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ISF clubroot project Brassica oleracea

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Validation of ISF differential table of *Plasmodiophora brassicae* (Pb) in *Brassica oleracea*

Abstract
New introgressions of resistance from other *Brassica* species into *Brassica oleracea* necessitate the use of a new host differential set for races of *Plasmodiophora brassicae,* the causal agent of the clubroot disease. The Expert Group Disease Resistance Terminology of the International Seed Federation (ISF EG DRT) has published a set of differentials and 4 denominated races. The ISF clubroot project group has confirmed the validity of the ISF differential set on the basis of ringtest data for 8 *P. brassicae*-isolates on 4 host genotypes in 14 laboratories, and decided to keep 4 validated differentials and the 4 denominated races, distinguishing all resistances currently used in *B. oleracea.*

Introduction

Clubroot, caused by the biotrophic protist *Plasmodiophora brassicae*, causes the formation of large galls on the roots. This abnormal growth of the root tissue impedes nutrient and water uptake and causes loss of yield in many *Brassica* crops, like *Brassica napus*, *B. juncea*, *B. carinata* and *B. oleracea*. The spores are soilborne and persistent in the soil for up to 20 years. The disease can spread mechanically, manually and by transplants using infected peat soils. Infections occur during wet and warm soil conditions. Isolates can be clustered into races by testing them on a host differential set. Host ranges are described by Williams (1966), Buczacki (1975), Somé (1996), Kuginuki (1999) and others.

Isolates are variable in genetics and mixes occur in commercial fields (Somé, 1996). Even in a single clubroot gall several races can be found. Field isolates may be unstable genetic mixtures, while single spore isolates can be considered as genetically pure isolates that will be stable during multiplication. Single spore isolates (SSI) are preferable for race denominations, but isolates that are proven to be stable are also acceptable.

Clubroot management depends strongly on host resistance. Only a limited set of resistance genes are available for breeding within the crop type *B. oleracea.* Clubroot management will benefit from race descriptions that are valid only for this crop type. After the introduction of introgressions from other *Brassica* species which naturally do not occur in *B. oleracea*, a differential set for *B. oleracea* was proposed by the European Seed Association. The ISF clubroot project group has investigated the completeness of this set from a global perspective, and organized a ringtest to validate candidates differentials and isolates with a standardized protocol.

The ISF project group consisted of representatives of the companies Bejo Zaden, Enza Zaden, HM Clause, Kaneko Seeds, NongwooBio, Nippon Norin Seed, Rijk Zwaan, Sakata Seed Corporation, Sakata Vegetables Europe, Syngenta, Takii, Tokita Seed and the institutes GEVES, Julius Kühn-Institute, Naktuinbouw, Warwick University (see **Annex 1**).
They discussed and validated the isolates in their labs with the standardized protocol, isolates and genetics.

Materials and methods

The differential set should reflect resistance genetics that are used in the market. The market for clubroot resistance has a worldwide dimension. The ISF differential set contains three different sources of resistance. At the beginning of the project, we were not sure about the completeness of the set. All participants investigated in their own company whether new candidate differentials were available. When it appeared that no new candidates could be mentioned, we concluded that the set is complete. This means that the results of a ringtest can help to reach worldwide consensus.

An inventory of 18 *P. brassicae*- isolates available for the project was grouped into 8 groups with a distinct reaction pattern on four differentials (**Table 1**). From this grouped list, 8 isolates (1 isolate from each group, marked yellow in the table) were selected for testing in the ringtest.

The use of one harmonized testing protocol is an important condition for exchange of data about races and resistance. In the meetings it became clear that different inoculation methods are currently used: Japanese companies use the seed slurry method described by Yoshikawa (1983), European companies and institutes use the pipetting method described by Williams (1966). We decided to use the pipetting method. Also, a distinct grade 2 (Resistant) phenotype was discussed and accepted as a necessary improvement. The complete protocol used in the ringtest can be found in the **Annex 2.**

A pre-ringtest was performed before the ringtest was started. The aim of the pre-ringtest was to compare laboratory performance and allow improvements if necessary. The pre-ringtest also allowed to learn more about transportation of inoculum to the Asian partners in the project. Due to logistical constraints, unfortunately the Asian partners were unable to perform a successful pre-ringtest.

The pre-ringtest was successfully performed with 2 isolates in 2 spore concentrations (10^5 and 10^7 spores per ml) by 8 laboratories. The ringtest was performed with 8 isolates divided over 14 labs, each lab receiving 4 of the 8 isolates. Isolate names were coded, names of the differentials were not coded.

The project group had 9 meetings, starting in February 2022 and the last meeting in November 2024 (see **Annex 3**).

Results and discussion

The pre-ringtest resulted in good concordance between most of the labs (**Table 2**), except for differential 2 (Bejo 051632) infected with Pb: 2. After discussion, it appeared that this problem can be solved by a full implementation of the adapted observation scale. The lower inoculum concentration of 10^7 spores per ml did not result in problems, so this lower concentration was chosen for the subsequent ringtest with fourteen laboratories.

The ringtest resulted in general in a good concordance between the labs, confirming the expected phenotypes of the 4 original isolates and the 4 differentials of the ISF differential table (**Table 3**). However, some non-conformities were noted and laboratories indicated these will be investigated. Moreover, based on the results of the ringtest, it appeared that the chosen isolates from group 3 (RZ2) and group 6 (RZ1) now belong to a different group, RZ1 to group 2, RZ2 to group 7. It was discussed and agreed not to retest other isolates from the two groups not represented finally.

It was also discussed and agreed to keep only 4 already denominated type-isolates and patterns (Pb: 0, 1, 2, 3) because these four are sufficient for differentiating the 4 different genotypes for resistance to clubroot in *B. oleracea.* Other isolates and other patterns will not be denominated as new races. Some of the isolates mentioned in the table are not available anymore, like SAK B and SAK C.

To investigate the possible influence of temperature and other factors on the non-conformities, the data were analysed in relation to the recorded temperatures during the test (**Figure 1**). The optimal temperature range appears to be 20-23°C. Possibly, temperatures below 20°C may lead to symptoms on resistant varieties. Temperatures above 23°C may lead to symptomless susceptible varieties. Other factors such as testing place, did not show any significant effect on the results of the ringtest.

Conclusions

The ringtest was concluded by consensus about the reliability of the testing protocol and the practical usefulness of the set for international harmonization between laboratories on October 28th 2024. The differential table in **Table 4**, with 4 differentials and 4 isolates will be proposed to the ISF EG DRT. The protocol in **Annex 4** will be proposed to UPOV and CPVO in the proposal to add clubroot as a characteristic to the relevant *B. oleracea* guidelines and protocols. It is slightly amended compared to the one used in the ringtest for more clarity in the wording.
The 4 type-isolates and the 4 differentials in the differential table will be maintained and available through the Naktuinbouw - Plantum isolate collection and the MATREF collection. Some other

isolates like the P1 isolate breaking all genetics, were proposed to be maintained and available for future use.

References

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Buczacki, S.T., H. Toxopeus, P. Mattusch, T.D. Johnson, G.R. Dixon, L.A. Hobolth, **1975**. Study of physiologic specialization in *Plasmiodophora brassicae*: proposals for attempted rationalization through an international approach. Transactions of the British Mycological Society 65: 295-303

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Kuginuki, Y., H. Yoshikawa, M. Hirai, **1999**. Variation in virulence of *Plasmodiophora brassicae* in Japan tested with Clubroot-resistant cultivars of Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*). European Journal of Plant Pathology 105(4): 327-332

Table 1. Inventory of 18 isolates grouped in 8 groups by their reaction pattern on the 4 differentials (yellow chosen for the ring test)

		Differential			
	Clubroot Isolate	Bartolo	Bejo 051632	Clapton	Lodero
Group		S1	S2	S3	S4
1	Pb: 0	S	R	R	R
2	Pb: 1	S	S	R	R
3	RZ2	S	R	S	R
	Sak B	S	R	S	R
	JP Group 2	S	IR	S	IR
4	Pb: 2	S	S	S	R
5	Pb: 3	S	R	R	S
	JP Group 3	S	R	R	S
	JP Group 4	S	IR	R	S
6	RZ1	S	S	R	S
	Sak C	S	S	R	S
	P1-	S	S	R	S
7	SAK A	S	R	S	S
	RZ3	S	R	S	S
	JP Group 1	S	R	S	S
8	P1	S	S	S	S
	P2	S	S	S	S
	HMClause	S	S	S	S

Table 2. Average disease indexes in the pre-ringtest with two inoculum strengths and two isolates and four differentials in eight laboratories.

				Lab							
Isolate Pb: 0		Differential	Expected	1	2	3	4	5	6	7	8
	high inoculum strength	Bartolo	S	0,67	1,00	0,83	0,99	1,00	1,00	1,00	0,98
		Bejo 051632	R	0,00	0,00	0,00	0,02	0,04	0,00	0,00	0,00
		Clapton	R	0,00	0,00	0,00	0,00	0,00	0,12	0,00	0,05
		Lodero	R	0,00	0,00	0,00	0,26	0,03	0,07	0,79	0,13
	low inoculum strength	Bartolo	S	0,93	0,85	0,63	1,00		0,95	0,89	0,95
		Bejo 051632	R	0,00	0,00	0,00	0,00		0,08	0,00	0,00
		Clapton	R	0,00	0,00	0,00	0,00		0,05	0,05	0,00
		Lodero	R	0,00	0,00	0,00	0,03		0,00	0,42	0,00
Isolate Pb: 2											
	high inoculum strength	Bartolo	S	0,94	1,00	0,90	1,00	1,00	1,00	1,00	0,72
		Bejo 051632	S	0,69	0,05	0,29	0,86	0,79	1,00	0,83	0,63
		Clapton	S	1,00	1,00	0,99	1,00	1,00	1,00	1,00	0,67
		Lodero	R	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	low inoculum strength	Bartolo	S	0,93	0,78	0,87	1,00		0,67	0,90	0,57
		Bejo 051632	S	0,74	0,00	0,11	0,88		0,67	0,42	0,31
		Clapton	S	1,00	0,50	0,70	1,00		0,67	1,00	0,57
		Lodero	R	0,02	0,00	0,00	0,00		0,00	0,00	0,00

Table 3. Inventory of 18 isolates grouped by their reaction pattern on the four differentials and results of the ringtest with 8 clubroot isolates. Empty cells: isolates not included in this ringtest. Yellow marked: isolates included in the differential table.

				Differential			
	Clubroot Isolate	% Non-conformity in ringtest	non-conform differentials	Bartolo	Bejo 051632	Clapton	Lodero
Pattern				S1	S2	S3	S4
1	Pb: 0	6%	S4	S	R	R	R
2	Pb: 1	14%	S2 S4	S	S	R	R
	RZ1	14%	S1 S2 S3	S	S	R	R
3	Sak B			S	R	S	R
	JP Group 2			S	IR	S	IR
4	Pb: 2	14%	S1 S2 S3	S	S	S	R
5	Pb: 3	0%		S	R	R	S
	JP Group 3			S	R	R	S
	JP Group 4			S	IR	R	S
6	Sak C			S	S	R	S
	P1-			S	S	R	S
7	RZ2	11%	S2 S3 S4	S	R	S	S
	SAK A	0%		S	R	S	S
	RZ3			S	R	S	S
	JP Group 1			S	R	S	S
8	P1	17%	S2 S4	S	S	S	S
	P2			S	S	S	S
	HMClause			S	S	S	S

Figure 1. Expected minus observed disease index (DI) plotted against average temperature during the experiment. At higher temperatures the difference is positive because the observed DI is lower than the expected DI. At lower temperatures there is an opposite tendency, indicating resistance breakdown.

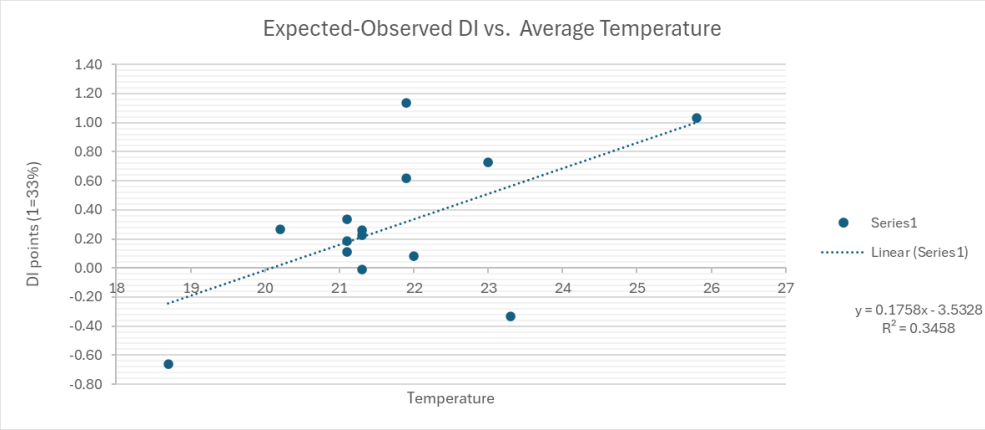

Table 4. Proposed differential set for *Plasmodiophora brassicae* (Pb), clubroot in *Brassica oleracea.*

Differentials		ISF Code and races	Pb: 0	Pb: 1	Pb: 2	Pb: 3
		Gene (s)				
Bartolo	White cabbage		S	S	S	S
051632	White cabbage		HR	S	S	HR
Clapton	Cauliflower		HR	HR	S	HR
Lodero	Red cabbage		HR	HR	HR	S

S = Susceptible; IR = Intermediate Resistance; HR= High Resistance

Annex 1: List of participants of the project

Company name	Contact person	Emailaddress
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Annex 2: Clubroot resistance testing protocol for the project

1. Pathogen	*Plasmodiophora brassicae*
2. Quarantine status	No quarantaine
3. Host species	*Brassica oleracea*
4. Source of inoculum	distributed by Naktuinbouw
5. Isolate	Pb:0, Pb:1, RZ1, Pb:2, Pb:3, RZ2, SAK A, P1
6. Establishment isolate identity	On differential host range
7. Establishment pathogenicity	On susceptible variety
8. Multiplication inoculum	
8.1 Multiplication medium	Plant roots
8.2 Multiplication variety	Bartolo, Granaat (*Brassica rapa*)
8.3 Plant stage at inoculation	Seedling, 1 week after sowing
8.4 Inoculation medium	Water
8.5 Inoculation method	1 ml spore suspension (10^8 sp/ml) Pipette to the base of each seedling.
8.6 Harvest of inoculum	Harvest roots 6-8 weeks after inoculation
8.7 Check of harvested inoculum	Microscopic count
8.8 Shelf life/viability inoculum	Frozen 3 years, room temp 1-2 days.
9. Format of the test	
9.1 # plants per genotype	30 plants per genotype (2 x 15 plants)
9.2 # replicates	2 replicates (2 x 15 plants)
9.3 Control varieties	Bartolo (no resistance) Bejo 051632 (resistant Pb:0 and Pb:3) Clapton (resistant Pb:0, Pb:1 and Pb:3) Lodero (resistant Pb:0, Pb:1, Pb:2)
9.4 Test design	
9.5 Test facility	Greenhouse
9.6 Temperature	20-22°C, log temperature data
9.7 Light	Natural, extended to 16 h if needed
9.8 Season	Not in winter, not in too warm conditions
9.9 Special measures	A moderate amount of water is required to prevent rotting, saturated soil in the first week, during plant growth not too dried out soil to lower soil temperature
10. Inoculation	
10.1 Preparation inoculum	Symptomatic roots are homogenized in ca. 1 min in a blender. Dilute clubs 1 :4 with demi water. Blender the mix for less than 1 minute. (Beware: Longer blendering may cause overheating of the suspension)
10.2 Quantification inoculum	10^7 spores per ml
10.3 Plant stage at inoculation	1 week old seedlings
10.4 Inoculation method	Pipetting on both side 1 ml to the base of each seedling, 2 ml per plant

10.7 Observation, evaluation and end of test	6 weeks after inoculation (destructive)

11. Observations

11.1 Method	Visual: observation of severe galling and growth retardation Destructive: observation on a 0-3 scale for galling
11.2 Observation scale	Grade 0: • no swellings or a few small spheroid galls Grade 1: • very slight swelling, usually confined to the lateral roots Grade 2: • moderate swelling on lateral and/or tap roots, or: • slight swelling of the main root and browning and ultimately death of all the lateral roots Grade 3: • severe swelling on lateral and/or tap roots
11.3 Validation of test	On standards

12. Interpretation of data in terms of UPOV characteristic states

Grade 0 and 1: resistance present
Grade 2 and 3: resistance absent (=susceptible)

The observation scale is described in figure 1 and 2.

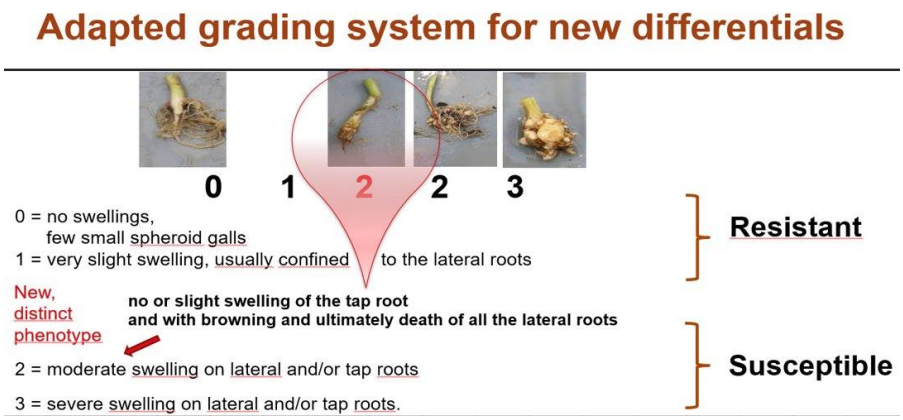

Figure 1. Grading system. 0 is resistant, 3 is susceptible (Figure made by NAKTuinbouw).

✓ Six weeks after inoculation (42 dpi), the roots of each plant is dug out, washed with tap water and examined for gall formation

✓ Club root severity is rated using a 0 to 3 scale as described previously by Horiuchi & Hori (1980), which was modified by Strelkov et al. (2006)

0 = no galling | 1 = a few small galls | 2 = moderate galling | 3 = severe galling

$$\text{Disease severity index (\%)} = \frac{\Sigma\,(n_0 \times 0 + n_1 \times 1 + n_2 \times 2 + n_3 \times 3)}{N \times \text{Number of classes with symptoms}} \times 100$$

Disease Threshold

DSI ≤ 25% : resistant

DSI > 25% : susceptible

n = the number of plants in each class; **N** = the total number of plants; and **0**, **1**, **2** and **3** = the symptom severity classes

Figure 2. Disease severity index (figure made by Julius Kühn Institute).

Annex 3: Meetings of the project group

Kick off meeting on 8 February 2022
Second meeting on 31 March 2022
Third meeting on 25 May 2022
Fourth meeting on 6 September 2022
Fifth meeting on 20 June 2023
Sixth meeting on 4 September 2023
Discussion on sending isolates on 20 November 2023 (part of the group)
Seventh meeting on 5 February 2024
Eighth meeting on 4 September 2024
Final meeting on 12 November 2024

Annex 4. Proposal for a draft updated UPOV guideline/CPVO protocol for the characteristic clubroot in *Brassica oleracea*

1.	Pathogen	*Plasmodiophora brassicae*
2.	Quarantine status	no
3.	Host species	*Brassica oleracea*
4.	Source of inoculum	Naktuinbouw[1] (NL)
5.	Isolate	Race Pb: 0, Pb: 1, Pb: 2 and Pb: 3
6.	Establishment isolate identity	with genetically defined differentials from Naktuinbouw (NL) The most recent table is available through ISF at https://www.worldseed.org/our-work/plant-health/differential-hosts/
7.	Establishment pathogenicity	symptoms on susceptible *Brassica oleracea*
8.	Multiplication inoculum	
8.1	Multiplication medium	Plant roots
8.2	Multiplication variety	Bartolo (WC), Granaat (CC) [2]
8.3	Plant stage at inoculation	Seedling, 1 week after sowing
8.4	Inoculation medium	Water
8.5	Inoculation method	2 ml spore suspension (10^7 sp/ml) Pipette to the base of each seedling.
8.6	Harvest of inoculum	Harvest roots 6-8 weeks after inoculation
8.7	Check of harvested inoculum	Microscopic count
8.8	Shelf life/viability inoculum	Frozen 3 years, room temperature 1-2 days
9.	Format of the test	
9.1	Number of plants per genotype	20 plants
9.2	Number of replicates	2 replicates (2 x 10)
9.3	Control varieties	Susceptible: Bartolo (WC) [2] Resistant to race Pb: 0 Bejo 051632 (WC), Clapton (CF), Lodero (RC) Resistant to race Pb: 1 Clapton (CF), Lodero (RC) Resistant to race Pb: 2 Lodero (RC) Resistant to race Pb: 3 Bejo 051632 (WC)
9.5	Test facility	Glasshouse or climatic chamber
9.6	Temperature	20-22°C
9.7	Light	Natural, extended to 16 h if needed
9.9	Special measures	A moderate amount of water is required to prevent rotting. Keep the soil saturated in the first week. During plant growth the soil should not be too dry to lower the soil temperature.
9.8	Season	Not in winter, not in too warm conditions if test performed in greenhouse
10.	Inoculation	

[1] Naktuinbouw: resistentie@naktuinbouw.nl

[2] WC=White cabbage, CC=Chinese cabbage, RC=Red cabbage, CF=Cauliflower

10.1	Preparation inoculum	Symptomatic roots are homogenized ca. 1 min in a blender. Dilute clubs 1:4 with demineralized water. Blender the mix for less than 1 minute. (Beware: longer blendering may cause overheating of the suspension)
10.2	Quantification inoculum	count spores; adjust to 10^7 spores per ml
10.3	Plant stage at inoculation	1 week old seedlings
10.4	Inoculation method	Pipette 1 ml on both sides at the base of each seedling, totalling 2 ml per plant.
10.7	Observation, evaluation and end of test	6 weeks after inoculation (destructive)
11.	Observations	
11.1	Method	Visual: observation of severe galling and growth retardation Destructive: observation on a 0-3 scale for galling
11.2	Observation scale	class 0 = no swellings or a few small spheroid galls class 1 = very slight swelling, usually confined to the lateral roots class 2 = moderate swelling on lateral and/or tap roots or slight swelling of the main root and browning and ultimately death of all the lateral roots class 3 = severe swelling on lateral and/or tap roots
11.3	Validation of test	evaluation of variety resistance should be calibrated with results of resistant and susceptible controls
12.	Interpretation of data in terms of UPOV characteristic states	absent [1] symptoms grade 2 and 3. present [9] symptoms grade 0 and 1
13.	Critical control points	Clubroot is a zoosporic pathogen. Keep isolates spatially well-separated.


0 = no galling


1 = a few small galls


2 = moderate galling


2 = slight swelling of the main root, no lateral roots


3 = severe galling