly supported cluster (bootstrap value of 100%) corresponding to clade 2b-a previously described by Newberry and his collaborators (2019). This cluster also gathered eight strains isolated from squash and watermelon (Newberry et al. 2019) and *P. syringae* pv. *syringae* HS191 isolated from millet (*Panicum miliaceum*). The closest type and pathotype strains to clade 2b-a were *P. syringae* pv. *syringae* CFBP 1392 and *P. syringae* pv. *aptata* CFBP 1617 isolated from lilac (*Syringa vulgaris*) and sugar beet (*Beta vulgaris*), respectively. A second highly supported cluster (bootstrap value of 99%) previously...
described as clade 2a (Berge et al. 2014), grouped 15 VCZ strains and four strains isolated from cantaloupe, squash, and watermelon (Newberry et al. 2019). The closest type and pathotype strains were *P. syringae* pv. *dysoxyli* CFBP 2356, *P. syringae* pv. *syringae* cit7 and *P. syringae* pv. *papulans* LMG 5076 isolated from kohekohe (*Dysoxylum spectabile*), orange (*Citrus sinensis*) and apple tree (*Malus domestica*), respectively. The last four VCZ strains (P136<sub>2b-S</sub>, P138<sub>2b-S</sub>, P108<sub>2b-S</sub> and P101<sub>2b-S</sub>) grouped in clade 2b but were clearly separated from clade 2b-a. Three strains isolated from watermelon (Newberry et al. 2016) were closely related to these four singletons.

According to the SH test, ML trees performed on individual gene and the concatenated four genes were not congruent suggesting recombination events among loci (Supplementary fig. 1). Specifically, trees built on gapA, gltA and rpoD were not congruent with the tree based on the concatenated dataset. The differences were limited between gltA and the concatenated dataset trees, while clusters behaved substantially differently when gapA or rpoD were analyzed separately.

**Pathogenicity of VCZ strains on zucchini.** All VCZ strains, *P. amygdali* pv. *lachrymans* CFBP 6463 and *P. syringae* pv. *aptata* CFBP 5427 were pathogenic on zucchini as demonstrated with the cotyledon rubbing test, but only VCZ strains succeed to induce severe stunting (Fig. 2, Supplementary table 2). More specially, 23 VCZ strains were evaluated on two zucchini cultivars (*C. pepo* subsp. *pepo* cvs. Tosca and Opale). All VCZ strains induced cotyledon necrosis and leaf necrosis on plantlets with little variability among the tested strains (Fig. 2). Short-live vein clearing symptom was observed in the days following the inoculation of all VCZ strains, except for strains P22<sub>2ba-A</sub> and P79<sub>2ba-A</sub> that failed to induce vein clearing on one or the other cultivar (Supplementary table 2).
Most VCZ strains induced severe stunting except strains P22_{2ba-A} and P79_{2ba-A} which were less aggressive than other strains. Strains *P. syringae* pv. *aptata* CFBP 5427 and *P. amygdali* pv. *lachrymans* CFBP 6463 previously reported as pathogenic on various cucurbits (Bradbury 1986; Morris et al. 2000) were used as controls. Both strains induced necroses on both cultivars that were similar to those induced by VCZ strains. Moreover, *P. syringae* pv. *aptata* CFBP 5427 was able to produce short-live vein clearing on zucchini cv. Opale but not *P. amygdali* pv. *lachrymans* CFBP 6463. Neither CFBP 5427 nor CFBP 6463 were able to induce severe stunting on inoculated zucchini. No symptom was observed on plantlets inoculated with water and *P. syringae* pv. *dysoxyli* CFBP 2356, which is a pathotype strain from clade 2a that is not pathogenic on cucurbits.

Seed soaking tests revealed different levels of aggressiveness for VCZ strains and overall, no differences in terms of aggressiveness on zucchini were linked to the genetic group of the strains (Fig. 2). With the seed soaking test in a suspension at $1 \times 10^6$ ufc/mL, VCZ strains were highly aggressive on cv. Opale and weakly aggressive on cv. Tosca (Fig. 2). With the seed soaking test in a higher concentrated suspension ($1 \times 10^7$ ufc/mL), VCZ strains were highly aggressive on both cultivars inducing necrosis on almost all plantlets. Strain P22_{2ba-A} was the only VCZ strain that failed to induce any stunting with this test (Supplementary table 2). *P. amygdali* pv. *lachrymans* CFBP 6463 and *P. syringae* pv. *aptata* CFBP 5427 induced limited necroses on a few plantlets using these seed soaking tests. No symptoms were observed on plantlets issued from seeds inoculated with water and *P. syringae* pv. *dysoxyli* CFBP 2356.

**Host range of VCZ strains.** All VCZ strains induced similar symptoms on zucchini and pumpkin (*C. pepo* subsp. *pepo*) cultivars (Fig. 3A). On zucchini (*C. pepo* subsp. *pepo* cvs.
Tosca and Opale) and on pumpkin (C. pepo susp. pepo cv. Mischief), the strains responsible for VCZ P99_{2ba-A}, P79_{2ba-A}, P66_{2a-E}, P77_{2a-D}, P73_{2a-E}, P108_{2b-S} induced water-soaking spots evolving in necrotic spots surrounded by a yellow halo at 7 dpi. Interestingly, P. syringae pv. aptata CFBP 5427 also induced this type of symptoms on these genotypes and could not be distinguished from the VCZ strains while P. amygdali pv. lachrymans CFBP 6463 induced smaller irregular necrotic lesions.

All VCZ strains were pathogenic on straightneck squash (C. pepo subsp. texana) and winter squashes (C. maxima, C. moschata) but different types of symptoms were observed according to the MLSA group (Fig. 3A). The yellow straightneck squash (C. pepo subsp. texana cv. Cheetah) seemed to be less susceptible to VCZ strains from clade 2b-a (P99_{2ba-A}, P79_{2ba-A}) which induced dried brown spots, while VCZ strains from clade 2a (P66_{2a-E}, P73_{2a-D}, P77_{2a-D}) induced water-soaked spots evolving in necrosis and were associated with chlorosis. On butternut (C. moschata cv. Sibelle) VCZ strains from clade 2b-a induced brown necrotic spots with a yellow halo that collapsed while strains from clade 2a induced large water-soaking lesions and necrotic spots surrounded by a yellow halo. On C. maxima cv. Rouge Vif d’Étampes, VCZ strains from clade 2b-a induced tiny yellow spots while VCZ strains from clade 2a induced water-soaked spots surrounded by a yellow halo.

Only VCZ strains from clade 2a and the singleton P108_{2b-S} were pathogenic on the cucumber, melons and watermelon cultivars that were tested (C. sativus, C. melo, C. lanatus) (Fig. 3). VCZ strains from clade 2a (P66_{2a-E}, P73_{2a-D}, P77_{2a-D}) were pathogenic on all three melons varieties (C. melo var. galia cv. Citrex, C. melo var. inodorus cv. Helios, C. melo var. cantaloupe cv. Perseus) and induced necrotic spots surrounded by a yellow
halo. On cucumber (C. sativus cv. Dahab), VCZ strains from clade 2a were highly aggressive and induced collapsing circular necrotic spots with large chlorosis. In clade 2a, only strain P66\textsubscript{2a-E} was able to induce necrotic spots surrounded by a little yellow halo on watermelon (C. lanatus cv. Livia). The tested cultivars of cucumber, melon and watermelon were not susceptible to the VCZ strains from clade 2b-a (P99\textsubscript{2ba-A}, P79\textsubscript{2ba-A}). Some tiny dry white spots were sometimes visible on inoculated leaves but plants remained healthy two weeks post inoculation. Singleton VCZ strain P108\textsubscript{2b-S} was pathogenic on all tested cucurbits but the symptoms were different from those caused by VCZ strains from clade 2a (P66\textsubscript{2a-E}, P73\textsubscript{2a-D}, P77\textsubscript{2a-D}) on some hosts. For instance, on watermelon, P108\textsubscript{2b-S} induced large dried necrotic spots while P66\textsubscript{2a-E} induced small spots with a discrete yellow halo. *P. amygdali* pv. *lachrymans* CFBP 6463 and *P. syringae* pv. *aptata* CFBP 5427 were pathogenic on almost all the tested genotypes, and the symptoms were sometimes different from those caused by VCZ strains. As expected, *P. amygdali* pv. *lachrymans* CFBP 6463 induced angular leaf spots on cucumber and *P. syringae* pv. *aptata* CFBP 5427 induced circular necrotic spots on melons. All plantlets inoculated with water remained asymptomatic 7 dpi.

**Hypersensitive response (HR) of different cucurbits toward VCZ strains.** Infiltration tests on cucumber, watermelon and melons confirmed that VCZ strains had different host range (Fig. 4). Starting from 48 hpi, spreading lesions that eventually collapsed with a water-soaked aspect on the abaxial leaf surface were noticed for all pathogenic controls on their hosts (*A. citrulli* on watermelon, *P. amygdali* pv. *lachrymans* on cucumber and *P. syringae* pv. *aptata* on melons). *P. syringae* pv. *dysoxyli* CFBP 2356, a non-pathogenic strain on cucurbits, induced HR with a dried brown necrosis limited to the infiltrated area.
As expected, strains P66\textsubscript{2a-E} and P108\textsubscript{2b-S} caused water-soaked lesions similar to that of the corresponding pathogenic controls on cucumber and the three melon varieties. In contrast, strain P99\textsubscript{2ba-A} caused the same HR-like necrosis than those of \textit{P. syringae pv. dysoxyli} CFBP 2356 on all tested genotypes confirming non-host reaction. On watermelon, strain P66\textsubscript{2a-E} was pathogenic while strains P73\textsubscript{2a-D} and P77\textsubscript{2a-D} induced HR-like, confirming pathogenic heterogeneity on this host for strains of the clade 2a (Supplementary fig. 2).

**VCZ strains genome sequence analyses.** Based on whole genome sequences, ANI\textsubscript{b} revealed strong relationships between VCZ strains from 2a and 2b-a groups (Fig. 5, Supplementary table 3). First, ANI\textsubscript{b} values between genome sequences of \textit{P. syringae} strains from clades 2a and 2b (except 2b-a strains) were below 95\% indicating that strains belonged to different species (Supplementary table 3). Interestingly, the genome sequences from the 23 VCZ strains and the other 14 cucurbit strains (Newberry et al. 2019) included in clades 2b-a and 2a were more similar with ANI\textsubscript{b}-value higher than 95\%.

Based on whole genome sequences, new clusters inside the main genetic groups (clades 2b-a and 2a) were highlighted by ANI\textsubscript{b} and shared \emph{k}-mers analyses (Fig. 5). Both dendrograms displayed similar phylogeny and showed three new clusters within each clade 2b-a and 2a (Fig. 5). Within clade 2b-a, the first cluster (A) gathered 15 VCZ strains and two cucurbit strains (Ps711\textsubscript{2ba-A}, 13-509A\textsubscript{2ba-A}) isolated from squash (Newberry et al. 2019). The second cluster (B) gathered the strain P12857\textsubscript{2ba-B} responsible for VCZ with six cucurbit strains isolated from melon CC457 (Baltrus et al. 2014) and watermelon (13-C2\textsubscript{2ba-B}, 13-140A\textsubscript{2ba-B}, 14-410\textsubscript{2ba-B}, 14-32\textsubscript{2ba-B}, 14-Gil\textsubscript{2ba-B}) and finally, cluster C did not
contain any VCZ strain but one strain (Zum35842ba-C) isolated from squash (Newberry et al. 2019). The strain *P. syringae* pv. *syringae* HS191 which was encompassed in clade 2b-a with MLSA was excluded from one of these clusters. Within clade 2a, the first cluster (D) comprised four VCZ strains (P772a-D, P732a-D, P782a-D, P872a-D) and two other strains (03-19A2a-D, 200-12a-D) isolated from melon and squash (Newberry et al. 2019). The second cluster (E) gathered two VCZ strains (P662a-E, P892a-E) with strains Bs21212a-E and Zum39842a-E isolated from squash (Newberry et al. 2019). The last cluster (F) was composed of two strains (13-139B2a-F, 13-1492a-F) isolated from watermelon (Newberry et al. 2019).

Specific signatures were found in genome sequences with SkIf that allow to distinguish the six clusters containing cucurbit strains within clades 2a and 2b-a. In genomic sequences of strains from clade 2b-a, a total of 108 CDSs with specific signatures were found to differentiate the strains of cluster A from strains of clusters B and C. For cucurbit strains from clade 2a, a total of 578 CDSs with specific signatures of cluster E were found and allowed to distinguish cluster E from clusters D and F. Many specific k-mers were identified on CDSs localized in an integrative and conjugative element (ICE) but other signatures were located elsewhere in the genome sequences. Among these latter CDSs, three were retained because they were widely distributed within the set of 116 *P. syringae* genome sequences and allowed phylogenetic analyses. These three CDSs corresponded to *Psyr3420, Psyr4880* and *Psyr3208* in strain B728a of *P. syringae*, encoding the nitrogen fixation protein FixH, a hypothetical protein and the NADH dehydrogenase subunit M, respectively. OrthoMCL analysis revealed proteins specifically present in each cluster.
comparatively to the others in the same clade. Therefore 13, 9, 190, 54, 75, and 384 proteins were specific of clusters A, B, C, D, E and F, respectively (data not shown).

**Proposal of a novel MLSA scheme dedicated to cucurbit pathogens.** Seven genes (\textit{gapA}, \textit{gltA}, \textit{gyrB}, \textit{rpoD}, \textit{Psyr3420}, \textit{Psyr4880} and \textit{Psyr3208}) were selected to highlight all clusters observed with whole genome analyses within clades 2b-a and 2a. The MLSA tree based on the sequences of this novel MLSA scheme including these seven genes was constructed for 23 VCZ strains, 14 strains isolated from cucurbits (Newberry et al. 2019) and 22 diverse strains from phylogroup 2 (Fig. 6A). Phylogenies were constructed for each gene individually and for the concatenated data set (Supplementary fig. 3) and their topologies were compared with the SH test. As expected individual phylogenies were not congruent with the one constructed on the concatenated dataset indicating that each gene fragment was bringing complementary information. The tree showed that cucurbit strains of clade 2b-a were separated into three distinct branches and VCZ strains were carried by two of them. The cucurbit strains from clade 2a split also into three separate branches and VCZ strains were carried by two of them. Overall, composition of each branch was coherent with the composition of clusters identified using whole genome sequence analyses.

**T3Es.** Strains responsible for VCZ presented different T3E repertoires according to their genetic group (Fig. 7). Using t-BLASTN and sequence alignment, a total of 23 different T3Es were identified in the genome sequences of the 23 VCZ strains. The composition of T3E repertoires was associated with strain clusters identified with whole genome sequence analysis/core-genome MLSA as all strains from a given cluster had homogeneous set of T3Es (Fig. 6B, Fig. 7) except two strains from the collection of
Newberry, strains 14-Gil$_2ba$-B and 13-139B$_{2a-F}$ which lacked one T3E. The 15 VCZ strains from the cluster A possessed a set of 18 T3Es and one of them (avrRpt2) was specific to that cluster. The strain P12857, which was the only VCZ strain from cluster B, possessed 16 T3Es in which 14 were common with the cluster A. The four VCZ strains from cluster D possessed 13 T3Es, of which one (hopAR1) was specific to strains from that cluster. The strains P89$_{2a-E}$ and P66$_{2a-E}$ from cluster E possessed 14 T3Es, 11 being common with cluster D. P108$_{2b-S}$ (singleton) had a smaller T3E repertoire (11 T3Es) with a unique composition but no specific T3E in comparison with the main VCZ clusters. Finally, eight T3Es (avrE1, hopAA1, hopAE1, hopAZ1, hopAG1, hopAH1, hopBA1 and hopl1) were found in all strains responsible for VCZ.

The different genetic groups of VCZ strains differing by their host ranges are associated with different T3E repertoires (Fig. 6, Fig. 7). As no pathogenicity data was available for VCZ strains from cluster B, we evaluated the behavior of P12857$_{2ba-B}$ on cucumber, melons and watermelon with the infiltration method (Supplementary fig. 4). P12857$_{2ba-B}$ induced spreading water-soaked necrosis in all leaves from these hosts, thus indicating that this strain was pathogenic on these cucurbits. Interestingly, homogeneous T3E repertoires among strains from the same cluster gave homogeneous pathogenicity results (Fig. 6 and 7). The tested strains (P99$_{2ba-A}$, P79$_{2ba-A}$) from cluster A (clade 2b-a) were not pathogenic on cucumber, melons and watermelon and were weakly pathogenic on yellow straightneck genotype. Interestingly, strains from cluster A possessed a unique T3E (avrRpt2) and did not have any copy of hopZ5 compared to all other VCZ strains from clusters B, D, E, which were highly pathogenic on these hosts. Strains P66$_{2a-E}$ (cluster E), P12857$_{2ba-B}$ (cluster B) and P108$_{2b-S}$ (singleton) were pathogenic on watermelon (C. 
lanatus cv. Livia). It is worth noticing that all these strains lacked hopC1 and hopH1, which were present in strains P99_{2ba-A} and P79_{2ba-A} from cluster A, and P73_{2a-D} and P77_{2a-D} from cluster D, strains that were not pathogenic and drove to a HR-like on watermelon.

**Discussion**

Facing the increase of VCZ in zucchini seed producing areas, we engaged a study to refine results obtained previously on this disease. The present study reveals that VCZ is currently caused by strains belonging to different phylogenetic lineages within the phylogroup 2 of the *P. syringae* species complex. Based on a MLSA using four housekeeping genes (*gapA, gltA, gyrB* and *rpoD*), VCZ strains are mainly grouped in two clades, namely clade 2b-a and clade 2a. A high-resolution analysis based on genome sequences of VCZ strains and cucurbit strains highlights six distinct clusters. VCZ strains belong to four of these six clusters: A and B being associated to clade 2b-a, while D and E are associated to clade 2a. Despite a common disease on zucchini (*C. pepo* subsp. *pepo*), VCZ strains induce different types of symptoms on other squashes (*C. pepo* subsp. *texana, C. moschata, C. maxima*) and have different host range of cucurbit genotypes. Genome sequences were mined to recover genes that matched those clusters. Three largely distributed genes (*Psyr3420, Psyr4880* and *Psyr3208*) were then selected to complete a novel MLSA scheme that can resolve clusters of strains pathogenic on cucurbits. The VCZ strains from each cluster share homogeneous T3E repertoires that are associated with pathogenicity features on cucurbit genotypes. Strains from cluster A have a narrow host range and are pathogenic on squashes (*Cucurbita* spp.), while VCZ strains from cluster B, D, E and singleton P108 have a wide host range including...
squashes, cucumber and melons. By having different host range extents, VCZ strain lineages represent different epidemiological risks in cucurbit producing areas.

Comparative genomic analyses led to proposing a novel MLSA scheme and differential T3Es that distinguish various lineages of strains that are responsible for diseases on cucurbits. VCZ strains group in four main lineages while a few strains remain isolated in the phylogeny. Other strains responsible for diseases on cucurbits that were previously characterized (Newberry et al. 2019) were included in our genomic analyses. Clearly, all these strains are distant from *P. amygdali pv. lachrymans* and cause different diseases.

One lineage of VCZ strains (cluster A) presents a narrower host range than other groups, including zucchini, yellow straightneck, butternut, and pumpkin. The presence of gene encoding AvrRpt2 and the absence of gene encoding HopZ5 characterize this group of VCZ strains. This lineage includes most strains previously named *P. syringae pv. peponis* (Manceau et al. 2011) and is included in the clade 2b-a, which is described as being the result of recombination events between *P. syringae* and *P. cerasi* (Newberry et al. 2019).

The three other groups of VCZ strains (clusters B, D and E) are distributed in clades 2a and 2b-a. Clade 2a corresponds to *P. cerasi* (Newberry et al. 2019). We show here that VCZ strains of these three groups are pathogenic not only on zucchini, but also on yellow straightneck, butternut, pumpkins, cucumber, melons and in some cases watermelon. These groups include also strains that were independently shown to induce angular leaf spot and bacterial leaf spot of watermelon, cantaloupe and squash by Newbery and colleagues (2016, 2018, 2019). The presence of the gene encoding HopZ5 and the absence of the gene encoding AvrRpt2 in their genome sequences characterize these lineages. Taking into account that, i) the diseases induced in zucchini by the four groups
of strains and the singleton (P108$_{2b-S}$ in clade 2b corresponding to *P. syringae sensu stricto*) are similar, while the host range of cluster A is different from the one of clusters B, D and E; ii) the distribution of these strains over different species and iii) the fact that we did not test on our cucurbit genotypes the pathotype strain of *P. syringae pv. syringae*, known to have a large host range, we cannot conclude that the four lineages truly form distinct pathovars. It should be noticed that the singleton P108$_{2b-S}$ is phylogenetically close to *P. syringae pv. aptata* that includes strains isolated from melons (Berge et al. 2014).

Strain CFBP 5427 from this pathovar induces symptoms on squash, cucumber and melons that are similar to those caused by P108$_{2b-S}$ strain. The only few differences are the inability of the *P. syringae pv. aptata* CFBP 5427 strain to induce stunting of zucchini plantlets with the seed soaking test and the susceptibility of watermelon to P108$_{2b-S}$ but not to CFBP 5427.

Specific T3Es could be used as genetic markers of differential host ranges of VCZ strains. In this study, we identified that presence of *hopZ5* and absence of *avrRpt2* are common characters of strains from the three clusters sharing a wide host range, and having in common to be pathogenic on melons and cucumber. In contrast cluster A strains have *avrRpt2* and lack *hopZ5* and are not pathogenic on these groups of genotypes. Further experimental work is necessary to decipher the role of these T3Es in host range extents.

Interestingly, it was also shown that strains from clusters C and cluster F harbor *hopZ5* and are pathogenic on squash, melon and watermelon (Newberry et al. 2019). HopZ5 was previously proposed to promote virulence among strains pathogenic on cucurbits and having different T3E repertoires (Newberry et al. 2019). Here we suggest that *hopZ5* could also be a good predictor of a wide host range on cucurbits. Cluster A strains (Ps711$_{2ba-A}$...
and 13-509A<sub>2ba-A</sub>), harboring <i>avrRpt2</i> and missing <i>hopZ5</i> have been previously reported to be weakly pathogenic on squash, melon and watermelon (Newberry et al. 2018, 2019). Their pathogenicity was evaluated on a yellow straightneck genotype but strains were not tested on cucumber nor on any other genotypes of squashes, including zucchini. Therefore, this weak pathogenicity on yellow straightneck for cluster A strains is coherent with the dried brown lesions we observed for strains P99<sub>2ba-A</sub> and P79<sub>2ba-A</sub> on yellow straightneck. Indeed, the dried lesions seemed less severe than the water-soaked lesions caused by strains of clusters B, D and E and the singleton. The gene <i>avrRpt2</i> is commonly found in <i>P. syringae</i> pv. <i>tomato</i> strains (Almeida et al. 2008) and has been shown to promote pathogenicity by stimulating key regulators in auxin signaling pathways (Cui et al. 2013). This effector leads to HR-like in <i>A. thaliana</i> through the cognate RPS2 resistance gene (Pike et al. 2005). Apart from the main genetic clusters including VCZ strains, we also characterized a singleton (P108<sub>2b-S</sub>), which is pathogenic on all tested cucurbits and induces VCZ. P108<sub>2b-S</sub> is close to <i>P. syringae</i> pv. <i>aptata</i> strains in the phylogenetic analysis. This singleton has fewer T3Es (11 T3Es) than other VCZ strains. Seven T3Es (<i>avrE1</i>, <i>hopAA1</i>, <i>hopAE1</i>, <i>hopAZ1</i>, <i>hopAG1</i>, <i>hopAH1</i>, <i>hopBA1</i> and <i>hopl1</i>) are common to all the VCZ strains, and might be associated to pathogenicity on zucchini. It is also interesting to note that Newberry and collaborators characterized singletons from clade 2b and 2d isolated from diseased cucurbits (Newberry et al. 2016, 2019). Previous studies also identified a potential large host range for strains from clade 2d (Monteil et al. 2016; Morris et al. 2019). Not all VCZ strains were pathogenic on watermelon cultivar Livia as tested strains from clusters A (P99<sub>2ba-A</sub> and P79<sub>2ba-A</sub>) and D (P73<sub>2a-D</sub> and P77<sub>2a-D</sub>) did not induce any disease
with the spray inoculation and induced HR-like necrosis when infiltrated in leaves. These results were also obtained with cv. Troubadour (data not shown). Based on genome analysis, strains Ps711ba-A and 13-509A2ba-A belong to cluster A and strains 200-1a-D and 03-19A2a-D belong to cluster D. Interestingly, these strains were previously tested with a similar spray inoculation and were shown to be weakly pathogenic or not pathogenic on watermelon cultivars Troubadour and Wrigley (Fig. 6B) (Newberry et al. 2016, 2017, 2019). These discrepancies on watermelon could be strain- or test-dependent. However, some effectors are associated to strains from cluster A and D which are weakly pathogenic or not pathogenic on watermelon as all possess complete genes of hopH1 and hopC1 in contrast to other aggressive strains from cluster B, C, E, F and singleton. This result is coherent with those previously reported (Newberry et al. 2019).

The use of several pathogenicity tests permitted a precise phenotypic characterization of strains responsible for VCZ. On zucchini, the cotyledon rubbing test successfully reproduces the typical symptoms (necroses on leaves and cotyledons, vein clearing and stunting) observed in seedling producing areas and caused by VCZ strains. The cotyledon rubbing test is quick and easy to perform, however it induces micro-injuries on plantlets by trichome breaks and thus creates openings. For this reason, the cotyledon rubbing test is quite invasive and can produce biases to study bacterial strains pathogenicity (Burdman et al. 2005; Klement et al. 1990). The seed soaking tests were appropriate to study VCZ strains on their host of origin and permitted to properly characterize strain aggressiveness. Seed soaking test is coherent with VCZ strains being described as seed-borne bacteria (Manceau et al. 2011). Seed soaking tests are commonly used to characterize aggressiveness of seed-borne bacterium such as A. avenae subsp. citrulli on cucurbits.
(Burdman et al. 2005). Then, the spray inoculation method was used to simulate natural
infection of leaves by pathogen and allowed bacterial entry through natural openings
(Klement et al. 1990). This test was particularly relevant to finely characterize symptoms
and host range. Finally, the HR test is an easy, fast and efficient method to define host
range of VCZ strains on different cucurbit genotypes. Host-incompatibility gives HR-like
reaction on leaves after 48 h post-inoculation while host-compatibility gives spreading
water-soaked necrosis.

The strains responsible for VCZ were isolated from plantlets issued from zucchini seed
lots produced in geographic distant regions over time. Only few studies so far deal with \( P. \)
\textit{syringae} strains transmitted by cucurbits seeds (Dutta et al. 2014; Meng et al. 2016; Shila
et al. 2013). Strains from \( P. \textit{syringae pv. aptata} \), \( P. \textit{syringae pv. syringae} \) and \( P. \textit{amygdali}
\textit{pv. lachrymans} \) had been reported to be seed disseminated and efforts to develop seed
detection tests contribute to improve pathogen control and disease management. We
identified specific markers (\textit{Psyr3420}, \textit{Psyr4880} and \textit{Psyr3208}) that can be used to design
specific VCZ strain primers. A specific detection tool is indeed needed to conduct
epidemiological studies of VCZ in worldwide zucchini seed production areas. Typing \( P. \)
\textit{syringae} isolates from diseased cucurbits with the new MLSA scheme would give insight
into worldwide dissemination of strains and origin of epidemics. The gain of knowledge on
VCZ strains and the survey of epidemics—are essential steps to implement an efficient
management of the various VCZ lineages, which do not represent the same risk as a
consequence of their different host range.

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TABLE 1. 58 VCZ strains used in this study

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**Fig. 1. Phylogenetic relationships of strains responsible for VCZ.** The diversity of 58 VCZ strains and 17 strains isolated from diverse cucurbits species (Newberry et al. 2016, 2019) was assessed through MLSA. Maximum likelihood (ML) trees were based on concatenated partial sequences of \textit{gapA}, \textit{gltA}, \textit{gyrB} and \textit{rpoD} (2007 bp). Bootstrap scores (1,000 replicates) are displayed at each node. (A) ML tree of typed strains representing the \textit{P. syringae} group. Phylogroups (PGs) are derived from Bull and Koike (2015) (B) Detail of the phylogroup 2 diversity including VCZ strains. Clades are indicated based on Berge et al. 2014; Bull and Koike 2015; and Newberry et al. 2019. Black points indicate VCZ strains.

**Fig. 2. Responses of two cultivars of zucchini to seed inoculation and cotyledon rubbing assay with \textit{P. syringae} strains.** \textit{Cucurbita pepo} subsp. \textit{pepo} cv. \textit{Opale} (orange) and cv. \textit{Tosca} (blue) plants or seeds were inoculated with 23 strains of \textit{P. syringae} responsible for VCZ and four control strains including CFBP 5427 (\textit{P. syringae} pv. \textit{aptata}), CFBP 6463 (\textit{P. amygdali} pv. \textit{lachrymans}), CFBP 2356 (\textit{P. syringae} pv. \textit{dysoxyli}) and water. Bars indicate the percentage of disease incidence over the number of germinated plantlets seven days post-inoculation for a given strain and a given test (cotyledon rubbing, number of tested plantlets \(n = 5\); seed soaking at 1x \(10^7\) CFU/mL, number of sown seeds \(n = 10\); seed soaking at 1x \(10^6\) CFU/mL, number of sown seeds \(n = 10\)). (*) SS for seed soaking test.

**Fig. 3. Host range test of VCZ strains.** Six \textit{P. syringae} strains responsible for VCZ and three controls including water, \textit{P. syringae} pv. \textit{aptata} CFBP 5427 and \textit{P. amygdali} pv. \textit{lachrymans} CFBP 6463 were spray inoculated (1 x \(10^7\) cfu/ml until runoff) on six genotypes of \textit{Cucurbita} sp., four genotypes of \textit{Cucumis} sp. and one genotype of \textit{Citrullus lanatus}. Water was sprayed as control. (A) Photography of the symptoms observed on the first leaf 7 dpi (B) Results of pathogenicity test, (+) disease symptoms, (-) absence of symptoms.

**Fig. 4. Hypersensitive response of diverse cucurbits following infiltration of VCZ strains.** Three VCZ strains (P99, P66 and P108) were infiltrated in leaves of four genotypes of \textit{Cucumis}...
sp. and one *Citrullus lanatus*. Symptoms were pictured 72 h post-inoculation. Pathogenic controls consisted in *A. citrulli* CFBP 4459 on *C. lanatus* cv. Livia, *P. syringae* pv. *aptata* CFBP 5467 on melon cultivars and *P. amygdali* pv. *lachrymans* CFBP6463 on cucumber. The HR control for all tested plants was *P. syringae* pv. *dysoxily* CFBP 2356. Water was used as a negative control. Within the inoculated area the red line delimits necrosis and the orange line delimits water-soaked lesion. The white line delimits the expansion of water-soaked symptoms beyond the inoculation point.

**Fig. 5. Relationships between genomic sequences of *P. syringae* from phylogroup 2 using AN Ib and k-mers approaches.** Dendrograms were built on distances calculated from AN Ib and k-mers matrices obtained with Ki-S (Briand et al. 2019). The color blocks represent the cucurbit clusters highlighted with both approaches.

**Fig. 6. Phylogeny of VCZ strains, host range and presence of some T3E encoding genes.**

(A) Phylogeny based on new MLSA scheme using seven genes (*gapA, gltA, gyrB, rpoD, Psyr3420, Psyr4880* and *Psyr3208*). Maximum likelihood tree was based on concatenated partial sequences (4431 pb). Bootstrap scores (1,000 replicates) were displayed at each node. Black points indicate VCZ strains. (B) Summarized results obtained from pathogenicity tests and presence of differential genes encoding T3Es recovered from genome sequence analysis. Columns in dark grey report results from Newberry et al. 2018, 2019 that were available for strains Ps711, 13-509A, 13-140A, 13-C2, 14-32, 14-410, 14-Gil, Zum3584, 13-139B, 13-429, Zum3984, Bs2121, 03-19A and 200-1. The light grey report the results obtained in this study. Colors are given as following: light or dark grey when a strain is not tested on a given host, red when a strain is pathogenic on a given host, yellow when a strain is weakly pathogenic on a given host and green when a strain is not pathogenic on a given host. A black box means a T3E presence. Cucurbit species and sub-species are reported as following, ZU for zucchini, YS for yellow Straightneck squash, PU for pumpkin, ME for melons and WM for watermelon.

Supplementary table 1. List of strains and their characteristics and list of genome sequences used in this study.

Supplementary table 2. Plantlets evaluation for the cotyledon rubbing test and seed soaking test at 1.10^7 cfu/mL. Colors refer to the incidence of a given symptom, with red for more than 80% of plantlets, orange 40 to 79%, yellow for 1 to 39% and green when none of the plantlets presented the symptom. For vein clearing, red color indicate that this symptom was observed at least at one date and green color when it had been never observed.

Supplementary table 3. ANIb-values calculated on genomic sequences.

Supplementary fig. 1. Phylogenies built on four individual housekeeping genes (gapA, gltA, gyrB and rpoD) and the concatenated dataset. Maximum-likelihood (ML) trees were generated with MEGA 7 (Kumar et al. 2016) using the Tamura-Nei model with gamma distributed with invariant sites (G+I), and 1000 bootstrap replicates. Blue color represents the clade 2b-a and red color the cucurbit clade 2a. P values determined using the Shimodaira-Hasegawa test for congruence of tree topologies run on each tree based on four housekeeping genes, individually and the concatenated dataset. Values an asterisk are significantly depart from the hypothesis of congruency.

Supplementary fig. 2. Response of watermelon (C. lanatus cv. Livia) to infiltration of P. syringae VCZ strains P66, P73, P77 and P79.

Supplementary fig. 3. Phylogenies built on seven individual genes (gapA, gltA, gyrB, rpoD, Psyr3208, Psyr3420, Psyr4880) and the concatenated sequences. Maximum-likelihood (ML) trees
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**Supplementary fig. 4.** Response of melons, cucumber and watermelon to infiltration of VCZ strain P12857 from clade 2b-a.
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527x294mm (72 x 72 DPI)
Supplementary fig. 1
Phylogenies built on four individual housekeeping genes (gapA, gltA, gyrB, and rpoD) and the concatenated dataset. Maximum-likelihood (ML) trees were generated with MEGA7 (Kumar et al. 2016) using the Tamura-Nei model with gamma distributed with invariant sites (G+I), and 1000 bootstrap replicates. Blue color represents the clade 2b-a and red color the cucurbit clade 2a. P values determined using the Shimodaira-Hasegawa test for congruence of tree topologies run on each tree based on four housekeeping genes, individually and the concatenated dataset. Values an asterisk are significantly depart from the hypothesis of congruency.

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Supplementary Fig. 2: Response of watermelon (C. lanatus cv. Livia) to infiltration of P. syringae VCZ strains P66, P73, P77 and P79
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