Development of a real-time PCR method for the identification of cucurbit seed-borne *Pseudomonas syringae* species

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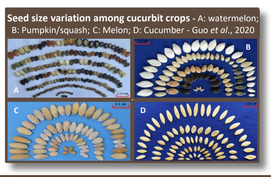
Pseudomonas syringae-induced diseases in cucurbits

➢ **Cucurbits:**
- Among the **most cultivated crops**
- Most **economically important** species: watermelon, cucumber, melon…
- Remarkable **diversity** in seeds and in fruit size, shape and colour

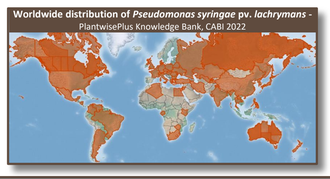
➢ ***Pseudomonas syringae* diseases:**
- Angular leaf spot (**ALS**) and Vein Clearing on Zucchini (**VCZ**)
- **Seed-borne diseases**
- Diseases management: resistant crop cultivars, cultural practices, chemical and biological control, and **seed detection test**

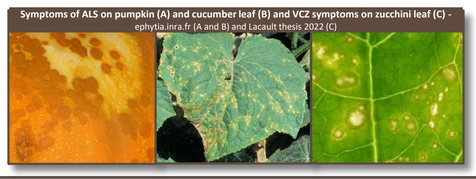
➢ **ALS caused by *P. syringae* pv. *lachrymans*:**
- **Most widespread** bacterial disease of all cucurbit crops
- **Significant yield reduction** in the number of fruits and fruit weight
- **Symptoms**: Angular spots with yellow halo on leaves and water-soaked tan, small circular spots on fruits

➢ **VCZ caused by several lineages of *P. syringae*:**
- Causal agent was provisionally named ***P. syringae* pv. *'peponis'***
- First observation: **2004**
- **Symptoms**: cotyledon and leaf necroses, vein clearing, and stunting



Seed size variation among cucurbit crops - A: watermelon; B: Pumpkin/squash; C: Melon; D: Cucumber - Guo *et al.*, 2020


Worldwide distribution of *Pseudomonas syringae* pv. *lachrymans* - PlantwisePlus Knowledge Bank, CABI 2022


Symptoms of ALS on pumpkin (A) and cucumber leaf (B) and VCZ symptoms on zucchini leaf (C) - ephytia.inra.fr (A and B) and Lacault thesis 2022 (C)

GEVES and seed health

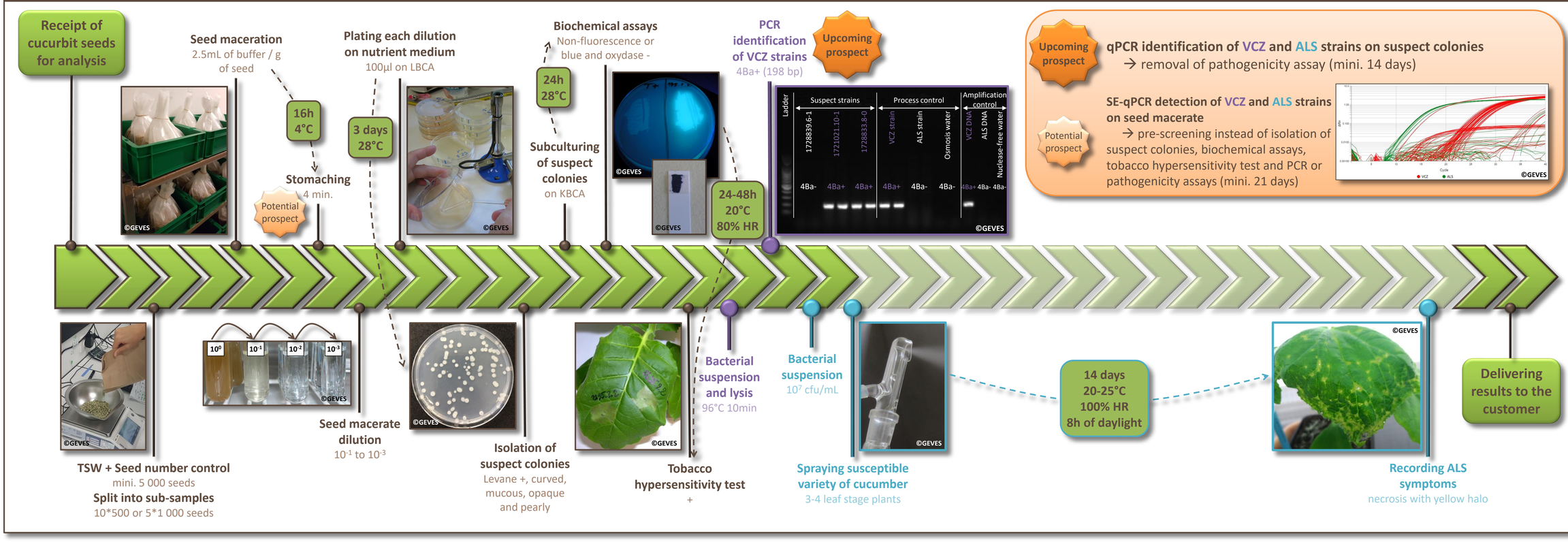
➢ **Missions:**
- Evaluating **new plant varieties**
- Analysing **seed quality**

➢ **Three main sectors:**
- Variety Study Department (**SEV**)
  - Field testing of new varieties for all cultivated plant species
  - Registration in the **Official Catalogue of plant varieties**
  - Issue of **Plant Variety Right**
- National Seed Testing Station (**SNES**)
  - Seed quality testing
  - Three laboratories: **Physical analysis**, **Germination** and **Pathology**
  - **National Reference Laboratory (NRL) for seed quality analysis**
- Laboratory for molecular biology and biochemistry (**BioGEVES**)
  - Analyses for seed certification and variety registration
  - Three units: **Genotyping**, **Biochemistry** and **Detection**
  - **NRL for GMO detection** in maize, soya, rapeseed and flax

➢ **Seed health:**
- Co-working of **'Pathology laboratory'** for microbiological, serological and morphological methods and **'Detection unit'** for biomolecular techniques
- Around **36 people** dedicated to seed health testing (**10 000 analyses per year**) and research projects
- Nearly **300 pathogens and pests detected** (bacteria, fungus, virus and nematodes)
- **3 800 strains** in collection, including 680 in reference collection and 70 in **MATREF** (network of reference material)

➢ **Bacteriological analysis of cucurbit seeds (VCZ and ALS):**
- Around **450 analyses per year** (≈20% of bacteriological analyses)
- Analysis performed on **5 000 seeds** and in **36 days**
- Detection by **dilution-plating** and identification by **biochemical**, **PCR** and **pathogenicity assays**

Detection and identification of VCZ and ALS strains on cucurbit seeds and GEVES protocol improvements



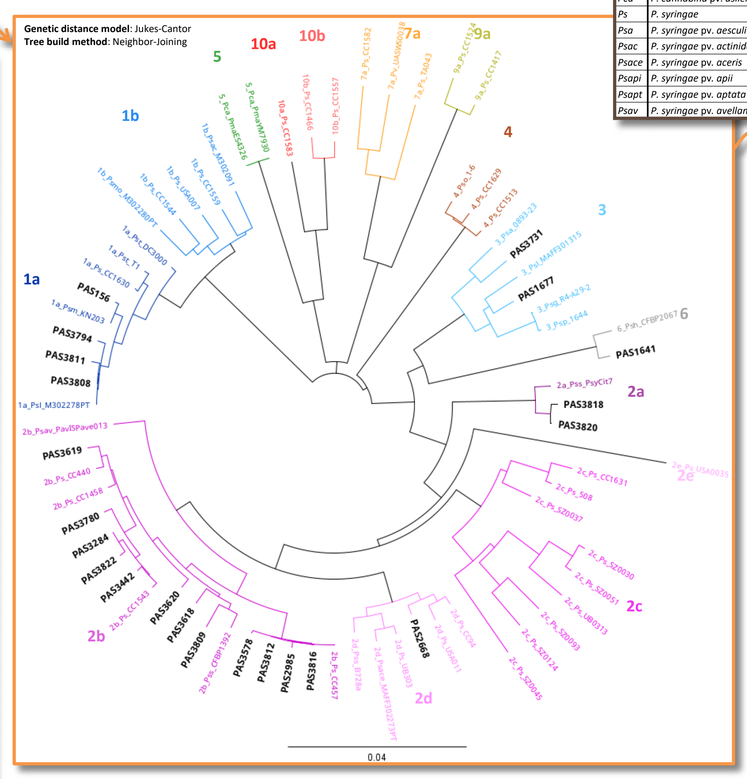
Development of a qPCR identification method for VCZ and ALS strains

➢ **Characterisation of the collection of target and non-target strains:**
- Use of the **VCZ and ALS strain identification protocol** described above, with a **pathogenicity assay adapted** according to bacterial species for non-target species
- Phylogroup determined with a **multilocus sequence analysis** based on concatenated partial sequences of ***cts***, ***gapA***, ***gyrB*** and ***rpoD*** from tested strains and from Berge *et al.*, 2014
- Use of specific **PCRs** and **qPCRs** to identify **2ba-A**, **2ba-B**, **2ba-C**, **2a-D** and **2a-E** phylogenetic clusters and to confirm **2b** and **2d** phylogroups (Lacault *et al.*, 2023)
  - Expected phylogroup for **ALS** strains: **1a** and **3**
  - Expected phylogroup for **VCZ** strains: **2a, 2ba**, **2b** and **2d**

Characterised strain collection:
- **18 target VCZ strains**
- **4 target ALS strains**
- **18 non-targeted strains**

and used to evaluate the primers specificity

General information						Identification confirmation assays							
Name	Other name	Origin	Year	Host	Expected species	Levane	Fluorescence	Oxidase	Tobacco hypersensitivity	PCR 4Ba	Pathogenicity assay	Phylogroup	Species identified
PAS1671				*Cucurbita pepo*	*Pspe*	+	NF/blue	-	+	+	*+ Pspe/Psl*	2ba-A	*Pspe*
PAS1789				*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-A	*Pspe*
PAS1790				*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-A	*Pspe*
PAS1791				*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-A	*Pspe*
PAS1792				*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-A	*Pspe*
PAS1793				*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-A	*Pspe*
PAS3812	P12855[1]	Chile	2007	*Cucurbita pepo*	*Pspe*					+	*+Pspe*[2]	2ba-A	*Pspe*
PAS3815	P69[1]	USA	2010	*Cucurbita pepo*	*Pspe*					+	*+Pspe*[3]	2ba-B	*Pspe*
PAS2985			2020	*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-C	*Pspe*
PAS3577			2022	*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-C	*Pspe*
PAS3578			2022	*Cucurbita pepo*	*Pspe*	+	NF	-	+	+	*+ Pspe/Psl*	2ba-C	*Pspe*
PAS3816	P88[1]	China	2011	*Cucurbita pepo*	*Pspe*					+	*+Pspe*[2]	2ba-C	*Pspe*
PAS3818	P77[1]	France	2010	*Cucurbita pepo*	*Pspe*					+	*+Pspe*[2]	2a-D	*Pspe*
PAS1670				*Cucurbita pepo*	*Pspe*					+	*+ Pspe/Psl*	2a-E	*Pspe*
PAS3820	P89[1]	France	2011	*Cucurbita pepo*	*Pspe*					+	*+Pspe*[2]	2a-E	*Pspe*
PAS3822	P108[1]	Chile	2011	*Cucurbita pepo*	*Pspe*					-	*+Pspe*[2]	2b	*Pspe*
PAS3824	P135[1]	India	2014	*Cucurbita pepo*	*Pspe*					-	*+Pspe*[2]	2d	*Pspe*
PAS3284			2021	*Cucurbita pepo*	*Psl*	+	NF/blue	-	+	-	*+ Pspe/Psl*	2b	*Pspe*
PAS 3808	CFBP2104	USA	1935	*Cucumis sativus*	*Psl*					-	*+ Pspe/Psl*	1a	*Psl*
PAS 3811	CFBP8534	USA	1935	*Cucumis sativus*	*Psl*					-	*+ Pspe/Psl*	1a	*Psl*
PAS1677	CFBP6463	Hungary	1958	*Cucumis sativus*	*Psl*	+	NF/blue	-	+	-	*+ Pspe/Psl*	3	*Psl*
PAS3731					*Psl*					-	to confirm	3	*Psl*
PAS3618			2022	*Cucurbita maxima*	*Psp*	+	NF/blue	-	+	-	*- Pspe/Psl*	2b	*Psp*
PAS3619			2022	*Cucurbita maxima*	*Psp*	+	NF/blue	-	+	-	*- Pspe/Psl*	2b	*Psp*
PAS3620			2022	*Cucurbita maxima*	*Psp*	+	NF/blue	-	+	-	*- Pspe/Psl*	2b	*Psp*
PAS1199				*Cucurbita pepo*	*Xc*					-	*+ Xc*		*Xc*
PAS2020					*Aac*					-	*+ Aac*		*Aac*
PAS156*				*Brassica oleracea*	*Psm*		NF/blue	-		-	*+ Psm*	1a	*Psm*
PAS3794			2023	*Raphanus sativus*	*Psm*		NF/blue	-		-	*+ Psm*	1a	*Psm*
PAS3442*			2017	*Beta vulgaris*	*Psapt*					-	*+ Psapt*	2b	*Psapt*
PAS3780*			2023	*Beta vulgaris*	*Psapt*	+	NF/blue	-	-	-	*+ Psapt*	2b	*Psapt*
PAS3781			2023	*Beta vulgaris*	*Psapt*	+	NF/blue	-		-	*+ Psapt*	2b	*Psapt*
PAS2668*			2020	*Phaseolus vulgaris*	*Pss*	+	blue	-		-	*+ Pss*	2b	*Pss*
PAS3048*	CFBP 4888	USA		*Phaseolus vulgaris*	*Pss*					-	*+ Pss*	2d	*Pss*
PAS1959	CFBP5321	France	1986	*Lycopersicon esculentum*	*Pc*	-	NF	+	-	-	*+ Pc*		*Pc*
PAS3750			2023	*Petroselinum crispum*	*Psapi*	-	NF/blue	-		-	*+ Psapi*		*Psapi*
PAS3574			2022	*Coriandrum sativum*	*Psc*	+	NF/blue	-		-	*+ Psc*		*Psc*
PAS1641	CFBP2067	Mexico		*Helianthus annuus*	*Psh*					+[4]	*+ Psh*	6	*Psh*
PAS185	CFBP6471	France	1999	*Phaseolus vulgaris*	*Psph*					-	*+ Psph*		*Psph*
PAS1554	CFBP2107	Switzerland	1927	*Phaseolus* sp.	*Pv*	-	yellow/blue	-	+	-	*+ Pv*		*Pv*



LEGENDS			
1	Strain from Lacault *et al.*, 2020	*Psc*	*P. syringae* pv. *coriandricola*
2	Pathogenecity assay realised in Lacault *et al.*, 2020	*Psci*	*P. syringae* pv. *cichorii*
3	Pathogenecity assay realised in Lacault thesis 2022	*Psg*	*P. syringae* pv. *glycinea*
4	3 amplicons including 1 of the expected size	*Psh*	*P. syringae* pv. *helianthi*
Psl	*P. syringae* pv. *lachrymans*	*Psm*	*P. syringae* pv. *maculicola*
Pspe	*P. syringae* pv. *'peponis'*	*Psmo*	*P. syringae* pv. *morprunorum*
Aac	*Acidovorax citrulli*	*Psmor*	*P. syringae* pv. *mori*
Pc	*P. corrugata*	*Pso*	*P. syringae* pv. *oryzae*
Pca	*P. cannabina* pv. *asilensis*	*Psp*	*Pseudomonas* sp.
Ps	*P. syringae*	*Psph*	*P. savastanoi* pv. *phaseolicola*
Psa	*P. syringae* pv. *aesculi*	*Pspi*	*P. syringae* pv. *pisi*
Psac	*P. syringae* pv. *actinidae*	*Pss*	*P. syringae* pv. *syringae*
Psace	*P. syringae* pv. *aceris*	*Pst*	*P. syringae* pv. *tomato*
Psapi	*P. syringae* pv. *apii*	*Psta*	*P. syringae* pv. *tabaci*
Psapt	*P. syringae* pv. *aptata*	*Pv*	*P. viridiflava*
Psav	*P. syringae* pv. *avellanea*	*Xc*	*Xanthomonas cucurbitae*

Phylogroups obtained:

VCZ strains:
- 2ba-A: 7
- 2ba-B: 1
- 2ba-C: 4
- 2a-D: 1
- 2a-E: 2
- 2b: 2
- 2d: 1

ALS strains:
- 1a: 2
- 3: 2

Non-targeted strains:
- 1a: 2 (*Psm*)
- 2b: 8 (*Psp, Psapt, Pss*)
- 2d: 1 (*Pss*)
- 6: 1 (*Psh*)

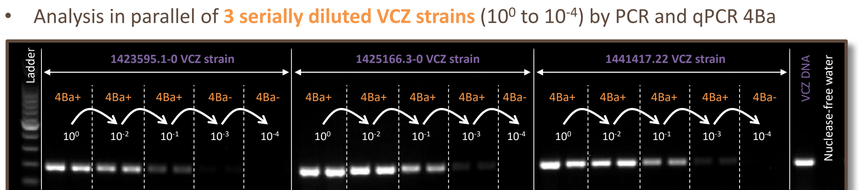
➢ **Evaluation of primer specificity:**
- Analysis of strain collection using the **qPCR method** developed for the **identification** of **VCZ** and **ALS** strains

VCZ primers = 4Ba (Lacault *et al.*, 2023)	Expected +	Expected -
Obtained +	18	4 (*Psapt* + Pss**)
Obtained -	0	18

ALS primers designed by GEVES	Expected +	Expected -
Obtained +	4	1 (*Psm**)
Obtained -	0	35

Primer specificity:
- **VCZ = 81.8%**
- **ALS = 97.2%**

➢ **Difference in sensitivity between PCR and qPCR 4Ba:**
- Analysis in parallel of **3 serially diluted VCZ strains** (10^0 to 10^{-4}) by PCR and qPCR 4Ba



PCR 4Ba negative

=

qPCR 4Ba Ct>24

qPCR 4Ba amplifies VCZ strains of 2b and 2d phylogroups

Conclusions

➢ **Identification of VCZ strains - from PCR to qPCR 4Ba:**
- **Increased sensitivity**
  - Detection of VCZ strains of **phylogroups 2b** and **2d**
    - Less severe symptoms than strains of phylogroups 2a and 2ba
- **Slightly decreased specificity**
  - Detection of several other strains of phylogroups 2b and 2d
    - Previously reported in Lacault *et al.*, 2023
    - ***P. syringae* pv. *syringae*: not pathogenic on cucurbits**
    - ***P. syringae* pv. *aptata*: pathogenic on cucurbits (melon) but not seed-borne**

➢ **qPCR identification for ALS strains:**
- **Good specificity**
  - **More strains** of ***P. syringae* pv. *lachrymans*** needed to confirm this specificity
  - Detection of another phylogroup 1a strain
    - ***P. syringae* pv. *maculicola*: not pathogenic on cucurbits**
- **Multiplexing** of VCZ and ALS strain identification
  - **Pathogenicity assays** to identify ALS strains **are no longer necessary**
  - **Save time** (at least 14 days)
  - **Reduced costs** for bacteriological analyses of cucurbit seeds

➢ **Next steps and prospects:**
- **Validation** of the method for identifying VCZ and ALS strains by qPCR
  - **Specificity** data to be completed for ALS primers if possible
  - Assessment of method **repeatability** and **reproducibility**
- **New service** available on the GEVES price list
  - Isolation on medium + **identification of suspect strains by qPCR**
  - Confirmation by **pathogenicity assay** of PCR-positive isolates as an **option**
- Development of a detection method directly from cucurbit seed macerates by **SE-qPCR**
  - Potential **ISF ISHI-Veg project** to come

10th International conference on *Pseudomonas syringae*
4-7 June 2024 - Porto