

# **Harmonization of resistance test to diseases for DUS testing - 2**

## **Final report of the project: June 2012-June 2015**

### **I Summary page:**

**Coordinator of the project:** GEVES (F)  
**Other partners involved:** Naktuinbouw (NL)  
INIA (SP)  
Central Institute for Supervising and Testing in Agriculture (CZ),  
Palacky University (CZ)  
Bundessortenamt (D)  
Julius Kühn-Institut (D)  
Central Agricultural Office (HU)  
Science and Advice for Scottish Agriculture (UK)  
ESA (UE)

**Name of the contact person:** Aurelia LUCIANI (GEVES)  
**Name of the scientific coordinator:** Valérie GRIMAUULT (GEVES)

**Duration of the project:** 3 years

**Total estimated cost:** 373641 €.

### **Summary of the project:**

Following the successful achievement of a first project co funded by CPVO on the harmonization of resistance tests on vegetables in the European Union, a new project was proposed on another set of host/race/pathogen combinations. Seven European countries involved in vegetables DUS testing participate: Czech Republic, France, Germany, Hungary, the Netherlands, the United Kingdom and Spain. ESA participates, in link with the project currently under progress at ESA on definition of resistance and susceptibility and to have a harmonization of reference material between breeding and official tests to avoid differences between declaration and DUS tests. Seven host/race/pathogen combinations are studied: *Bremia lactucae*/lettuce, *Fusarium oxysporum* f. sp. *pisi* race 1/pea, *Ascochyta pisi* race C/pea, TMV: 0/pepper- PMMoV: 1.2/pepper - PMMoV:1.2.3/pepper- -PVY: 0/pepper. The objective of this new project is to harmonize the resistance tests corresponding to the selected host/pathogen combinations in terms of reference material (isolates and varieties), test conditions and notation scales, and to propose new harmonized and robust protocols to CPOV. A kick off meeting was organized in 2012, the first steering committee was organized the 13-14<sup>th</sup> of May 2013, and hosted by GEVES in Angers, France, the second steering committee was organized the 15-16 of April 2014 and hosted by Naktuinbouw in Roelofarendsveen, The Netherlands and the third steering committee was organized the 21-22 of April 2015 and hosted by INIA in Madrid. Results of the third year of the project and conclusions are presented.

### **Objectives of the project:**

The project aims at harmonizing, at the European level, resistance tests to seven vegetable diseases: *Bremia lactucae*/lettuce, *Fusarium oxysporum* f. sp. *pisi* race 1/pea, *Ascochyta pisi* race C/pea, TMV: 0/pepper- PMMoV: 1.2/pepper - PMMoV:1.2.3/pepper- PVY: 0/pepper. For each of them, the detailed objectives are:

- definition and validation of reference isolates, of maintainers varieties for obligate pathogens, of resistant/susceptible controls and of differentials to identify race/pathotypes of pathogens;

- definition and validation of tests conditions: environmental parameters (temperature..), strains, controls for a test to be used in all laboratories;
- definition and validation of notation scales;
- validation of reproducibility and repeatability of the developed tests;
- proposal for robust harmonized protocols.

## List of partners

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- Mr.: Semon Sergio (CPVO), Smilde Diederik (Naktuinbouw), Ludlage Richard (Naktuinbouw ), Kovács Ferenc (NEBIH), Tóbiás István (Julia-NKI), Allersma Tom (Monsanto), Kaagman Willem (ENZA), Jaunet Thierry (HM Clause), Willem Kaagman (Enza), Lebeda Ales (UPOL).

<b>II Detailed description of the project:</b>
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**1. Relevance for the system**

Genetic resistance to diseases is one of the major focuses for breeding programs of vegetables and many resistances to bacteria, fungi, viruses and nematodes have been introduced in commercial varieties. Resistance tests are used as a character for DUS testing and also as a grouping character and published in UPOV guidelines and CPVO protocols. Some resistance tests are compulsory, while others are not, but a lot of countries in the European Union cannot afford to apply them. It appears that each country does not use the same protocol and particularly the published one, by habit or because protocol conditions are not robust enough to

be used in different laboratories conditions. As a result, different isolates, resistance and susceptible controls and test conditions are used in the different European countries involved in the testing of a given disease. This can make comparisons of results between countries very difficult. Some efforts were therefore needed to harmonize protocols in order to obtain robust and reliable tests useable by all countries. Reliable resistance tests used as grouping characters should also enable to reduce the cost of the experiments and the time spent in comparisons of varieties.

From 2004 to 2006, three national examination offices in France, Spain and the Netherlands worked together to harmonize 7 protocols on tomato and bean: *Fusarium oxysporum* f. sp. *lycopersici* race0/tomato, *Fusarium oxysporum* f. sp. *lycopersici* race1/tomato, *Verticillium*/tomato, ToMV/tomato, BCMNV/bean, *Pseudomonas savastanoi* pv. *phaseolicola*/bean, *Colletotrichum lindemuthianum*/bean. This project was followed by one more year of validation for the *Pseudomonas savastanoi* pv. *phaseolicola*/bean test. Updated harmonized protocols have been proposed to CPVO and published on their website in the technical protocols section. For these protocols, common reference isolates, resistance and susceptible controls, differentials and test conditions have been validated. Protocols have also been updated compared to the bibliography (taxonomy, new races...). They should allow having better coherence of results between countries and between declarations of breeders and official tests, better definition and exchange of reference material (isolates, controls and differentials) and in the medium term some improvement in the management of the collections.

We now propose a new project for harmonization of resistance tests for DUS testing in the EU. We will focus on lettuce, pea and pepper pathogens.

## 2. Quality of the project

The priority host/pathogen combinations that will be studied have been selected in collaboration with partners of the project. These diseases were chosen according to the following criteria:

- they were compulsory
- they were commonly used as a grouping character for DUS testing
- the number of entries to be tested for these diseases was significant
- the protocols were known to be difficult and to give slightly different results depending on the test conditions
- they were of a high interest for the largest number of countries involved.

- *Bremia lactucae*/lettuce

This host pathogen combination was chosen because 6 countries out of 8 were interested in it, and because it was compulsory in CPVO protocols. For this host pathogen combination, differentials used as controls and strains have been harmonized at IBEB and will therefore be used as harmonized reference material in this project, but the protocols used can be different and varieties used as maintainers of isolates are not defined. When IBEB worked on the introduction of Bl: 27 it was shown that maintainer variety was very important to define as reference material. During official tests, we also observe differences of results compared to declarations and one explanation could be the protocol used (substrate, delay for notation). In the present project we propose to work with one Bl-x, to be defined at the start of the program.

- *Fusarium oxysporum* f. sp. *pisi* race 1/pea

Here again, this host pathogen combination was chosen because 6 countries out of 8 were interested in it, and because it was compulsory in CPVO protocols. To our knowledge, controls, differentials and isolates are not harmonized between laboratories. We will survey the different protocols used and perform appropriate ring tests.

- *Ascochyta pisi* race C/pea

This host pathogen combination was chosen because 4 countries out of 8 were interested. This protocol is not compulsory in CPVO protocols, but this choice allowed UK to participate to more than one ring test. To our knowledge, controls, differentials and isolates are not harmonized between laboratories. There are also differences in environmental conditions, methods of inoculation and notation scales, between laboratories including seed companies. We will list the different protocols used and test them by organising adapted ring tests.

- TMV:0/pepper- PMMoV:1.2/pepper - PMMoV:1.2.3/pepper- -PVY:0/pepper

These host/pathogen combinations were chosen because 4 countries out of 8 were interested and because it was compulsory in CPVO protocols. To our knowledge, controls, differentials and isolates are not harmonized between laboratories. We will make a survey of the different protocols used and perform the appropriate ring tests.

## **Project description**

The project is structured in 4 phases.

### *Phase 1: Description and comparison of the existing tests*

We have first got a better knowledge of the available protocols. For each test, we have gathered information from laboratories experienced since many years on the tests on:

- host/pathogen combination and the genetic of the corresponding resistance (resistance or partial resistance, influence of genetic background...)
- isolates used (virulence, culture, stability in culture)
- resistant and susceptible controls used
- environmental conditions of the test.

### *Phase 2: Selection of common reference material*

We have conducted inter laboratory comparative tests (CT), in laboratories experienced since many years on the tests, on isolates and controls used in the different countries to be able to validate common reference material.

### *Phase 3: Harmonisation of protocols*

We have conducted inter laboratory comparative tests (CT), in laboratories experienced since many years on the tests, with the reference material chosen in phase 2 to compare tests conditions and notation scales.

### *Phase 4: Validation of harmonized protocols*

Based on these results, we have selected/defined harmonized protocols. If some test conditions were equivalent, the protocols have taken this parameter into account to give a range of flexibility providing results on reference material are equivalent. These protocols have then been validated by specific inter laboratory comparative tests (CT).

Not all partners have participated to all CTs. The table below indicates the proposed ring tests and the corresponding participants for validation of protocols.

**Table 1: participants for ring test**

Species	Pathogen	Pathogen abbrev.	CZ	DE	ES	F	HU	NL	UK	ESA	participants per ring test
Lettuce	<i>Bremia</i>	Bl	Bl:x	Bl:x	Bl:x	Bl:x		Bl:x		Bl:x	6
Pea	<i>Fusarium</i>	Fop			race 1	race 1		race 1	race 1	race 1	5
	<i>Ascochyta</i>	Ap				race c			race c	race c	3
Pepper	Tobamoviruses				TMV : 0, PMMoV : 1.2 ; 1.2.3	TMV : 0, PMMoV : 1.2 ; 1.2.3	TMV : 0, PMMoV : 1.2 ; 1.2.3	TMV : 0, PMMoV : 1.2 ; 1.2.3		TMV : 0, PMMoV : 1.2 ; 1.2.3	5
	PVY	PVY			PVY:0	PVY:0	PVY:0	PVY:0		PVY:0	5
Ring tests per participant			1	1	6	7	4	6	2	7	

For ESA, 1 to 3 laboratories have been involved in the first ring tests of the protocols, and up to 4 laboratories have participated to the second step of validation of the harmonized protocols.

### III Report on year 3 activities:

Year 1 and 2 were focused on phase 1, phase 2 and phase 3.

#### *Phase 1: Description and comparison of the existing tests*

Test plans for comparative tests were set up based on the survey analyzed during the kick off meeting (annex 1 to 5).

#### *Phase 2: Selection of common reference material*

- Results of the ring test in 2012-2013
- Comparison of protocols
- Preparation of ring tests for 2013-2014
  - exchange of strains and differential hosts
  - quantity of seeds
  - date of sending

#### *Phase 3: Harmonisation of protocols*

- Results of the ring test in 2013-2014
- Choice of protocols
- Preparation of ring tests for 2014-2015
  - exchange of strains and differential hosts
  - quantity of seeds
  - date of sending

Year 3 was focused on phase 4.

#### *Phase 4: Validation of harmonized protocols*

- Results of the ring test in 2014-2015
- Validation of protocols
- Choice of reference materials



**Table 2 : Details of phases 1-3: survey of the pathogens and protocols used within the partners and preparation of the CTs**

	Year	Date	Who?	Actions
Phase 1	2011	Mid of June	GEVES	Sending of the draft questionnaire for definition of laboratories participating to first comparative tests (CTs)
		End of June	All partners	Comments about participation to CTs
		End of July	GEVES	Sending of the table summarizing the participation of the partners for each host/pathogen couple
	2012	Beginning of June	GEVES	Sending of the questionnaire on test based on protocols
		End of June	All partners	Comments about the draft questionnaire written by GEVES
		June 28th	GEVES	Sending of the completed questionnaires to all partners
		June 29 <sup>st</sup>	All partners	Kick-off meeting in France: Analysis of the questionnaire to define controls, isolates and protocols and preparation of ring tests (exchanges of seeds and hosts, calendar of setting up of tests)
		July	GEVES	Redaction of test plans
		August 7th	GEVES	Sending of test plan for the CT for Harmores2 tobamo, PVY pepper and <i>Fusarium</i> and <i>Ascochyta</i> Pea
		August 13th	GEVES	Sending of the test plan for the CT for Harmores2 <i>Bremia</i> 1
		August to October	All partners	Sending of isolates and seeds to GEVES
		October-November	GEVES	Preparation of materials for CTs
		October to December	GEVES	Sending of materials for CTs to partners
	2013	January to March	All partners	Realisation of CTs
		End of March-April	All partners	Sending of results of CTs to GEVES
		April	GEVES	Interpretation of results
		May 13-14 <sup>th</sup>	All partners	1st meeting in France: Results of the first ring test and preparation of second ring tests (exchanges of seeds and hosts, calendar of setting up of tests)
		August	GEVES-SNES	1st intermediate report
Phase 3	2013-2014	July to September	All partners	Realisation of pepper CTs
		September to October	All partners	Realisation of pea/ <i>Ascochyta</i> CTs
		October and March	All partners	Realisation of pea/ <i>Fusarium</i> CTs
		February to March	All partners	Realisation of lettuce CTs
		September to November	All partners	Sending of results of pepper CTs to GEVES
		March	All partners	Sending of results of pea/ <i>Ascochyta</i> CTs to GEVES
		December and May	All partners	Sending of results of pea/ <i>Fusarium</i> CTs to GEVES
		February to March	All partners	Sending of results of lettuce CTs to GEVES

Phase 4	2014- 2015	January to April	GEVES	Interpretation of results
		April 15-16 <sup>th</sup>	All partners	2nd meeting in The Netherlands: Results of the second ring test and preparation of third ring tests (exchanges of seeds and hosts, calendar of setting up of tests)
		August	GEVES-SNES	2nd intermediate report
		September	All partners	Realisation of pepper/PVY CTs
		August to December	All partners	Realisation of pepper/Tobamo CTs
		October to December	All partners	Realisation of pea/ <i>Ascochyta</i> CTs
		5 <sup>th</sup> November	All partners	Realisation of pea/ <i>Fusarium</i> WS
		December to March	All partners	Realisation of pea/ <i>Fusarium</i> CTs
		4 <sup>th</sup> November	All partners	Realisation of lettuce WS
		January to February	All partners	Realisation of lettuce CTs
		September to 6 <sup>th</sup> of March	All partners	Sending of results of pepper/PVY CTs to GEVES
		December to 6 <sup>th</sup> of March	All partners	Sending of results of pepper/Tobamo CTs to GEVES
		December to 6 <sup>th</sup> of March	All partners	Sending of results of pea/ <i>Ascochyta</i> CTs to GEVES
		6 <sup>th</sup> of March	All partners	Sending of results of pea/ <i>Fusarium</i> CTs to GEVES
		February to 6 <sup>th</sup> of March	All partners	Sending of results of lettuce CTs to GEVES
		6 <sup>th</sup> of March	All partners	Deadline for sending of results
		6 <sup>th</sup> of March to 3 <sup>rd</sup> of April	GEVES	Interpretation of results
		3 <sup>rd</sup> of April	GEVES	Sending of raw data to all partners
		April 21-22 <sup>nd</sup>	All partners	3rd meeting in Spain: Results of the third ring tests and validation of protocols
		June	GEVES-SNES	Final report

Main conclusions, decisions and remarks taken during the steering committee meeting are reported below. The presentation updated during the meeting is attached.



## I. Statistical analysis

Data were analysed using a statistical model derived from ISO 16140 for analysis of accuracy and reproducibility. This analysis is based on comparison of expected results defined by the variety description during kickoff meeting and results obtained by each partner on each isolate and variety during the ring test. The expected result + is for a variety expected with a susceptible comportment and expected result – is for a variety expected with a resistant comportment (tab. 3).

**Table 3: comparison records positive and negative agreement or positive and negative deviation**

	<b>expected result + (S)</b>	<b>expected result – (R)</b>
Obtained result +	positive agreement +/+ (PA)	positive deviation -/+ (PD)
Obtained result -	negative deviation +/- (ND)	negative agreement -/- (NA)

Calculation of accuracy was performed according to the following mathematical formulas: (no result, HG, Low Germination were considered as ND):

$$\text{Accuracy} = (\Sigma NA + \Sigma PA) / (\Sigma PA + \Sigma NA + \Sigma PD + \Sigma ND)$$

An accuracy of 1 showed that all labs obtained expected results.

Reproducibility count between laboratories the numbers of pairs both + or – (i.e. Number of accords) and number of possible pairs (total number of possible accords)

- The number of pairing/accords between n items is :  $n(n-1) / 2$
- Reproducibility = number of accords / number of possible accords between laboratories

A reproducibility of 1 showed that all labs obtained the same result.

For each disease, we presented the results of each strain for controls and differential hosts.

## II. Differentials host (HD)

Differential hosts are sets of plant varieties used to define strains of plant pathogens based on susceptible and resistant reactions. Reference strains are known characterized isolates of a given pathogen.

A differential set for a pathogen is defined by varieties from within one or several plant species that are hosts to the pathogen.

For each host/pathogen couple tested, differentials were added to controls to validate isolates as the expected pathotypes of pathogens.

### III. Pepper

According to the rules noted in the CPVO protocol (TP/076/2), one off-type plant is allowed for testing the uniformity of a variety (hybrid). Therefore the varieties interpreted heterogeneous by labs (with for example 1 susceptible plant out of 22) were judged resistant in the following results. This proposal by France was accepted by the steering committee to determine respectively the conformity of resistant and susceptible cultivars.

#### A. Pepper/Tobamovirus TMV: 0

##### 1. Materials and methods

One isolate of *Tobacco mosaic virus* (TMV) race 0: Vi-6, selected in phase 3, was tested in the labs. This isolate was:

- ✓ provided and multiplied by GEVES
- ✓ validated by ELISA by GEVES

Following phase 3, one critical control point in the future protocol is to specify that because tomato and tobacco *Nicotiana* Samsun have large leaves and can produce a lot of inoculum, they are recommended for the multiplication of TMV: 0.

The comparative test was performed on a panel made up of the same six controls (three susceptible + three resistant) as phase 3. Turia was suspected to have either a special genetic background or different resistance genes than Yolo Wonder and Feherozon. There was no addition of hot pepper varieties because in phase 3, it was confirmed that there was no atypical necrosis and mosaic observed on these varieties.

The isolate was tested in one lab on all the controls (= panel and differentials), differentials used during CTs are highlighted in yellow (tab.4).

**Table 4: Proposal differentials for Pepper Tobamovirus (TMV and PMMoV) ISF working group**

Variety /Pathotype	Gene present	Strain			
		TMV:0	TMV:1	PMMoV: 1-2	PMMoV: 1-2-3
		P0	P1	P1-2	P1-2-3
Lamu, Early Carlwonder	-	S	S	S	S
Tisana, Yolo Wonder	L1	R	S	S	S
Tabasco	L2	R	R	S	S
Solario F1, <b>Novi 3</b> , PI159236	L3	R	R	R	S
<b>Tom4</b> , PI260429	L4	R	R	R	R

Tests were performed at two stages of inoculation (cotyledons and 1st leaf), on twenty plants per variety.

Symptoms to record were defined in phase 3:

- Symptoms of susceptibility
  - Mosaic (aucuba)
  - Growth reduction
  - Death of plants
- Symptoms of resistance
  - Local necrotic lesions
  - Systemic necrosis
  - Vein necrosis
  - Stem necrosis

If a plant has no symptoms, it was considered as escape from inoculation.

Two dates of notation were recorded: at 4 to 7 days post-inoculation for notation of local necrosis lesions and at 2 weeks post-inoculation (with an optional extension to 3 weeks if necessary), depending on symptoms on controls or heterogeneous comportment.

## 2. Results

### a) Date of notation

Two dates of notation were recorded: 4 to 7 and 14 (optional 21) days post inoculation. In the tables, the numbers in bold indicate the date chosen for interpretation of results.

The labs 1, 10 and 14 concluded at second notation at cotyledon stage and did not need a third notation (tab. 5).

**Table 5: comparison of periods of notation for TMV: 0 at cotyledon stage**

Dpi	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	
1st notation	7	4	3	7	<b>11</b>	6	4	7	[3 to 11]
2nd notation	<b>18</b>	7	10	14	18	<b>14</b>	7	<b>11</b>	[7 to 18]
3rd notation	/	11	<b>17</b>	21	25	/	12	/	[11 to 25]

The lab 1 concluded at second notation at first leaf stage and did not need a third notation (tab. 6).

**Table 6: comparison of periods of notation for TMV: 0 at 1<sup>st</sup> leaf stage**

Dpi	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	
1st notation	7	4	3	7	7	7	4	4	[3-7]
2nd notation	<b>14</b>	7	<b>10</b>	14	14	<b>14</b>	7	7	[7-14]
3rd notation	/	9	17	21	21	21	12	<b>11</b>	[9-21]

At cotyledon stage, only two out of eight labs have followed the expected dates of notation. At first leaf stage, only four out of eight labs have followed the expected dates of notation. There was a significant difference between expected dates and the effective dates of notation between labs depending of the symptoms on the susceptible control. One critical control point in the future protocol is to specify that recommended dates of notation should be adapted depending of expression of symptoms on controls.

### b) Comparison of stage of inoculation

The comparison of stages of inoculation was done at the date of final interpretation per labs (tab. 7 and 8).

## (1) Cotyledon stage

**Table 7: results for TMV: 0 at cotyledon stage**

Variety	Expected Results	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	Accuracy					Reproducibility			
										PA	PD	NA	ND		PA	NA	TA	
Turia	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Fehérözön	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Yolo Wonder	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Piquillo	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Pepita	S	S	S	NT	S	S	S	NT	S	6	0			1	15		15	1
Lamu	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Tom 4	R	R																
Novi 3	R	R																

R: resistant; S: susceptible; NT: not tested

There was a good concordance between labs. All labs obtained expected results. Following these results, due to its stability, the strain Vi-6 was validated at cotyledon stage. The protocol was validated at cotyledon stage.

## (2) 1st leaf stage

**Table 8: results of TMV: 0 at 1st leaf stage**

Variety	Expected Results	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	Accuracy					Reproducibility			
										PA	PD	NA	ND		PA	NA	TA	
Turia	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Fehérözön	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Yolo Wonder	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Piquillo	S	S	S	S	R	S	S	S	S	7	1			0.88	21		28	0.75
Pepita	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Lamu	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Tom 4	R	R																
Novi 3	R	R																

R: resistant; S: susceptible;

There was a good concordance between labs. All labs obtained expected results, except one lab for one variety Piquillo. Because of the consistency of results in phase 3 and the consistency of the results of the other labs in phase 4, we have not interpreted Piquillo as non-conform. This result was due to this test in this lab.

Following these results, due to its stability, the strain Vi-6 was validated at first leaf stage. The protocol was validated at first leaf stage.

### ***c) Summary of results***

#### **(1) Strains**

The strain Vi-6 showed mosaic symptoms type “aucuba” easy to read and a strong mosaic (fig. 1).

Taking into account results and the symptoms of this strain, the strain Vi-6 was validated at both stages of inoculation: cotyledon and first leaf stage.



**Figure 1: strain Vi-6**

#### **(2) Stage of inoculation**

Both stages of inoculation selected in phase 3 were validated on controls in phase 4.

#### **(3) Date of notation and symptoms observed**

The three dates of notation selected in phase 3 were validated in phase 4:

- 4 to 7 dpi: local necrotic lesions on inoculated organ, risk of leaf/cotyledon drop after 7 days
- 14 dpi: symptoms of susceptibility (mosaic, growth reduction or death of plants)
- 21 dpi: optional, same symptoms as 14 days













The third date of notation need to be retained but only as optional. The varieties will be tested in different conditions some in climatic chambers, some in greenhouse and this has an effect on the expression of symptoms. Expression of susceptibility could be spread over time

One critical control point in the future protocol is to specify that environmental conditions can have an effect on the expression of symptoms over time. In this case a third notation could be necessary.

#### (4) Controls












The six controls were validated at both stages of inoculation (tab. 9 & 10).

**Table 9: susceptible controls for TMV: 0**

Varieties	Symptoms observed					Comments
Pepita						white spots with mosaic
Lamu						1 <sup>st</sup> leaf : mosaic and plant death due to strong mosaic development
Piquillo						No remark

The three susceptible varieties were proposed as reference material.

**Table 10: resistant controls for TMV: 0**

Varieties	Symptoms observed					Comments
Turia						No remark
Fehérözön						No remark
Yolo Wonder						No remark

No difference were observed between Turia and Yolo Wonder on one side and Feherozon on the other side, so no conclusion can be drawn on any differences between these 3 varieties.

The three resistant varieties were proposed as reference material.



### **3. Proposal harmonized protocol for TMV: 0**

The phase 4 of validation of protocol allowed defining the elements for the future harmonized protocol.

➤ Strains:

The strain Vi-6 of TMV: 0 was selected.

➤ Stage of inoculation:

Both stages of inoculation were validated.

➤ Varieties:

The six controls selected in phase 3 were validated in phase 4. It will be specified to choose in test one of the selected one:

- Susceptible controls: Pepita or Lamu or Piquillo
- Resistant controls: Turia or Feherozon or Yolo Wonder

The three resistant controls (Turia, Feherozon, Yolo Wonder) are susceptible to PMMoV: 1.2 and PMMoV: 1.2.3 and allow to confirm the race of Tobamovirus.

➤ Notation:

The dates of notation selected in phase 3 were validated in phase 4:

- First notation will be done at 4-7 days post-inoculation for observation of local necrosis.
- Second notation will be done at two weeks post-inoculation and could be extended at three weeks if necessary (depending on symptoms on controls or heterogeneous comportment).

The notation will be performed based on the symptoms of susceptibility and resistance defined in phase 3 and validated in phase 4:

- Symptoms of susceptibility
  - Mosaic (aucuba in case of aucuba strain)
  - Growth reduction
  - Death of plants
- Symptoms of resistance
  - Local necrotic lesions which can lead to leave drop
  - Systemic necrosis
  - Vein necrosis
  - Stem necrosis

One critical control point in the future protocol is to specify that plants with no symptoms at all have to be interpreted as escapes of inoculation.



## B. Pepper/Tobamovirus PMMoV: 1.2

### 1. Materials and methods

One isolate of *Pepper mild mottle virus* (PMMoV) race 1.2: nt203, selected in phase 3, was tested in the labs. This isolate was:

- ✓ provided by the partner
- ✓ multiplied by partner or by GEVES
- ✓ validated by ELISA by GEVES

The comparative test was performed on a panel made up of the same seven controls (three susceptible + four resistant) as phase 3.

Feherozon was included in comparison with Turia to bring to light either a special genetic background or different resistance genes.

Tests were performed at one stage of inoculation (cotyledons), on twenty plants per variety.

Symptoms to record were defined in phase 3:

- Symptoms of susceptibility
  - Mosaic (sometimes death of plants)
  - Growth reduction
- Symptoms of resistance
  - Local necrosis lesions which can lead to leaf dropping
  - Systemic necrosis

Two dates of notation were recorded: at 4 to 7 days post-inoculation for notation of local necrosis lesions and at 2 weeks post-inoculation (with an optional extension to 3 weeks if necessary), depending on symptoms on controls or heterogeneous comportment.

### 2. Results

#### a) Date of notation

Two dates of notation were recorded: 4 to 7 and 14 (optional 21) days post inoculation. In the tables, the numbers in bold indicate the date chosen for interpretation of results.

The labs 1, 2 and 14 concluded at second notation at cotyledon stage and did not need a third notation (tab. 11).

**Table 11: comparison of periods of notation for PMMoV: 1.2 at cotyledon stage**

Dpi	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	
1st notation	7	5	3	7	<b>12</b>	7	4 & 7	5	<b>[3 to 12]</b>
2nd notation	<b>15</b>	<b>15</b>	10	14	19	14	12	<b>12</b>	<b>[10 to 19]</b>
3rd notation	/	/	<b>17</b>	21	26	<b>21</b>	<b>18</b>	/	<b>[12 to 26]</b>

At cotyledon stage, only five out of eight labs have followed the expected dates of notation. There was a significant difference between expected dates and the effective dates of notation between labs depending of the symptoms on the susceptible control. One critical control point in the future protocol is to specify that recommended dates of notation should be adapted depending of expression of symptoms on controls

### ***b) Validation of stage of inoculation and dates of notation***

The validation of stage of inoculation was done at the date of final interpretation done per labs (tab. 12).

**Table 12: Results PMMoV: 1.2 at cotyledon stage**

Variety	Expected Results	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	Accuracy					Reproducibility			
										PA	PD	NA	ND		PA	NA	TA	
Novi 3	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Candella	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Internal R check ESA	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Ferrari	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Turia	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Fehérözön	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Yolo wonder	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Lamu	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1

R: resistant; S: susceptible;

It was shown a good concordance between labs. All labs obtained expected results. Following these results, due to its stability, the strain nt203 was validated at cotyledon stage. The protocol was validated at cotyledon stage.

### ***c) Summary of results***

#### **(1) Strain**

The strain nt203 showed symptom of local necrosis and was easy to read.

Taking into account results and the symptoms of this strain, the strain nt203 was validated at cotyledons stage of inoculation.

#### **(2) Date of notation and symptoms observed**

The three dates of notation selected in phase 3 were validated in phase 4:

- 4 to 7 dpi: local necrotic lesions which can lead to cotyledon drop. After this date these necrosis can hardly be seen on fallen cotyledons
- 14 dpi: symptoms of susceptibility (mosaic (green), growth reduction or rarely death of plants)
- 21 dpi: optional, same symptoms as 14 days







The third date of notation need to be retained but only as optional. The varieties will be tested in different conditions some in climatic chambers, some in greenhouse and this has an effect on the expression of symptoms. Expression of susceptibility could be spread over time.

One critical control point in the future protocol is to specify that environmental conditions can have an effect on the expression of symptoms over time. In this case a third notation could be necessary.

### (3) Controls

The seven controls were validated at cotyledon stage of inoculation (tab. 13 & 14).




**Table 13: susceptible controls for PMMoV: 1.2**

Varieties	Symptoms observed	Comments
Turia		No remark
Fehérözön	 	No remark
Yolo Wonder	 	No remark
Lamu		Weak mosaic symptoms

No difference were observed between Turia and Feherozon on the other side, so no conclusion can be drawn on any differences between these 2 varieties.

The four susceptible varieties were proposed as reference material.

**Table 14: resistant controls for PMMoV: 1.2**

Varieties	Symptoms observed	Comments
Novi 3		No remark
Candella		No remark
Internal R check ESA		No remark

Ferrari		Die in one lab
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Only three resistant varieties (Novi 3, Candella and Ferrari) were proposed as reference material, because internal R check was not decoded.

### 3. **Proposal harmonized protocol for PMMoV: 1.2**

The phase 4 of validation of protocol allowed defining the elements for the future harmonized protocol.

➤ **Strain:**

The strain nt203 of PMMoV: 1.2 was validated.

➤ **Stage of inoculation:**

Cotyledon stage of inoculation selected in phase 3 was validated in phase 4.

➤ **Varieties:**

The seven controls selected in phase 3 and Fehérözön were validated in phase 4 but only seven varieties were kept. It will be specified to choose in test one of the selected one:

- Susceptible controls: Turia or Fehérözön or Yolo Wonder or Lamu
- Resistant controls: Novi 3 or Candella or Ferrari

The three susceptible controls are (Turia, Feherozon and Yolo Wonder are resistant to TMV: 0 and the three resistant controls (Novi 3, Candella, Ferrari) are susceptible to PMMoV: 1.2.3 and this allow to confirm the race of Tobamovirus.

➤ **Notation:**

The dates of notation selected in phase 3 were validated in phase 4:

- First notation will be done at 4-7 days post-inoculation for observation of local necrotic lesions which can lead to cotyledon drop. After this date these necrosis can hardly be seen on fallen cotyledons.
- Second notation will be done at two weeks post-inoculation for observation of symptoms of susceptibility (mosaic (green), growth reduction or rarely death of plants) and could be extended at three weeks if necessary (depending on symptoms on controls or heterogeneous comportment).

The notation will be performed based on the symptoms of susceptibility and resistance defined in phase 3 and validated in phase 4:

- Symptoms of susceptibility
  - mosaic (green)
  - growth reduction
  - rarely death of plants
- Symptoms of resistance
  - local necrotic lesions which can lead to cotyledon drop
  - Systemic necrosis

## C. Pepper/Tobamovirus PMMoV: 1.2.3

### 1. Materials and methods

Three isolates (Eve, Samsun latens and Nt204) of *Pepper mild mottle virus* (PMMoV) race 1.2.3 were compared in the labs. Each isolate was:

- ✓ provided by the partner
- ✓ multiplied by partner or by GEVES
- ✓ validated by ELISA by GEVES

The comparative test was performed on a panel made up of the same seven controls (three susceptible + four resistant) as phase 3 and Lamu and Novi 3 in one lab as differentials.

Tests were performed at one stage of inoculation (cotyledons), on twenty plants per variety.

Symptoms to record were defined in phase 3:

- Symptoms of susceptibility
  - Mosaic (sometimes death of plants)
  - Growth reduction
- Symptoms of resistance
  - Local necrosis lesions which can lead to leaf dropping
  - Systemic necrosis

Two dates of notation were recorded: at 4 to 7 days post-inoculation for notation of local necrosis lesions and at 2 weeks post-inoculation (with an optional extension to 3 weeks if necessary), depending on symptoms on controls or heterogeneous comportment.

### 2. Results

#### a) Date of notation

Two dates of notation were recorded: 4 to 7 and 14 (optional 21) days post inoculation. In the tables, the numbers in bold indicate the date chosen for interpretation of results.

The labs 1, 2, 10 and 14 concluded at second notation at cotyledon stage for the strain Eve and did not need a third notation (tab. 15).

**Table 15: comparison of periods of notation for PMMoV: 1.2.3 at cotyledon stage for strain Eve**

Dpi	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	
1st notation	7	7	3	7	<b>11</b>	5	4	5	[3 to 11]
2nd notation	<b>15</b>	<b>15</b>	<b>10</b>	14	18	<b>16</b>	7	<b>12</b>	[7 to 18]
3rd notation	/	/	17	22	25	/	<b>12</b>	/	[12 to 25]

The labs 1, 2 and 14 concluded at second notation at cotyledon stage for the strains Samsun latens and Nt204 and did not need a third notation (tab. 16).

**Table 16: comparison of periods of notation for PMMoV: 1.2.3 at cotyledon stage for strains Samsun latens and Nt204**

Dpi	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	
1st notation	7	7	3	7	<b>11</b>	5	4	5	[3 to 11]
2nd notation	<b>15</b>	<b>15</b>	<b>10</b>	14	18	<b>16</b>	7	<b>12</b>	[7 to 18]
3rd notation	/	/	17	22	25	/	<b>12</b>	/	[12 to 25]

For the three strains, only three out of eight labs have followed the expected dates of notation.

There was a significant difference between expected dates and the effective dates of notation between labs depending of the symptoms on the susceptible control.  
One critical control point in the future protocol is to specify that recommended dates of notation should be adapted depending of expression of symptoms on controls.

### ***b) Comparison of strains***

The comparison of strains was done at the date of final interpretation done per labs (tab. 17, 18 & 19).

**Table 17: results of strain Eve of PMMoV: 1.2.3 at cotyledon stage**

Variety	Expected Results	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	Accuracy				Reproducibility			
										PA	PD	NA	ND	PA	NA	TA	
Bisonte	R	R	R	R	R	R	R	R	R			8	0	1	28	28	1
Century	R	R	R	R	R	R	R	R	R			8	0	1	28	28	1
Friendly	R	R	NG	R	R	R	R	R	R			7	0	1	21	21	1
Tom 4	R	R	R	R	R	R	R	R	R			8	0	1	28	28	1
Candella	S	S	S	S	S	S	S	S	S	8	0			1	28		1
Ferrari	S	S	S	S	S	S	S	S	S	8	0			1	28		1
Yolo Wonder	S	S	S	S	S	S	S	S	S	8	0			1	28		1
Lamu	S	S															
Novi 3	S	S															

R: resistant; S: susceptible; NG: no germination

It was shown a good concordance between labs. All labs obtained expected results. Following these results, due to its stability, the strain Eve of PMMoV: 1.2.3 was validated at cotyledon stage. The protocol was validated at cotyledon stage.

**Table 18: results of strain Samsun latens of PMMoV: 1.2.3 at cotyledon stage**

Variety	Expected Results	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	Accuracy				Reproducibility			
										PA	PD	NA	ND	PA	NA	TA	
Bisonte	R	R	R	R	R	R	R	R	R			8	0	1	28	28	1
Century	R	R	R	R	R	R	Seg?	R	R			7	1	0.88	21	28	0.75
Friendly	R	R	R	R	R	R	R	R	R			8	0	1	28	28	1
Tom 4	R	R	R	R	R	R	Seg?	R	R			7	1	0.88	21	28	0.75
Candella	S	S	S	S	S	S	S	S	S	8	0			1	28		1
Ferrari	S	S	S	S	S	S	NT	S	S	7	0			1	21		1
Yolo Wonder	S	S	S	S	S	S	S	S	S	8	0			1	28		1
Lamu	S	S															
Novi 3	S	S															

R: resistant; S: susceptible; NT: not tested; Seg: segregation

It was shown a good concordance between labs. All labs obtained expected results excepted one lab for the varieties Century and Tom 4. The working group concluded that this result was due to this test in this lab. Following these results, due to its stability, the strain Samsun latens of PMMoV: 1.2.3 was validated at cotyledon stage. The protocol was validated at cotyledon stage.

**Table 19: results of strain Nt204 of PMMoV: 1.2.3 at cotyledon stage**

Variety	Expected Results	Lab 1	Lab 2	Lab 4	Lab 5	Lab 9	Lab 10	Lab 11	Lab 14	Accuracy					Reproducibility			
										PA	PD	NA	ND		PA	NA	TA	
Bisonte	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Century	R	R	R	R	R	R	Seg?	R	R			7	1	0.88		21	28	0.75
Friendly	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Tom 4	R	R	R	R	R	R	R	R	R			8	0	1		28	28	1
Candella	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Ferrari	S	S	S	S	S	S	S	S	S	8	0			1	28		28	1
Yolo Wonder	S	NG	S	S	S	S	S	S	S	7	0			1	21		21	1
Lamu	S	S																
Novi 3	S	S																

R: resistant; S: susceptible; NG: no germination; Seg: segregation

It was shown a good concordance between labs. All labs obtained expected results excepted one lab for the variety Century. The working group concluded that this result was due to this test in this lab. Following these results, due to its stability, the strain Nt204 of PMMoV: 1.2.3 was validated at cotyledon stage. The protocol was validated at cotyledon stage.

### ***c) Summary of results***

#### **(1) Strains**

Taking into account results and the symptoms of this strain, the three strains (Eve, Samsun latens and Nt204) were validated at cotyledons stage of inoculation. But during the phase 4, it was observed one lab effect linked to Century and Tom 4 with strains Samsun latens and Nt204. Only the strain Eve will be proposed for the harmonized protocol.

#### **(2) Date of notation and symptoms observed**

The three dates of notation selected in phase 3 were validated in phase 4:

- 4 to 7 dpi: local necrotic lesions which can lead to cotyledon drop. After this date these necrosis can hardly be seen on fallen cotyledons
- 14 dpi: symptoms of susceptibility (mosaic (green), growth reduction or rarely death of plants)
- 21 dpi: optional, same symptoms as 14 days

The third date of notation need to be retained but only as optional. The varieties will be tested in different conditions some in climatic chambers, some in greenhouse and this has an effect on the expression of symptoms. Expression of susceptibility could be spread over time.




One critical control point in the future protocol is to specify that environmental conditions can have an effect on the expression of symptoms over time. In this case a third notation could be necessary.

#### **(3) Controls**

The seven controls were validated at cotyledon stage of inoculation (tab. 20 & 21).






**Table 20: susceptible controls for PMMoV: 1.2.3**

Varieties	Symptoms observed	Comments
Candella		No remark
Ferrari		Rough leaf surface
Yolo Wonder		Rough leaf surface

The three susceptible varieties were proposed as reference material.

**Table 21: resistant controls for PMMoV: 1.2.3**

Varieties	Symptoms observed	Comments
Bisonte		No remark
Century		Necrosis on leaves
Friendly		Plant with leaf dropping and retarded growth (I); dead plants due to systemic necrosis. The presence of an abscission layer can be

		tested by touching the dead leaf
Tom 4		Plant with local necrosis

The four resistant varieties were proposed as reference material.

### 3. **Proposal harmonized protocol for PMMoV: 1.2.3**

The phase 4 of validation of protocol allowed defining the elements for the future harmonized protocol.

➤ **Strain:**

The strain Eve of PMMoV: 1.2.3 was validated and selected.

➤ **Stage of inoculation:**

Cotyledon stage of inoculation selected in phase 3 was validated in phase 4.

➤ **Varieties:**

The seven controls selected in phase 3 were validated in phase 4. It will be specified to choose in test one of the selected one:

- Susceptible controls: Candella, Ferrari, Yolo Wonder
- Resistant controls: Bisonte, Friendly, Tom 4

We advice to chose differentials (Candella or Ferrari) as susceptible controls because they are resistant to PMMoV: 1.2 or to add the differentials in tests to confirm the race.

➤ **Notation:**

The dates of notation selected in phase 3 were validated in phase 4:

- First notation will be done at 4-7 days post-inoculation for observation of local necrotic lesions which can lead to cotyledon drop. After this date these necrosis can hardly be seen on fallen cotyledons.
- Second notation will be done at two weeks post-inoculation for observation of symptoms of susceptibility (mosaic (green), growth reduction or rarely death of plants) and could be extended at three weeks if necessary (depending on symptoms on controls or heterogeneous compartment).

The notation will be performed based on the symptoms of susceptibility and resistance defined in phase 3 and validated in phase 4:

- Symptoms of susceptibility
  - mosaic (green)

- growth reduction
  - rarely death of plants
- Symptoms of resistance
  - local necrotic lesions which can lead to cotyledon drop
  - Systemic necrosis

## D. Pepper/PVY: 0

### 1. Materials and methods

One isolate of *Potato virus Y* (PVY) race 0: zb6, selected in phase 3, was tested in the labs. This isolate was:

- ✓ provided by the partner
- ✓ multiplied by partner or by GEVES
- ✓ validated by ELISA by GEVES

Following phase 3, one first critical control point in the future protocol is to specify that because problem of stability of PVY: 0 after shipment by BOS, it is recommended to send fresh leaves. One second critical control point in the future protocol is to specify that because tobacco *Nicotiana tabacum* cv. *xanthi* NC have large leaves and can produce a lot of inoculum and have a faster multiplication, they are recommended for the multiplication of PVY: 0.

The comparative test was performed on a panel made up of the same six controls (four susceptible + two resistant) as phase 3 but Yolo Y because seeds of this variety were not available for phase 4.

Tests were performed at cotyledon stage of inoculation, on twenty plants per variety.

Three conditions of tests selected in phase 3 were compared: greenhouse with shade, greenhouse without shade and climatic chamber in each lab according its facilities (tab. 22).

**Table 22: condition tested in labs for PVY: 0**

	Lab 1	Lab 2	Lab 4	Lab 5	Lab 8	Lab 9	Lab 10	Lab 11	Lab 14
Green house with shade	X		X	X	X		X		
Green house without shade	X	X			X	X	X		
Climatic chamber	X	X					X	X	X

Symptoms to record at 3 weeks post-inoculation were defined in phase 3:

- Symptoms of susceptibility
  - Mosaic
  - Growth reduction
  - Vein banding
- Symptoms of resistance
  - No symptoms
  - Necrosis

In two labs, controls were tested on different races of Potyvirus (at least PVY races 0, 1 and 1.2) to determine the resistance gene involved.

## 1. Results

### a) Date of notation

One date of notation was recorded: 21 days post inoculation (tab. 23). In the tables, the numbers in bold indicate the date chosen for interpretation of results.

**Table 23: date of notation for PVY: 0**

Lab 1	Lab 2	Lab 4	Lab 5	Lab 8	Lab 9	Lab 10	Lab 11	Lab 14	Dpi
<b>21</b>	<b>21</b>	<b>21</b>	13; <b>28</b>	<b>21</b>	<b>22</b>	18; <b>27</b>	11;15; <b>18</b>	<b>21</b>	<b>[21 to 28]</b>

### b) Comparison of condition of test

The comparison of condition of test was done at the date of final interpretation done per labs (tab. 24, 25 & 26).

#### (1) Greenhouse with shade

**Table 24: results for PYV: 0 for Greenhouse with shade**

Variety	Expected Results	Lab 1	Lab 4	Lab 5	Lab 8	Lab 10	Accuracy					Reproducibility			
							PA	PD	NA	ND		PA	NA	TA	
Yolo wonder	S	R	S	S	S	S	4	1			0.80	6		10	0.60
internal S check	S	S	S	S	S	R	4	1			0.80	6		10	0.60
Piquillo	S	S	S	S	S	S	5	0			1	10		10	1
Ferrari	S	S	S	S	S	DI	4	1			0.80	6		10	0.60
Vidi	R	LG	R	R	R	DI			3	1	0.75		3	6	0.50
Andalus	R	R	R	R	R	R			5	0	1		10	10	1

R: resistant; S: susceptible; LG: low germination; DI: difficult to interpret

Three out of five labs obtained expected results.

#### (2) Greenhouse without shade

**Table 25: results for PYV: 0 for Greenhouse without shade**

Variety	Expected Results	Lab 1	Lab 2	Lab 8	Lab 9	Lab 10	Accuracy					Reproducibility			
							PA	PD	NA	ND		PA	NA	TA	
Yolo wonder	S	S	S	S	S	S	5	0			1	10		10	1
internal S check	S	S	S	S	S	S	5	0			1	10		10	1
Piquillo	S	S	S	S	S	S	5	0			1	10		10	1
Ferrari	S	S	S	S	S	S	5	0			1	10		10	1
Vidi	R	LG	R	R	R	R			4	0	1		6	6	1
Andalus	R	R	R	R	R	R			5	0	1		10	10	1

R: resistant; S: susceptible; LG: low germination;

It was shown a good concordance between labs. All labs obtained expected results. Following these results, due to its stability, the condition greenhouse without shade and the strain zb6 of PVY: 0 were validated at cotyledon stage. The protocol was validated at cotyledon stage.

### (3) Climatic chamber

**Table 26: results for PVY: 0 for climatic chamber**

Variety	Expected Results	Lab 1	Lab 2	Lab 10	Lab 11	Lab 14	Accuracy					Reproducibility			
							PA	PD	NA	ND		PA	NA	TA	
Yolo wonder	S	S	S	S	S	S	5	0			1	10		10	1
internal S check	S	S	S	S	Nec S	S	5	0			1	10		10	1
Piquillo	S	S	S	S	S	S	5	0			1	10		10	1
Ferrari	S	S	S	S	S	R	4	1			0.80	6		10	0.60
Vidi	R	R	R	R	R	R			5	0	1		10	10	1
Andalus	R	R	R	R	R	R			5	0	1		10	10	1

R: resistant; S: susceptible; Nec: necrosis

It was shown a good concordance between labs. All labs obtained expected results, excepted one lab on Ferrari.

Following these results, due to its stability, the condition climatic chamber and the strain zb6 of PVY: 0 were validated at cotyledon stage. The protocol was validated at cotyledon stage.

### c) Summary of results

#### (1) Strain

Taking into account results and the symptoms of this strain, the strain zb6 of PVY:0 was validated at cotyledon stage of inoculation.

One critical control point in the future protocol is to specify that because problem of stability of PVY: 0 shipments are recommended to be done with fresh infected leaves.

#### (2) Conditions of test

Two out of three conditions of tests selected in phase 3 (tab. 26) were validated on controls in phase 4 (tab. 27).

**Tableau 27: comparison of condition of tests for PVY: 0 in phase 3**

	Greenhouse with shade	Greenhouse without shade	Climatic chamber
Yolo Wonder	3S/3	1S/1	2S/2
Ferrari	3S/3	1S/1	2S/2
Internal S check	3S/3	1S/1	2S/2
Piquillo	3S/3	1S/1	2S/2
Andalus	3R/3	1R/1	2R/2
Vidi	3R/3	1R/1	2R/2
Yolo Y	3R/3	1R/1	2R/2

R: resistant; S: susceptible



The tests of phase 3 were performed in summer (September).

**Tableau 28: comparison of condition of tests for PVY: 0 in phase 4**

	Greenhouse with shade	Greenhouse without shade	Climatic chamber
Yolo Wonder	4S/1R	5S	5S
Ferrari	4S/1 DI	5S	4S/1R
Internal S check	4S/1R	5S	4S/1Nec S
Piquillo	5S	5S	5S
Andalus	5R	5R	5R
Vidi	3R / 1 DI/1 LG	4R/1LG	5R

R: resistant; S: susceptible; DI: difficult to interpret; LG: low germination; Nec: necrosis













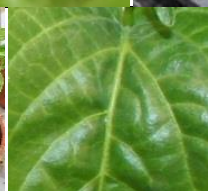

The tests of phase 4 were performed in Autumn (November-December).

Because of the consistency of results in phase 3 and the consistency of the results of the other conditions of test in phase 4, we have not interpreted the results of the condition greenhouse with shade as non-conform. This result was certainly due to the low daylight in the autumn period. The protocol is validated in the three conditions (condition depending of period).

### (3) Controls

The six controls were validated at the three conditions of test (tab. 29 & 30).





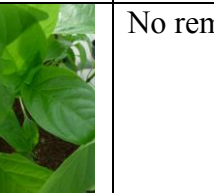


**Table 29: susceptible controls for PVY: 0**

Varieties	Symptoms observed				Comments
Yolo Wonder					Mosaic
Ferrari					Mosaic
Internal S check					Red vein, growth reduction, vein banding
Piquillo					Growth reduction, vein banding, mosaic

Only three out of four susceptible varieties (Yolo Wonder, Ferrari and Piquillo) tested in phase 4 were proposed as reference material, because internal S check was the variety Yolo Wonder.



**Table 30: resistant controls for PVY: 0**

Table 36: Resistant controls for T + T: 0						
Varieties	Symptoms observed					Comments
Andalus						No remark
Vidi						No remark
Yolo Y						Not tested in phase 4 but validated in phase 3

Both resistant varieties tested in phase 4 were proposed as reference material. Because of the consistency of results of Yolo Y in the previous phases of test, this variety was also proposed as reference material.

#### (4) Resistance gene involved in controls

Two labs (Lab 1 in climatic chamber and Lab 4 in greenhouse) have tested the controls on different races of Potyvirus (at least PVY races 0, 1 and 1.2) to determine the resistance gene involved (tab. 31).

**Table 31: determination of resistance gene involved in controls for PVY**

	PVY: 0	PVY: 1	PVY: 1.2		
	Phase 4 results (zb6)	Lab 1 (Sicile 15)	Lab 1 (SON41)	Lab 4 (nt163)	Pvr gene
Yolo Wonder	S	S	S	S	<i>Pvr0</i>
Ferrari	S	S	S	S	<i>Pvr0</i> *
Piquillo	S	S	S	S	<i>Pvr0</i> *
Yolo Y	R	S	S	/	<i>pvr 1<sup>1</sup> (pvr 2<sup>1</sup>)</i>
Florida VR2	R	R	S	/	<i>pvr 1<sup>2</sup> (pvr 2<sup>2</sup>)</i>
Andalus	R	R	S	S	<i>pvr 1<sup>2</sup> (pvr 2<sup>2</sup>)</i> *
Vidi	R	R	S	S	<i>pvr 1<sup>2</sup> (pvr 2<sup>2</sup>)</i> *

\* Resistance gene supposed according to the phenotypic comportment of the variety compared with the differentials.

According to the results in both labs, the varieties Ferrari and Piquillo are supposed to have the same genetic (*Pvr0*) as Yolo Wonder and the varieties Andalus and Vidi are supposed to have the same resistance gene *pvr I<sup>2</sup>* (*pvr 2<sup>2</sup>*) as Florida VR2.

## **2. Proposal harmonized protocol for PVY: 0**

The phase 4 of validation of protocol allowed defining the elements for the future harmonized protocol.

### ➤ Strains:

The strain zb6 of PVY: 0 was selected.

### ➤ Stage of inoculation:

Cotyledon stage of inoculation selected in phase 3 was validated in phase 4.

### ➤ Varieties:

Only six controls selected in phase 3 were tested and validated in phase 4. Yolo Y was previously validated in phase 3. Internal S check is the variety Yolo Wonder and so it is not selected.

It will be specified to choose in test one of the selected one:

- Susceptible controls: Yolo Wonder or Ferrari or Piquillo
- Resistant controls: Andalus or Vidi or Yolo Y

If Andalous or Vidi are used as resistant control without Yolo Y, PVY:0 can be confused with PVY:1.

One critical control point in the future protocol is to specify to chose Yolo Y as resistant control or to add the differentials in tests to be able to observe a possible contamination by PVY: 1 or 1.2.

### ➤ Conditions of test

The three conditions of tests (climatic chamber and greenhouse with and without shade) were validated.

One critical control point in the future protocol is to specify that in case of test in greenhouse during period of low daylight, shade should not be used.

### ➤ Notation:

The date of notation at 21 days post-inoculation selected in phase 3 was validated in phase 4.

The notation will be performed based on the symptoms of susceptibility and resistance defined in phase 3 and validated in phase 4:

- Symptoms of susceptibility
  - Mosaic (can be very light/faint)
  - Growth reduction
  - Vein banding and vein necrosis
- Symptoms of resistance
  - No symptoms

## IV. Lettuce

Previous ring test results on comparison of different substrates and different notation scales showed comparable results between laboratories. Sand and blotter were less aggressive than soil. The three substrates (soil, sand and blotter) were validated in phase 3.

One critical control point in the future protocol is to specify that in case of heterogeneity or non conformity on paper or sand a confirmation on soil was needed.

But different assessors did not record the same results for some varieties. Moreover, we observed that different official notation scales (CPVO, UPOV and IBEB) were described.

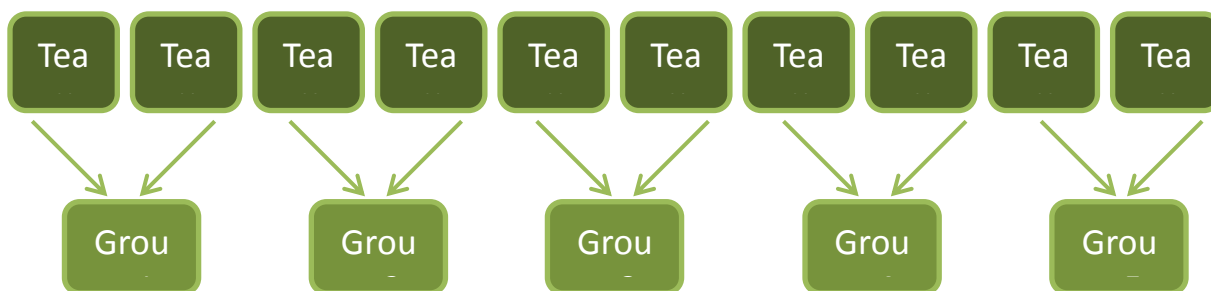
In the framework of Harmores 2, GEVES organized in autumn 2014 a workshop to draw the line between resistance and susceptibility around light or sparse sporulation, and to harmonize a common notation scale and interpretation of observed symptoms. Twenty five people from national examination offices and ESA representatives of seven European countries participated.

### A. Workshop Lettuce/*Bremia lactucae*

#### 1. Workshop results

During this workshop, a panel of twenty-one “difficult to judge” varieties from the reference collection, which gave different results when retested, was compared on three substrates (soil, sand and blotter). One hundred thirty-two boxes, each with seventy plants, were prepared in total.

The first step of the workshop was the notation of plants for each substrate. Symptoms observed and notations of each participant (scoring on a group basis for a first phase of common interpretation fig.2) were compared to identify the origin of differences of interpretation.



**Figure 2: Notation of plants by team of two people, Interpretation by groups of 2/3 teams**

The second step of the workshop was the common work of harmonization done based on the interpretation and on the symptoms observed by all participant and merged in a final table combining all the results (tab. 32). Each case of variety with different interpretations between groups was discuss, plants were observed with a camera placed on a binocular magnifier. For each variety, based on symptoms, the working group defined a common interpretation.

**Table 32: example of varieties with different interpretations between groups in the WS *Bremia***

				Proposition																
				Soil					Sand					Paper						
	Box	Varieties	Expected	Gp 1	Gp 2	Gp 3	Gp 4	Gp 5	Gp 1	Gp 2	Gp 3	Gp 4	Gp 5	Gp 1	Gp 2	Gp 3	Gp 4	Gp 5		
Bl: 24	5	TS	S	S	S	S	S	S	S	<u>S</u>	S	S	S	S	S	S	S	S		
		A	R	R	R	R	R	HG*	R	<u>HG</u>	R	R	R	R	R	R	R	R		
		B	R	R*	<u>S*</u>	<u>R*</u>	R*	?	R	R	R	R	R	R	R	R	R	R		
		C	R	R*	<u>S*</u>	S	R*	?	R	R	R*	R	R	R	R	R	R	R		
	6	F	R	R	R	R	?	R	R	HG	R*	R*	HG	R	R	R	R	R		
		G	R	<u>S</u>	0	0	s	?	R	R	R	R	R	R	R	R	R	R*		
		H	R	R*	<u>S*</u>	S	R	?	R	R	R*	R*	R	R	R*	R	R	R*		
	7	TS	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
		TR	R	R	R	R*	R	R*	R	R	R	R	R	R	R	R	R	R		
		K	R	S	<u>S*</u>	S	?	S*	R	R	R	R*	R	R	R	R	R	R*		
		K	R	S	S*	S	?	S*	R	R	R	R*	R	R	R	R	R	R*		
	Bl: 26	12	N	R	R	<u>S*</u>	S	?	HG*	R	R	R	R	R	R	R	R	R	R	
O			R	S	S*	R*	R	?	R	R	R	R	R	R	R	R	R	R		
P			R	R	S*	<u>R*</u>	?	?	R	R	R	R	R	R	R	R	R	R		
Q			R	R	R*	R*	R	R	R	R	R*	R*	?	R	R	R	R	R		
13		T	R	HG	<u>S*</u>	R*	R*	<u>S*</u>	R	R	R	R	R	R	R	R	R	R*		
		U	R	R	<u>S*</u>	S*	R*	<u>S*</u>	?	R	R	R*	R*	R	R	R	R	R		
		V	R	R*	R	R*	R*	R	R	R	HG	R*	R*	R	R	R	R	R		
14		Y	R	S	S*	S*	R*	?	R	R	R	R	R	R	R	R	R	R		
		Y	R	S	S*	S*	R*	?	R	R	R	R	R	R	R	R	R	R		
		Z	R	S	S*	S*	R*	S*	R	R	R	<u>S*</u>	R	R	R	R	R	R		
		Z	R	<u>S</u>	<u>S*</u>	S*	R*	S*	R	R	R	S*	R	R	R	R	R	R		
		AA	R	<u>S</u>	<u>S*</u>	S*	R	HG	R	R	R	R	R	R	R	R	R	R		

Gp X: group composed of two teams of two people; TS: susceptible control; TR: resistant control;

S : susceptible; R: resistant; \*: to be discussed; HG: heterogeneous; ?: not interpretable.

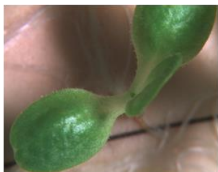




Results revealed that soil substrate favors the growth of plants and the sporulation of *Bremia*. Assessors, who were not used to test on soil, could misinterpret these strong symptoms. The interpretation was more homogeneous between assessors with the sand and blotter substrates. With the sand substrate, spores could be confused with sand.

A harmonized notation scale was established at the end of the workshop. Only two level of resistance were defined: absence and presence (fig. 3&4). All symptoms observed for both interpretations were reported.

Following the workshop a draft of illustration (from the pictures taken during the WS) of the new notation scale was sent to each participant for validation and to propose if necessary another picture for each note from their own photo library. Based on the participant feedback, a final harmonized notation scale with illustration was defined (fig 3, 4 & 5).




**Figure 3: symptoms of susceptibility to *Bremia lactucae***

Resistant				
				
No sporulation + no necrosis	No sporulation + necrosis	Weak sporulation (much less than susceptible control) + necrosis	Weak sporulation less than susceptible control not evolving between second and third notation + necrosis	In some cases very sparse sporulation can occur (without necrosis) and does not evolve between 2 <sup>nd</sup> and 3 <sup>rd</sup> notation

**Figure 4: symptoms of resistance to *Bremia lactucae***

One critical control point in the future protocol is to specify that if symptoms are not interpretable (normal sporulation with necrosis = other case), another test on bigger plants or on another substrate must be undertaken (fig. 5).

Other case	
Normal sporulation (same level as susceptible control) with necrosis <b>→ in this case no interpretation, another test on bigger plants or other substrate must be undertaken</b>	

**Figure 5: Other case on *Bremia lactucae***

## 2. Workshop dissemination

During the workshop, it was decided that the first dissemination of the harmonized notation scale would be the presentation of a three slide mini-presentation and a poster during the Eucarpia Leafy Vegetables congress in April 2015.

Based on the results of the workshop, a common work with the Naktuinbouw was done for the revision of the Lettuce UPOV guideline, which was presented and accepted during the UPOV Technical Working Party for Vegetables in June 2015.

A revision of the CPVO Lettuce protocol will be proposed during the Vegetable Expert Meeting of November 2015.

## B. Comparative tests Lettuce/*Bremia lactucae*

Following the workshop, a comparative test was performed using the new notation scale and the new rule of interpretation. This comparative test including Harmores 2 partners was extended to other breeding companies.

### 1. Materials and methods

Two races of *Bremia lactucae* Bl: 24 and Bl: 26 were compared in sixteen labs. Each race was:

- ✓ provided by GEVES
- ✓ multiplied by GEVES

Each lab used its own substrate (soil, sand or blotter/paper).

The comparative test was performed on twenty plants per variety, on a panel of 6 varieties: resistant, susceptible or with problems of interpretation in previous tests (tab. 33).

**Table 33: varieties tested in comparative test for *Bremia lactucae***

	<b>Bl: 24</b>	<b>Bl: 26</b>
<b>Resistant</b>	Bedfrod RYZ-line (Murai-res)	Bedfrod RYZ-line (Murai-res)
<b>Susceptible</b>	Green Towers Colorado	Green Towers Discovery
<b>Difficult to judge</b>	Design Femke	Diola Galavia

A first notation was performed as soon as when susceptible control sporulated well. Second and third notations were performed 3-4 days after the first notation and 14 days after inoculation.

### 2. Results

#### a) Dates of notation

Seven labs did not make the first notation (tab. 34).

**Table 34: comparison of dates of notation for *Bremia lactucae***

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 7	Lab 9	Lab 10	Lab 12	Lab 13 paper	Lab 13 soil	Lab 14	Lab 15	Lab 16	Lab 17	Lab 18	Lab 19	Lab 20	<b>Dpi</b>
Bl:24/Bl: 26																		
1st notation	7	7	7	11	7	6	8	10	6	6	7	8	9	11	7	11	7	[6-11]
2nd notation	11	9	11	13	10	9	11	/	8	8	10	13	12	14	10	/	10	[8 to 14]
3rd notation	14	/	14	14	14	/	/	/	11	11	14	/	/	22	14	/	14	[11 to 22]

The rule of the first notation date has not been followed in five labs where the susceptible control was not validated (judged resistant or not interpretable). That is why the comparison of results by notation cannot be presented.

Not every lab gave interpretation for each date of notation. The comparison of results by days post inoculation cannot be presented.

a) ***Bremia lactucae* race 24**

The results will be presented (tab. 35) based on the last interpretation done by labs (highlighted in the table 34).

**Table 35: Results presented for final interpretation of labs for Bl: 24**

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 7	Lab 9	Lab 10	Lab 12	Lab 13	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Lab 18	Lab 19	Lab 20	
BI: 24	Soil	Soil	Soil	Blotter	Paper	Paper	Soil	Sand	Paper	Soil	Soil	Soil	Soil	Paper	Paper	paper	Paper	
Green Tower	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	
Colorado	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
Design	R	R	R	S	R	R	R	S	R	HG	S	S	R	R	R	S	R	S
Femke	R	R	R	R	R	R	R	R	R	R	HG	R	R	R	R	R	R	R
RYZ-line (Murai-res)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
Bedford	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

S: susceptible; R: resistant; HG: heterogeneous; NI: not interpreted

Nine out of seventeen labs obtained expected results for Bl: 24.

Seven labs judged the variety Design (expected resistant) as susceptible or heterogeneous. Moreover, one lab judged the variety Femke as heterogeneous.

Lab 19 judged the susceptible control as resistant, an inversion with RYZ-line could not be confirmed or infirmed.

In the interpretation of results by some labs, the varieties with normal sporulation with necrosis “other case” were judged susceptible or heterogeneous unlike the decision rule. That is why the GEVES presented the results at second and third notation including the other cases (tab. 36).

**Table 36: Results presented for 2nd and 3rd interpretation (including other case) for Bl: 24**

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 7	Lab 9	Lab 10	Lab 12	Lab 13	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Lab 18	Lab 19	Lab 20
Bl: 24	Soil	Soil	Soil	Blotter	Paper	Paper	Soil	Sand	Paper	Soil	Soil	Soil	Soil	Paper	Paper	paper	Paper
Green Tower	S	S	S	S	S/OC	S	S	S	HG/S	S	S	S	S	S	OC	R	S/OC
Colorado	S	S	S	S	HG/OC	S	S	S	S	S	S	S	S	OC	S/OC	S	S/OC
Design	R	R	R	S	R	R	R	S	R/HG	OC	S	R	R	R	OC	R	OC
Femke	R	R	R	R	R	R	R	R	R	R/HG	R	R	R	R	R	R	R
RYZ-line (Murai-res)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Bedford	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

S: susceptible; R: resistant; HG: heterogeneous; NI: not interpreted; OC: other case

X: same interpretation at 2<sup>nd</sup> and 3<sup>rd</sup>; X: 2<sup>nd</sup> interpretation; X: 3<sup>rd</sup> interpretation; X: 1<sup>st</sup> interpretation

Using the rule of interpretation established during the workshop, seven out of seventeen labs obtained expected results for Bl: 24.

Two times out of three, when labs judged the susceptible control as other case at the third notation, the control was conform at the second notation.



Two times out of four, when labs judged the variety Colorado as other case at the third notation, the variety was conform at the second notation.  
 One time out of seven, when labs judged the variety Design as other case, susceptible or heterogeneous at the third notation, the variety was conform at the second notation.  
 When the variety Femke was judged as heterogeneous at the third notation, the variety was conform at the second notation.

### b) *Bremia lactucae* race 26

As for BI: 24, the results will be presented (tab. 37) by the last interpretation done by labs (highlighted in the table 34).

**Table 37: Results presented for final interpretation of labs for BI: 26**

		Lab 1	Lab 2	Lab 3	Lab 4	Lab 7	Lab 9	Lab 10	Lab 12	Lab 13	Lab 13	Lab 14	Lab 15	Lab 17	Lab 18	Lab 19	Lab 20
BI: 26		Soil	Soil	Soil	Blotter	Paper	Paper	Soil	Sand	Paper	Soil	Soil	Soil	Paper	Paper	paper	Paper
Green Tower	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		S
Discovery	S	S	S	S	S	R	R	S	S	S	S	S	S	R	S	S	S
Bedford	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Diola	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R		S
Galavia	R	R	R	R	R	R	R	R	R	HG	R		R	S	R	R	S
RYZ-line (Murai-res)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

S: susceptible; R: resistant; HG: heterogeneous; NI: not interpreted

Ten out of seventeen labs obtained expected results for BI: 26.

Three labs judged the variety Discovery (expected susceptible) as resistant. Three labs judged the variety Diola (expected resistant) as susceptible. Moreover, three labs judged the variety Galavia as susceptible or heterogeneous.

In the interpretation of results by some labs, the varieties with normal sporulation with necrosis “other case” were judged susceptible or heterogeneous unlike the decision rule. That is why the GEVES presented the results at second and third notation including the other cases (tab. 38).

**Table 38: Results presented for 2nd and 3rd interpretation (including other case) for BI: 26**

		Lab 1	Lab 2	Lab 3	Lab 4	Lab 7	Lab 9	Lab 10	Lab 12	Lab 13	Lab 13	Lab 14	Lab 15	Lab 17	Lab 18	Lab 19	Lab 20
BI: 26		Soil	Soil	Soil	Blotter	Paper	Paper	Soil	Sand	Paper	Soil	Soil	Soil	Paper	Paper	paper	Paper
Green Tower	S	S	S	S	S	OC	S	S	S	S	S	S	S	S	OC		S
Discovery	S	S	S	S	S	HG	R	S	S	S	S	S	S	R	S	S	S
Bedford	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Diola	R	R	R	R	R	R	R	R	R	HG/OC	HG/OC	R	R	R	R		HG/OC
Galavia	R	R	R	R	R	R	R	R	R	HG	R		R	HG	R	S	HG/OC
RYZ-line (Murai-res)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

S: susceptible; R: resistant; HG: heterogeneous; NI: not interpreted; OC: other case

X: same interpretation at 2<sup>nd</sup> and 3<sup>rd</sup>; X: 2<sup>nd</sup> interpretation; X: 3<sup>rd</sup> interpretation; X: 1<sup>st</sup> interpretation

Using the rule of interpretation established during the workshop, nine out of seventeen labs obtained expected results for BI: 26.

Two labs judged the susceptible control as other case.

Three labs judged the variety Discovery (expected susceptible) as resistant.  
Three labs judged the variety Diola (expected resistant) as heterogeneous or other case.  
Moreover, three labs judged the variety Galavia as heterogeneous or other case.

### ***c) Summary of results***

#### **(1) Varieties**

The susceptible varieties (Green Tower and Colorado for Bl : 24 and Green Tower and Discovery for Bl: 26) gave unexpected results on soil and paper substrates for both races.

Some varieties expected difficult to interpret are always difficult to judge with the new notation scale and the new decision rule:

- Design (expected resistant) was judged susceptible, heterogeneous or other case in seven labs not depending on substrate used.
- Femke (expected resistant) was judged heterogeneous only in one lab.
- Diola and Galavia (expected resistant) were judged heterogeneous or other case mainly on paper and blotter

These results confirmed the limits identified in a previous IBEB ring test and in the Harmores comparative tests. There are varieties, which, due to their genetic background, will always give different results from one test to the next. Therefore, tests with inoculation at cotyledon stage may not be suitable for varieties with this genetic background. Development of test with an inoculation at a later stage (5-7 leaf stage) might help in judging these varieties.

#### **(2) Date of notation**

The working group decided to keep the first notation (as soon as when susceptible control sporulated well) and the second notation (3-4 days after the first notation) and specified that it was important to comply to these stage of notation to be able to interpret results.

In the case in which the interpretation of the variety is possible at second notation, the third notation is not necessary. In the other case, if the interpretation of the variety is not possible at the second notation, a third notation (14 days after inoculation) is recommended.

### **3. Proposal harmonized protocol for *Bremia lactucae***

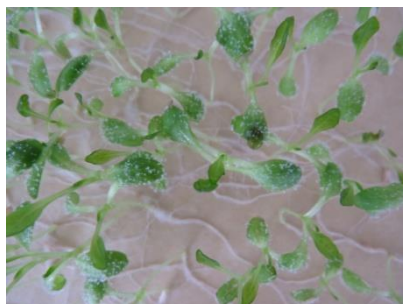
The comparative test following the workshop allowed defining the elements for the future harmonized protocol.

#### **➤ Substrate**

The three substrates (soil, sand and blotter/paper) were validated. Pictures from tests were chosen to illustrate the symptoms of susceptibility on each substrate (fig 6, 7 & 8).



**Figure 6: symptoms of susceptibility for *Bremia lactucae* on sand**



**Figure 7: symptoms of susceptibility for *Bremia lactucae* on blotter**



**Figure 8: symptoms of susceptibility for *Bremia lactucae* on soil**

➤ Notation scale

The notation scale and the decision rule defined during the workshop were validated. One critical control point in the future protocol is to specify that it is important to test differentials in each test. With differentials (particularly Design), it allows to avoid the risk of misinterpretation of certain resistance. If it is not validated on differentials, the test has to be repeated.

➤ Date of notation

The dates of notation defined during the workshop were validated. It is important to start the first notation when the susceptible control is sporulating well. The second notation is done 3-4 days after the first notation. In case of doubt or “other case” category a third notation can be done 3-4 days after the second notation.

## V. Pea

### A. Pea/Ascochyta pisi race C

#### 1. Materials and methods

Two strains of *Ascochyta pisi* (strains race C GEVES 2007 and race C 21A.13 from SASA) were compared in three labs. Each strain was:

- ✓ provided by the partner
- ✓ multiplied by GEVES
- ✓ validated on differentials (highlighted in yellow tab. 39) in one lab.

**Table 39: Physiological races of *A. pisi* and differentials (Gallais et Bannerot, 1992)**

Physiological races (Dr Hubbeling)	D	–	–	–	C	B	E
Strains	N°1	several isolates	N°4	N°14	Tézier	–	–
<b>Gullivert</b>	R	R	R	R	S	R	R
<b>Rondo</b>	R	R	S	TLS	R	R	S
<b>Finale</b>	R	R	S	LS	R	-	-
<b>Kelvedon Wonder</b>	R	S	S	S	S	R	R
<b>Dark-skinned Perfection</b>	S	S	S	S	S	R	S
<b>Arabal. Cobri, Starcovert, sucovert, Vitalis</b>	S	S	S	S	S	S	S

R = resistant; S = susceptible, TLS = very lightly susceptible, LS = lightly susceptible

The comparative test was performed on the same controls validated in phase 3:

- Kelvedon Wonder (S) and Crecerelle (S, indication of aggressiveness of test)
- Rondo and Madonna (IR, low level)
- Nina (R, high level)

A panel of four varieties (Elise, Vertige, Jymy and Alvesta) expected with an intermediate resistant comportment was added.

Tests were performed at the stage of inoculation (spraying two weeks seedlings by a suspension of spore) validated in phase 3, on twenty plants per variety.

The notation scale on five notes was selected in phase 3:

- 0: no symptoms
- 1: few small superficial necrosis
- 2: bigger darker and deep necrosis
- 3: necrosis at each level of the plant
- 4: serious symptoms surrounding the stem

#### 2. Results

##### a) Notation scales

One date of notation was recorded: 12 days post inoculation.

The lab 1 has not validated the susceptible control before 18 days post-inoculation (tab. 40).

**Table 40: date of notation of *Ascochyta pisi* for both strains**

	Lab 1	Lab 6	Lab 12
Strain F (GEVES 2007)	18 dpi	10 dpi	12 dpi
Strain G (21A.13)	18 dpi	11 dpi	12 dpi

***b) Comparison of strains***

Both strains were compared with the notation scale 1 and with the method of inoculation by spraying (tab. 41 & 42).

**Table 41: results of strain GEVES 2007 of *Ascochyta pisi***

Varieties	Expected result	Lab 1	Lab 6	Lab 12	Accuracy					Reproductibility			
					PA	PD	NA	ND		PA	NA	TA	
Crecerelle	S	S	S	S	3	0			1	3		3	1
Kelvedon	S	S	S	S	3	0			1	3		3	1
Madonna	IR	R	R	R			3	0	1		3	3	1
Rondo	IR	R	IR/R	R			3	0	1		3	3	1
Nina	R	R	R	R			3	0	1		3	3	1
Alvesta	IR	S	IR	S			1	2	0.33		1	3	0.33
Elise	IR		S/IR	I			1	1	0.5		1	2	0.5
Jymy	IR	R	IR	R			3	0	1		3	3	1
Vertige	IR	R	R	R			3	0	1		3	3	1
Cobri	S	S											
Darkskin Perfection	S	S											
Finale	R	S											
Gullivert	S	S											

R: resistant; IR: intermediate resistant; S: susceptible;

All labs obtained expected results, excepted for two varieties (expected intermediate resistant) Alvesta and Elise judged susceptible in some labs. The strain GEVES 2007 was validated on differentials but Finale. This susceptible comportment of Finale was previously observed for race C.

**Tableau 42: results of strain 21A.13 of *Ascochyta pisi***

Varieties	Expected result	Lab 1	Lab 6	Lab 12	Accuracy					Reproducibility			
					PA	PD	NA	ND		PA	NA	TA	
Crecerelle	S	S	S	S	3	0			1	3		3	1
Kelvedon	S	S	S	S	3	0			1	3		3	1
Madonna	IR	R	R	R			3	0	1		3	3	1
Rondo	IR	R	IR	R			3	0	1		3	3	1
Nina	R	R	R	R			3	0	1		3	3	1
Alvesta	IR	S	S/IR	S			0	3	0		3	3	1
Elise	IR	R	R/IR	R			3	0	1		3	3	1
Jymy	IR	R	R	R			3	0	1		3	3	1
Vertige	IR	R	R	R			3	0	1		3	3	1
Cobri	S	S											
Darkskin Perfection	S	S											
Finale	R	R											
Gullivert	S	S											

R: resistant; IR: intermediate resistant; S: susceptible;

All labs obtained expected results, excepted for one variety (expected intermediate resistant) Alvesta tested susceptible in all labs. The strain 21A.13 was validated on differentials. The susceptible compartment of Finale was not observed for strain 21A.13.



### c) Summary of results

#### (1) Strains

Both strains of *Ascochyta pisi* race C (21A.13 and GEVES 2007) were validated. But due to the results on the differential Finale, the strain of *Ascochyta pisi* race C 21A.13 was selected.

#### (2) Controls

Following the results, the controls selected in phase 3 were validated in phase 4.

Crecerelle and Kelvedon Wonder were validated as susceptible controls and Nina was validated as high level of resistance control. Madonna and Rondo were judged resistant or intermediate resistant, with both strains, depending of aggressiveness of test.

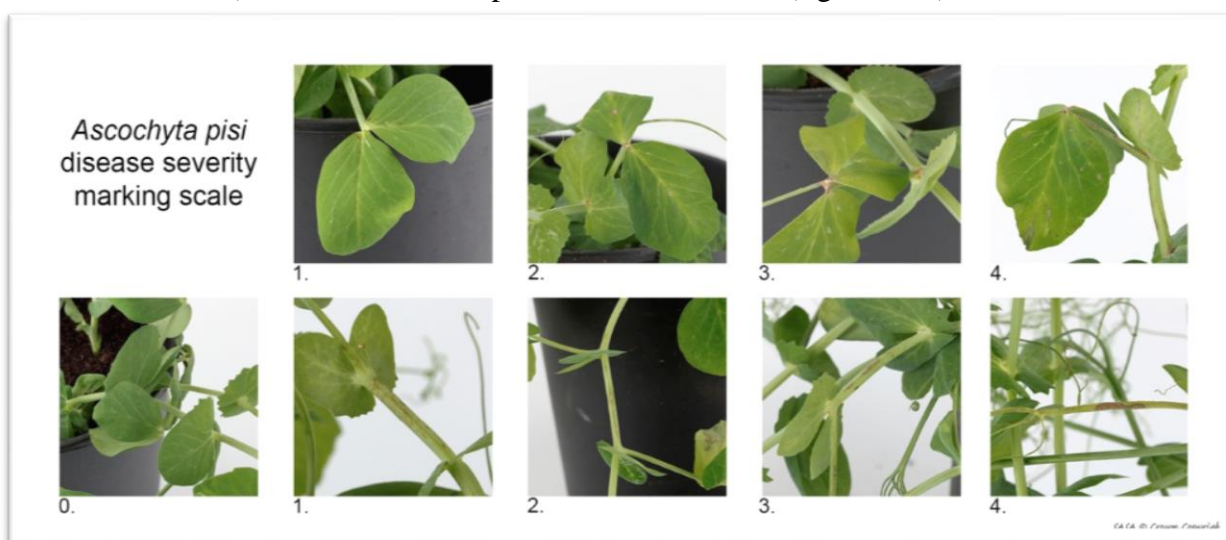
The steering committee decided to propose to spread the controls as susceptible (Crecerelle and Kelvedon Wonder) and resistant (Nina, Madonna and Rondo).

#### (3) Notation scales

The notation scale 1 (five note) was validated in phase 4:

- 0: no symptoms
- 1: few small superficial necrosis
- 2: bigger darker and deep necrosis
- 3: necrosis at each level of the plant
- 4: serious symptoms surrounding the stem

But due to the lower level of symptoms compared to inoculation by sowing (absence of plants at note 4 in all labs), two labs have adapted the notation scale (fig. 9 & 10).



1-2 Superficial necrosis on stem, petioles, leaves and tendrils; 2-3 Necrosis at junction of leaf petiole and leaf base, also present at junction of leaf petiole with stem axil. Leaf base necrosis severe enough to cause some leaf drop.

**Figure 9: notation scale for *Ascochyta pisi* modified by lab 12**



0: no symptoms; 1: few small superficial necrosis (or 1 or 2 level dried); 2: 1 to 3 level dried and/or necrotic spot; 3: necrosis at each level of the plant and on stem; 4: serious symptoms surrounding the stem

**Figure 10: notation scale for *Ascochyta pisi* modified by lab 1**

The working group proposed to merge notes 3 and 4 in a common note 3: necrosis at each level of the plant or serious symptoms surrounding the stem.

One critical control point in the future protocol is to specify to add a non inoculated control for each variety.

### **3. Proposal harmonized protocol for *Ascochyta pisi***

The phase 4 of validation of protocol allowed defining the elements for the future harmonized protocol.

#### **➤ Strains**

The strains 21A.13 was validated.

#### **➤ Controls**

The five controls selected in phase 3 were validated in phase 4. It will be specified to choose in test one of the selected one:

- Susceptible controls: Crecerelle or Kelvedon Wonder
- Resistant controls:
  - Nina and
  - Madonna or Rondo

#### **➤ Inoculation**

The inoculation by spraying 2 weeks seedlings selected in phase 3 was validated in phase 4.

#### **➤ Number of plants:**

At least 20 plants inoculated and 5 plants non inoculated per variety.

➤ Notation scale:

The notation scale selected in phase 3 was modified by the working group according to the results of phase 4 :

- 0: no symptoms
- 1: few small superficial necrosis
- 2: bigger darker and deep necrosis
- 3: necrosis at each level of the plant or serious symptoms surrounding the stem

➤ Interpretation:

Following the results, the rule of interpretation (fig. 11) was defined in phase 4:



**Figure 11: rule of interpretation for *Ascochyta pisi* race C**

Rondo, Madonna and Nina will be resistant controls, varieties with same level of resistance than Nina and/or Rondo and Madonna will be interpreted as resistant. Kelvedon Wonder and Crecerelle will be susceptible controls, varieties with a lower level of resistance than Nina and/or Rondo and Madonna will be interpreted as susceptible.

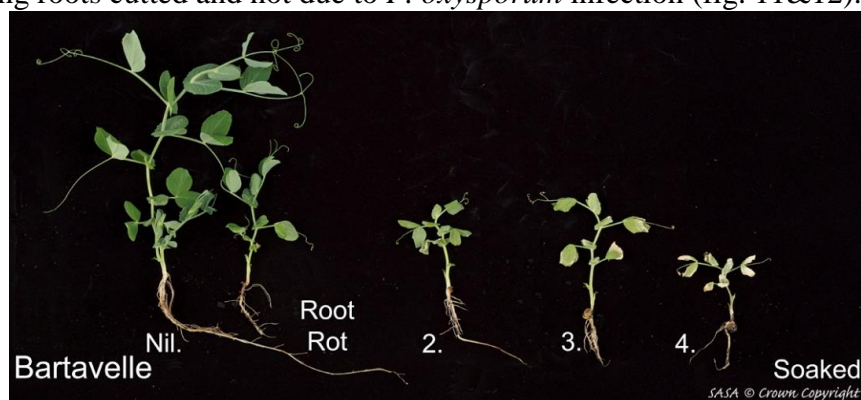
Evaluation of variety resistance should be calibrated with results of resistant (Nina Rondo and Madonna) and susceptible (Kelvedon Wonder and Crecerelle) controls.

## B. Pea/*Fusarium oxysporum* f. sp. *lisi* race 1

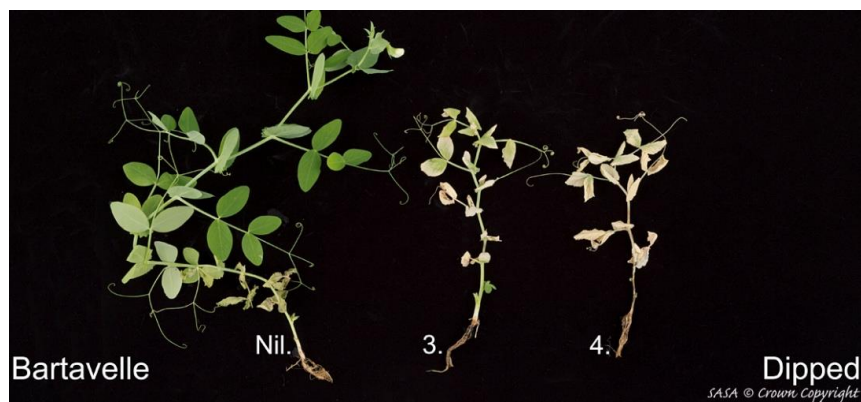
Previous ring test results on comparison of different substrates and different inoculation methods showed comparable results between laboratories with both inoculation methods: sowing on contaminated substrate and dipping roots. One critical control point in the future protocol is to specify that each lab have to define the best method in its lab depending of controls results.

The strain of *Fusarium oxysporum* f. sp. *lisi* race 1 (strains race 1 from GEVES) was validated in phase 3.

A common notation scale was defined in five notes. One critical control point in the future protocol is to specify that it is important to compare any plants with a negative control of the same sample to allow to interpret symptoms of root rot or senescence or 'wilting' due to the stress of having roots cutted and not due to *F. oxysporum* infection (fig. 11&12).



**Figure 12: symptoms of root rot with inoculation by sowing on contaminated substrate**



**Figure 13: symptoms of senescence or 'wilting' with inoculation by dipping roots**

Following the phase 3, a common decision rule was established (fig. 13):



**Figure 14: decision rule of interpretation of results for *Fusarium oxysporum* f. sp. *lisi***

The varieties with same level of resistance than Nina and/or New Era have to be interpreted as resistant.

The working group decided to organize a workshop at GEVES to harmonize the notation scale on both inoculation methods and to compare the notation scale (5 notes) to a reduced notation scale (3 notes).

## **1. Workshop Pea/ *Fusarium oxysporum* f. sp. *pis*i race 1**

During this workshop, a panel made up of controls selected in phase 3 (Nina, New Era and Bartavelle), varieties Borderlines and three varieties expected resistant or susceptible, was compared on two methods of inoculation:

- Sowing in contaminated substrate: soil based substrate
- Dipping roots :
  - Sowing in mix on vermiculite + soil or soil based substrate
  - Transplantation in soil based substrate

Both notation scale (extended: 5 notes or reduced: 3 notes) were compared on symptoms observed and notations of each participant (scoring on a group basis for a first phase of common interpretation).

The working group defined a harmonized notation scale validated during the workshop based on the notation scale 2 on three notes (0 to 2) (fig. 14):

- 0: no symptoms or equivalent to negative control, 1 or 2 senesced lower leaves and slight reduction in growth compared to negative control of same variety are acceptable
- 1: Range from a few chlorotic or wilting/senesced leaves not present on, or more than on the negative control, up to many leaves with symptoms of senescence or wilting, some leaf drop, upper part of the plant still green and growing
- 2: Range from most of the plant senesced or wilted but still alive, to plants brown and dead with stem collapsed



Notes 0 and 1 are judged resistant.  
Note 2 is judged susceptible.

The evaluation of variety resistance should be calibrated with results of resistant (Nina and New Era) and susceptible (Bartavelle) controls.

**Figure 15: reduced notation scale (0 to 2) for *Fusarium oxysporum* f. sp. *pis*i**

## **2. Comparative test**

Following the workshop, a comparative test was performed using the new notation scale and the new rule of interpretation.

### **a) Materials and methods**

One strain of *Fusarium oxysporum* f. sp. *pis*i race 1 (race 1 from GEVES) was compared in four labs. The strain was:

- ✓ provided and multiplied by GEVES
- ✓ validated on differentials (highlighted in yellow tab. 43) in one lab.

**Table 43: Races of *Fusarium oxysporum* f. sp. *pisi* (ISF differentials)**

<i>Fusarium oxysporum</i> f. sp. <i>pisi</i> (Fop) Pea Cultivar	Race 1	Race 2	Race 5	Race 6
Little Marvel	S	S	S	S
Darkskin Perfection	R	S	S	S
New Era	R	R	S	S
New Season	R	R*	S	R
WSU 23	R	R	R	S
WSU 28	R	S	R	R
WSU 31	R	R	R	R

S: susceptible R: resistant

The comparative test was performed on twenty plants per variety (more 5 non inoculated plants), on a panel made up of controls, varieties tested in phase 3 and varieties difficult to judge in phase 3 (tab. 44).

	Controls	Varieties tested in phase 3	Varieties difficult to judge in phase 3
<b>Resistant</b>	Nina New Era	Bordeline 3 Bordeline 4 KWS Paradisio	Bordeline 1 Bordeline 2
<b>Susceptible</b>	Bartavelle	Jantar Pionier	

Two methods of inoculation were compared:

- ✓ dipping roots in suspension of spores,
- ✓ sowing in contaminated substrate

Test was recorded at two four weeks post inoculation with the notation scale defined during the workshop.

## ***b)* Results**

### **(1) Date of notation**

One date of notation was recorded: 28 days post inoculation (tab. 45).

**Tableau 44: date of notation for Fop with both methods of inoculation**

Dpi	Lab 1	Lab 2	Lab 4	Lab 6	
Dipping roots	30	23	28	28	[23 to 30]
Sowing in contaminated substrate	31	31	28	28	[28 to 31]

The differences observed between expected dates and the effective dates of notation between labs were due to the lab conditions of test. The notation was done when the susceptible control was validated for each lab.



## (2) Comparison of inoculation methods

Both inoculation methods were compared at four weeks post-inoculation (tab. 45&46).

**Tableau 45: results for inoculation by dipping roots for Fop**

Varieties	Expected result	Lab 1	Lab 2	Lab 4	Lab 6	Accuracy					Reproductibility			
						PA	PD	NA	ND		PA	NA	TA	
Bartavelle	S	S	S	S	S	4	0			1	6		6	1
Jantar	S	S	S	S	S	4	0			1	6		6	1
Pionier	S	S	S	S	S	4	0			1	6		6	1
KWS Paradisio	R	R	R	R	R			4	0	1		6	6	1
New Era	IR	R	R	R	R			4	0	1		6	6	1
Nina	R	R	R	R	R			4	0	1		6	6	1
Borderline 1	R/IR	R	R	R	R			4	0	1		6	6	1
Borderline 2	S	R	R	R	R/IR	0	4			0		6	6	1
Borderline 3	IR	R	R	R	R			4	0	1		6	6	1
Borderline 4	IR	R	R	R	R			4	0	1		6	6	1
Darkskin Perfection	R	R												
WSU 23	R	R												
WSU 28	R	R												
Little Marvel	S	S												

R: resistant; IR: intermediate resistant; S: susceptible;

All labs obtained the expected results, excepted on the variety Borderline 2.

The strain race 1 GEVES of *Fusarium oxysporum* f. sp. *pisi* was validated on differentials with inoculation by dipping roots.

The protocol was validated with inoculation by dipping roots.

**Tableau 46: results for inoculation by sowing in contaminated substrate for Fop**

Varieties	Expected result	Lab1	Lab 2	Lab 4	Lab 6	Accuracy					Reproductibility			
						PA	PD	NA	ND		PA	NA	TA	
Bartavelle	S	S	S	S	S	4	0			1	6		6	1
Jantar	S	S	S	S	S	4	0			1	6		6	1
Pionier	S	S	S	S	S	4	0			1	6		6	1
KWS Paradisio	R	R	R	S ?	R			3	1	0,8		3	6	0,5
New Era	IR	R	R	R	R			4	0	1		6	6	1
Nina	R	R	R	R	R			4	0	1		6	6	1
Borderline 1	R/IR	R	R	R	R			4	0	1		6	6	1
Borderline 2	S	R	R	R	R	0	4			0		6	6	1
Borderline 3	IR	R	R	R	R			4	0	1		6	6	1
Borderline 4	IR	R	R	R	R			4	0	1		6	6	1
Darkskin Perfection	R	R												
WSU 23	R	R												
WSU 28	R	R												
Little Marvel	S	S												

R: resistant; IR: intermediate resistant; S: susceptible;

All labs obtained the expected results, excepted on the variety Borderline 2 in all labs and the variety KWS Paradiso in one lab.

The strain race 1 GEVES of *Fusarium oxysporum* f. sp. *pisi* was validated on differentials with inoculation by sowing in contaminated substrate.

The protocol was validated with inoculation by sowing in contaminated substrate.

### c) Summary of results

#### (1) Strain

The strain race 1 GEVES of *Fusarium oxysporum* f. sp. *pisi* was validated with both methods of inoculation.

#### (2) Controls and varieties

The controls selected in phase 3 (Nina, New Era and Bartavelle) were validated in phase 4. The varieties Bordelines 1, 2, 3 and KWS Paradiso, Jantar and Pioner were judged as expected by all labs with both methods of inoculation.

The variety Bordeline 2 obtained unexpected results by all labs with both methods of inoculation but results were in concordance between labs. The conclusion of the working group was that the expected result was false.

#### (3) Inoculation methods

Both methods of inoculation were validated on controls and varieties with strain race 1 GEVES. The working group decided to propose both methods for harmonized protocol depending of test conditions of laboratories.

#### (4) Date of notation

The notation around four weeks post-inoculation was validated for both methods of inoculation.

#### (5) Notation scales

The reduced notation scale (from 0 to 2) defined during the workshop was validated on controls and varieties with both methods of inoculation.

#### (6) Interpretation of results

The interpretation rule defined on phase 3 was validated in phase 4.

### 3. Proposal harmonized protocol for Fop

The phase 4 of validation of protocol allowed defining the elements for the future harmonized protocol.

➤ Strains:

The strain race 1 GEVES of *Fusarium oxysporum* f. sp. *lisi* was selected.

➤ Varieties:

The three controls selected in phase 3 were validated in phase 4.

- Susceptible control: Bartavelle
- Resistant controls: Nina, New Era

➤ Methods of inoculation:

Both methods were validated in phase 4.

One critical control point in the future protocol is to specify that labs have to define the best method in their lab depending of controls' results.

Another critical control point in the future protocol is to specify that inoculation by sowing in contaminated method can in some cases lead to germination problems. No conclusion can be done in this case, and the test should be repeated.

➤ Notation:

The notation at 4 weeks post-inoculation with the notation scale from 0 to 2 was validated.

- 0: no symptoms or equivalent to negative control, 1 or 2 senesced lower leaves and slight reduction in growth compared to negative control of same variety are acceptable
- 1: Range from a few chlorotic or wilting/senesced leaves not present on negative control, or more than on the negative control, up to many leaves with symptoms of senescence or wilting, some leaf drop, upper part of the plant still green and growing
- 2: Range from most of the plant senesced or wilted but still alive, to plants brown and dead with stem collapsed

Notes 0 and 1 are Resistant

Note 2 is Susceptible

➤ Interpretation:

Following the results, the rule of interpretation (fig. 15) defined in phase 3 was validated in phase 4:



**Figure 16: rule of interpretation for *Fusarium oxysporum* f. sp. *lisi* race 1**

New Era and Nina will be resistant controls, varieties with same level of resistance than Nina and/or New Era will be interpreted as resistant. Bartavelle will be susceptible control, varieties with a lower level of resistance than Nina and/or New Era will be interpreted as susceptible. Evaluation of variety resistance should be calibrated with results of resistant (Nina and New Era) and susceptible (Bartavelle) controls.

## **VI. Conclusion**

For pea/*Fusarium* and lettuce/*Bremia*, two workshops were scheduled in November 2014 to describe a harmonized notation scale and define a common interpretation. These workshops, including Harmores 2 partners and extended to other breeding companies, allowed to have the same interpretation of varieties on difficult cases.

Harmonized protocols defined in phase 3 and during the workshops were validated in phase 4 for the seven host pathogen combinations (pepper/TMV: 0, pepper/PMMoV: 1.2, pepper/PMMoV: 1.2.3, pepper/PVY: 0, Lettuce/*Bremia*, pea/*Ascochyta* and pea/*Fusarium*) with:

- Reference strains,
- Reference controls,
- Stages and methods of inoculation,
- Conditions of tests,
- Substrates,
- Notation scales,
- Decision rules defined.

For the full host/pathogens combinations harmonized in Harmores 2 project, the working group decided that all that has been validated during phase 4, will be retained in the protocols to allow everyone the freedom to use what suits him best.

The working group will propose to CPVO updated robust protocols validated in different laboratories.

## **VII. Maintainance of reference material (strains and control)**

During the last meeting, a general rule for maintainance of isolates and controls was defined which will become applicable to reference material defined during this meeting.

For each isolate, a list of volunteers for maintainance was established (see in annex)

All referenced isolates shall be available in the network of maintainers. The owner of isolates does a MTA to maintainers stating that they can distribute the isolate freely in his network but not sell it to a third party. In case of request from a third party, the request will be transferred to the owner. GEVES has sent in May an example of MTA to volunteers for maintainance, for sharing and harmonization.

For the controls, it will be mentioned in each protocol “this protocol has been validated on...” and it will be indicate which ones are available.

As reminder, maintainers have the responsibility to control isolates on differentials and controls twice every 5 years.

## VIII. Harmores 3

Before the final meeting of Harmores 2, GEVES has circulated a table to list the priorities of different potential partners and criteria of choice for a new project of harmonization of resistance tests for the host/pathogen chosen in terms of quantitative results and difficulties for interpretation. This table was completed by partners, depending of priorities of the national agencies and breeders via ESA (European Seed Association).

Following the feedback of partners, a table was defined with for each host/pathogen combination, if it is compulsory or not in the CPVO protocol, the priority for each partner and the number of partners interested. Based on this table, the working group established the priorities as regards to pathogen races/isolates to study for a future Harmores 3 R&D project according to the following criteria:

- they were compulsory and/or concerning an intermediate resistance (IR)
- they were commonly used as a grouping character for DUS testing
- the protocols were known to be difficult and to give slightly different results depending on the test conditions
- they were of a high interest for the largest number of countries involved.

Seven vegetable diseases were prioritized:

- *Meloidogyne incognita*/ tomato
- *Fusarium oxysporum* f. sp. *lycopersici* race 0 (ex 1) and race 1 (ex 2)/tomato
- *Erysiphe pisi*/pea
- Powdery mildew/melon
- *Fusarium oxysporum* f. sp. *melonis* race 1.2/melon
- *Fusarium oxysporum* f. sp. *melonis* race 2/melon (with also *Fusarium oxysporum* f. sp. *melonis* race 0 and 1 in a second time).

One partner asked to include CMV/cucumber, which had been discussed in the meeting, in the priorities, a consultation by email is under progress to identify if partners would be interested.

This proposal will be presented to the Annual meeting CPVO at the end of November.

In case this project is be accepted, it will be important to take into account the availability of reference material, and to include hybrids in validation to be sure that controls are representative of the market.



## IX. Annexes

### A. Raw data

The raw data of the third year of project are attached in Excel files.

### B. Maintainers of isolates

**Table 47: Harmonization of resistance test to diseases for DUS testing – 2, maintainers of isolates**

Host	Pathogen	Strain uncoded	Provided for Harmores by	Availability	Volunteer for maintainance
Pepper	TMV:0	Vi -6	GEVES	MATREF	GEVES( MATREF) INIA Naktuinbouw
	PMMoV: 1.2	Nt203	Naktuinbouw	Naktuinbouw	Naktuinbouw INIA GEVES( MATREF)
	PMMoV: 1.2.3	Eve	GEVES	MATREF	GEVES( MATREF) INIA Naktuinbouw
	PVY	Zb6	Naktuinbouw	Naktuinbouw	Naktuinbouw INIA GEVES( MATREF)
Pea	<i>Fusarium oxysporum</i> <i>f. sp. pisi</i>	Race 1 FR	GEVES	MATREF	GEVES( MATREF) INIA SASA
	<i>Ascochyta pisi</i>	Race C 21A.13 UK	SASA		SASA
		Race C GEVES	GEVES	MATREF	GEVES( MATREF)
Lettuce	<i>Bremia lactucae</i>	Bl: 24	GEVES	MATREF	GEVES( MATREF) INIA Naktuinbouw
		Bl: 26	GEVES	MATREF	GEVES( MATREF) INIA Naktuinbouw