Development of tests for resistance/tolerance to virus yellows in sugar beet

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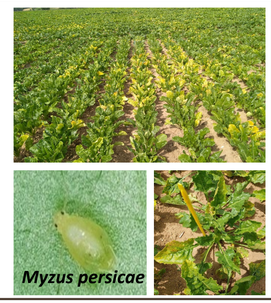
Yellows Resistbeet: PNRI Axis 2 project → P4.2-012

Context & aim

- **In order to find alternative solutions after the ban of neonicotinoids (NNI) in the fight against viruliferous aphids, the National Research and Innovation Plan (PNRI**) has launched several projects, which have to be effective and environmentally friendly by 2024.
- The **Yellows Resistbeet** project (2021-2024), led by GEVES, in partnership with ITB, aims to develop **a protocol for assessing varietal resistance/tolerance to Virus Yellows (VY) present in the EU**, transmitted by aphids, mainly *Myzus persicae :* **Beet Yellows Virus (BYV), Beet Chlorosis Virus (BChV), Beet Mild Yellowing Virus (BMYV) and Beet Mosaic Virus (BtMV).** The aim is to rapidly promote the inclusion of these tolerant/resistant varieties **in the French Catalogue**..



2021: Development of inoculation methods for Polerovirus and BYV in field

1. Production of plants with viruliferous aphids

Green aphid: Myzus persicae

Sowing
- Susceptible variety
- In tray

Transplantation
- 14 days post sowing
- In pots

Inoculation
- At 4 leaf stage
- Deposit of 20 aphids per plant

Check
- 28 dpi
- ELISA or RT-qPCR multiplex on aphids and plants

Use of inoculum
- 35 dpi
- Leaf pieces
- Pot

Ensured by GEVES, ITB & breeders:

2. Method of inoculation in field

Modalities

- **non inoculated**
- **BYV**
- **BChV**

→ **100** plants/plot, **4** reps/modality on a susceptible variety

Inoculation (2-4 leaf stage)

- **0%** Check
- **3%** inoculated **plants**
- **9%** inoculated **plants**
- **1 pot** by **plot**

→ leaf pieces

Date of aphicides in post inoculation (pi):

- **4 weeks pi**
- **6 weeks pi**

3. Parameters tested

- **Virus identification and spread by ELISA tests or RT qPCR**
- **Visual notations of symptoms: rate of infected surface area**
- **Sugar yield parameters**

4. Virus identification and spatio temporal spread in a plot

Method of inoculation	Inoculum density	BYV : % detection by ELISA		BChV : % detection by ELISA	
		Aphicide position		Aphicide position	
		4 weeks pi	6 weeks pi	4 weeks pi	6 weeks pi
% plants inoculated/plot	0%	0%	0%	8%	22%
	3%	8%	12%	65%	61%
	9%	33%	46%	93%	68%
Pot/plot	1 pot/plot	18%	26%	93%	61%

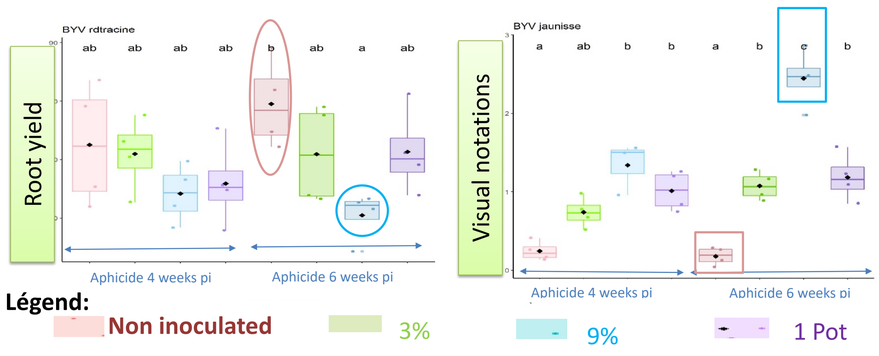
- **BChV : better detection & spread** with **Leaf pieces at 9% & 1 pot**
- **BYV: only with 9%**

5. Impact of BYV and BChV virus concentration on sugar yield

Method of inoculation	Inoculum density	BYV : Loss of sugar yield		BChV : Loss of sugar yield	
		Aphicide position		Aphicide position	
		4 weeks pi	6 weeks pi	4 weeks pi	6 weeks pi
% plants inoculated/plot	0%	0%	0%	0%	0%
	3%	3%	13%	-5%	21%
	9%	16%	30%	9%	29%
Pot/plot	1 pot/plot	12%	12%	15%	24%

For BYV & BChV: significant higher sugar yield loss (30%) with Leaf fragments on 9% inoculated plants, with an aphicide at 6 weeks pi

6. Correlations between yellow notations and root yield for BYV



- **BYV: variable partial negative correlation between Root yield & yellow notations:** up to -0.60
- **Idem for BChV**: up to -0.77

Conclusion: 9 % plants inoculated by leaf pieces, at stage 2-4 stage

2022: Evaluation of varietal resistance /tolerance (R/T) in tunnels and in field

1. Inoculation in tunnels & field

5 tunnels :
1) non inoculated
2) BYV
3) BChV
4) BMYV
5) Co-inoc BChV+BYV

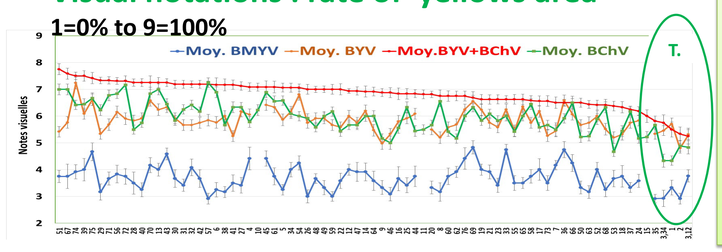
6 VCU trials :
1) non inoculated
2) BYV
3) BChV
4) BMYV

77 varieties inoculated with the modality 10% plant infected by leaf pieces

2. Evaluations

- **Visual notations : rate of yellows area**

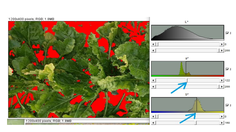
1=0% to 9=100%



- Significant differences in yellow symptoms,
- Less yellows in the 4 tolerant controls (T.)
- More symptoms with BYV+BChV co-inoculation than with BYV or BChV alone

- **RGB imaging with the « Phenoman » perch, by using Image J**

1. Leaf segmentation

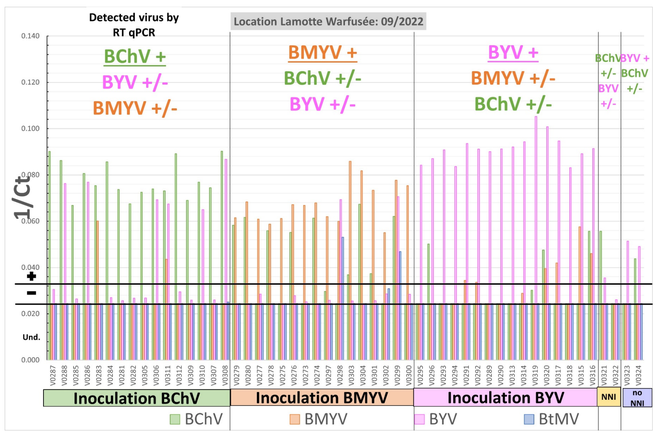


2. Yellows quantification

→ **Algorithm on going**

- **Control of virus identification by RT qPCR multiplex to 4 viruses: BYV, BChV, BMYV, BtMV at 2 dates:** July & september **(more details on P4.2-012)**

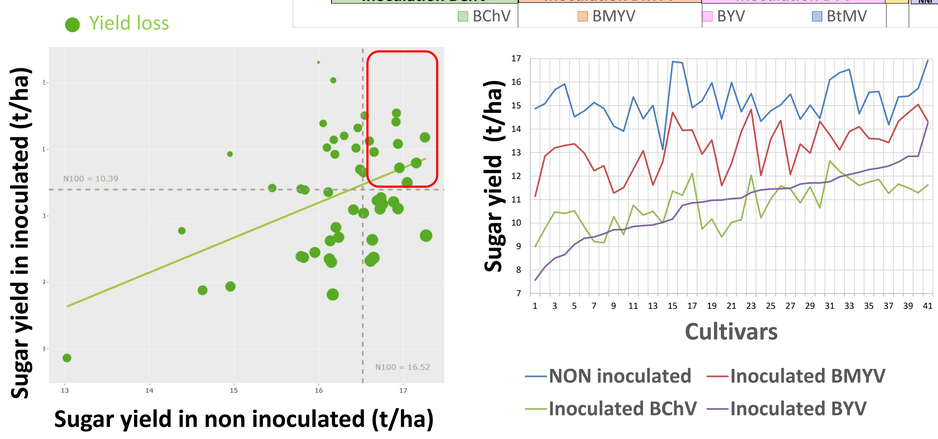
Tunnel inoculation could be complementary to field inoculation, in the case of secondary cross-contamination in the field



- **Sugar yield performance**

for BYV, BChV, BMYV vs non inoculated

Main criteria of decision for breeders & registration: Sugar yield performance in both inoculated & non inoculated conditions



Conclusion & prospect

- This project has enabled the development of a method for producing inoculum from viruliferous aphids, to define the parameters of inoculation ensuring a homogeneous virus infestation and significant discrimination of symptoms and yield between inoculated and non-inoculated modalities, to develop a multiplex RT-qPCR method for detecting and identifying these 4 viruses, and to study the most relevant criteria for assessing varietal tolerance, based mainly on productivity data.
- In 2023, R/T varietal evaluation in the field is ongoing, with a greater number of trials inoculated with BChV, BYV & BMYV, in order to identify new varieties with high sugar yield performance, under both inoculated and non-inoculated conditions. The development of this varietal tolerance/resistance assessment protocol will provide a genetic solution to NNI ban. This genetic lever will be offered to experimental pilot farms to develop an integrated pest management method in an agro-ecological context.

Poster ref. P1.3-009

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A new diagnostic tool for the identification of four beet yellows viruses by multiplex RT-qPCR

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Yellows Resistbeet: PNRI Axis 2 project
→ **P1.3-009**

Context

Virus yellows diseases in sugar beet (VY)

- Viral diseases that can cause yield losses of up to 50 %
- In Europe, complex of different viruses belonging to three viral *genera* present as mono- or co-infections
- Viruses transmitted by aphids whose populations can be controlled by the use of neonicotinoids (NNIs)

National Research and Innovation Plan (PNRI)

- NNIs banned since 2018 and 30 % yield losses in 2020
- Launch of a PNRI by the French government to fund projects with the aim of finding operational alternatives to NNIs against VY
- PNRI Yellows Resistbeet project is developing a sugar beet varietal evaluation protocol against VY (more details on **P1.3-009**)

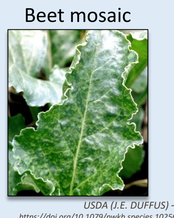
Virus detection and identification method

- Essential to have detection and identification test of the different viruses for varietal evaluation assays
- ELISA available, but not all viruses can be accurately identified.
- RT-PCR method available, but simultaneous identification of all viruses requires the development of multiplex RT-qPCR

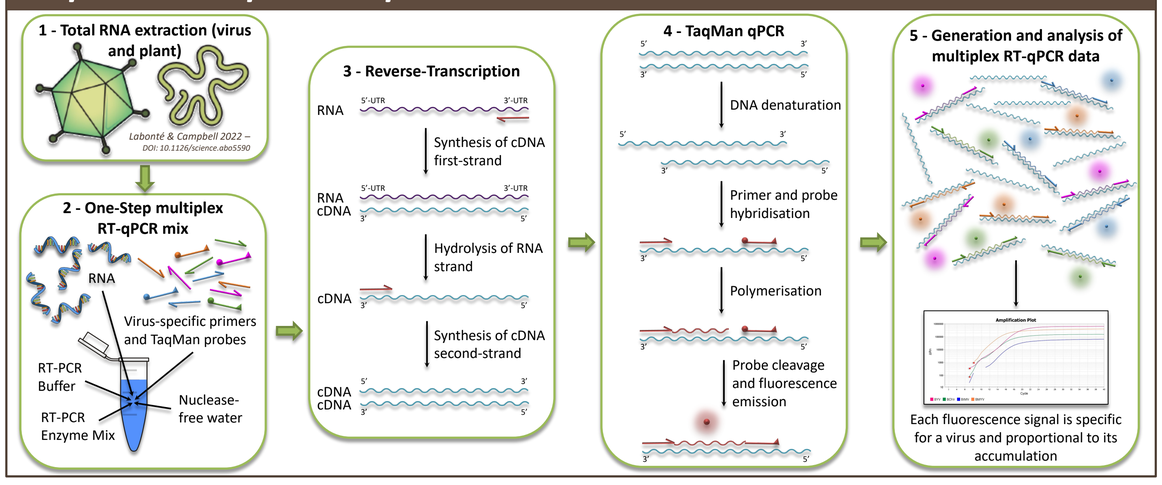
Virus yellows diseases in sugar beet

VY in sugar beet is caused in Europe by a complex of four different virus species present as mono- or co-infections: BChV, BMYV, BYV and BtMV. All viruses are mainly transmitted by the aphid *Myzus persicae.*

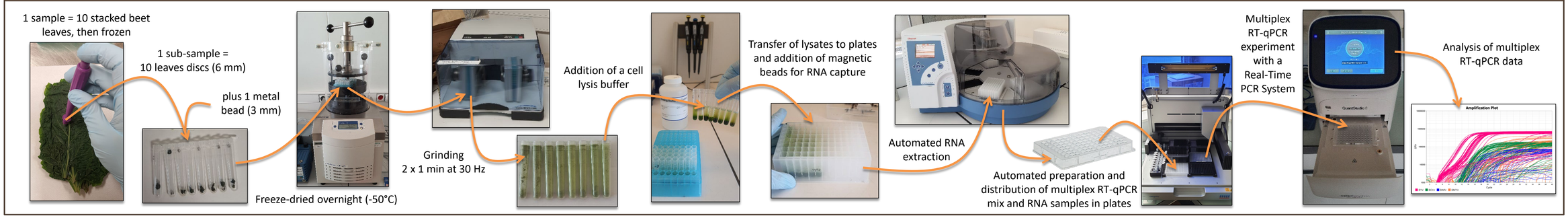
Myzus persicae - ITB - www.itbfr.org

BChV beet chlorosis virus	BMYV beet mild yellowing virus	BYV beet yellows virus	BtMV beet mosaic virus
Polerovirus		Closterovirus	Potyvirus
Persistent acquisition: 12-72h - retention: aphid all life		Semi-persistent acq.: few hours - ret.: 48h-72h	Non-persistent acq.: few min. - ret.: few min.
around 30 % yield loss		40-50 % yield loss	low yield loss
Moderate beet yellowing		Severe beet yellowing	Beet mosaic
			
GEVES – PNRI Yellows Resistbeet		*ITB – PNRI Yellows Resistbeet*	*USDA (J.E. DUFFUS) – https://doi.org/10.1079/pwkb.species.10256*

TaqMan multiplex RT-qPCR



Detection and identification method of the 4 viruses responsible for sugar beet yellows



Analytical specificity

Definition: Ability of an assay to detect the targeted pathogens (inclusivity) while excluding the non-targeted ones (exclusivity)

Method:

- Beet leaf samples for ***in vitro* testing**:
  - Infected with ≠ isolates of the 4 targeted viruses
  - Healthy
  - Infected with 2 non-targeted viruses
- For ***in silico* testing** with Primer-BLAST, all sequences belonging to :
  - Polerovirus
  - Closterovirus
  - Potyvirus

Sample tested \ Virus detected	BChV	BMYV	BYV	BtMV
BChV	+	-	-	-
BMYV	-	+	-	-
BYV	-	-	+	-
BtMV	-	-	-	+
Healthy beet	-	-	-	-
BWYV	-	-	-	-
TuYV	-	-	-	-
Inclusivity	100 %	100 %	100 %	100 %
Exclusivity	100 %	100 %	100 %	100 %
Analytical specificity	100 %	100 %	100 %	100 %

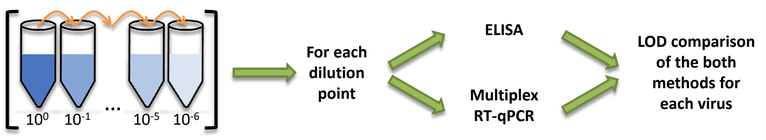
Conclusion: Inclusivity and exclusivity of all primers and TaqMan probes = 100 %

Multiplex RT-qPCR enables the specific detection of each of the four targeted beet yellows viruses

Analytical sensitivity

Definition: Smallest amount of the targeted pathogen that can be detected i.e. limit of detection (LOD)

Method:



Virus	Method	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	LOD
BChV	ELISA	2 + and 1 -	3 -	3 -	3 -	3 -	3 -	NA	$> 10^0$
	RT-qPCR	**3 +**	**3 +**	**3 +**	2 + and 1 -	1 + and 2 -	3 -	NA	10^{-2}
BMYV	ELISA	2 + and 1 -	3 -	3 -	3 -	3 -	3 -	NA	$> 10^0$
	RT-qPCR	**3 +**	**3 +**	**3 +**	**3 +**	1 + and 2 -	1 + and 2 -	3 -	10^{-3}
BYV	ELISA	**3 +**	**3 +**	1 + and 2 -	3 -	3 -	3 -	NA	10^{-1}
	RT-qPCR	**3 +**	**3 +**	**3 +**	**3 +**	**3 +**	2 + and 1 -	1 + and 2 -	10^{-4}
BtMV	ELISA	2 + and 1 -	2 + and 1 -	3 -	3 -	3 -	3 -	NA	$> 10^0$
	RT-qPCR	**3 +**	**3 +**	**3 +**	**3 +**	**3 +**	**3 +**	1 + and 2 -	10^{-5}

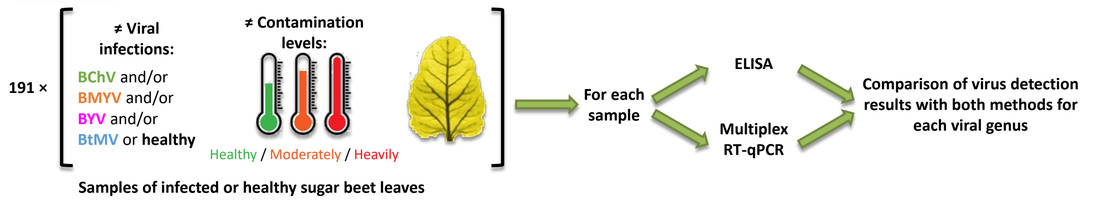
Conclusion: Multiplex RT-qPCR is always more sensitive than ELISA

Depending on the virus, LOD for multiplex RT-qPCR is 100 to 10,000 times lower than LOD for ELISA

Diagnostic sensitivity

Definition: Capacity to give a positive result when the pathogen is present (no false-negatives)

Method:



BChV + BMYV	RT-qPCR +	RT-qPCR -	Diagnostic sensitivity
ELISA +	58	0	100 %
ELISA -	61	40	

BYV	RT-qPCR +	RT-qPCR -	Diagnostic sensitivity
ELISA +	74	0	100 %
ELISA -	3	77	

BtMV	RT-qPCR +	RT-qPCR -	Diagnostic sensitivity
ELISA +	9	0	100 %
ELISA -	15	40	

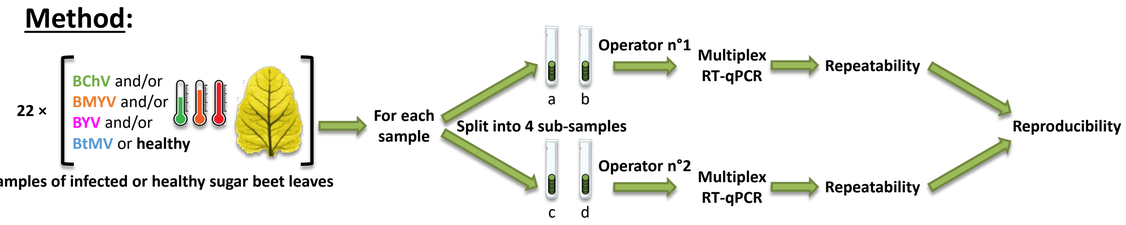
Conclusion: Diagnostic sensitivity for the four targeted viruses = 100 %

Multiplex RT-qPCR produces no false-negative results for all targeted viruses

Repeatability and Reproducibility

Definition: Capacity to produce the same result in the same lab by the same operator (repeatability) and the same result under different experimental conditions (reproducibility)

Method:



Virus detected	BChV	BMYV	BYV	BtMV
Repeatability n°1 (a+b)	100 %	100 %	100 %	100 %
Repeatability n°2 (c+d)	94.21 %	100 %	98.85 %	99.28 %
Reproducibility (1+2)	96.97 %	100 %	95.83 %	97.92 %

Conclusion: Repeatability > 94 % (for both operators) and Reproducibility > 95 %

For all targeted viruses, multiplex RT-qPCR is repeatable and reproducible

Prospects

Yellows Resistbeet project

- Control of *inocula* (upstream) and of inoculations (downstream) for varietal evaluation assays (more details on **P1.3-009**)
- Possibility valuation of using multiplex RT-qPCR semi-quantitative data and corresponding to virus accumulation to characterise the resistance or tolerance to VY of the sugar beet varieties

Within GEVES

- Providing services for customers
- Control tool for the Value for Cultivation, Use, and Sustainability testing (VCUS) for the registration of new sugar beet varieties in the French Catalogue, on the proposal of the Permanent Technical Committee for Plant Breeding (CTPS)

Further achievements

- Publication in a scientific journal in collaboration with INRAE
- Potential use of multiplex RT-qPCR as part of epidemiological surveillance to monitor the development of beet yellows in the field over time

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