Validation of the MALDI-TOF MS test for the identification of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*	
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1 Abstract

The MALDI-TOF MS test for the identification of *Xanthomonas oryzae* pv. *oryzae* and pv. *oryzicola* was validated. New MSP's were created successfully. The identification of a *X. oryzae* isolate at the pathovar level includes an additional sybtyping step where two peaks at around 4585 m/z and 9167 m/z, unique to *X. oryzae* pv. *oryzicola,* can be used to distinguish between the two pathovars. The robustness of the MALDI-TOF MS test is good when bacteria are grown on Wilbrink medium at 28°C for two or three days. The analytical specificity of the MALDI-TOF MS test is good since all *X. oryzae* isolates were identified correctly on the pathovar level and none of the other isolates resulted in score values ≥2 for the species *X. oryzae*.

2 Introduction

Xanthomonas oryzae pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc) are the causal agents of the diseases bacterial leaf blight of rice and bacterial leaf streak of rice, respectively [1]. Both bacteria are quarantine organisms in the European Union. The MALDI-TOF MS test would represent a very fast, accurate and cost-effective identification test for these bacteria. Part of the validation will be the generation of new MSP's (reference spectra) of both pathovars to make the identification possible.

Scope of this validation: to validate the MALDI-TOF MS test for the identification of pure cultures of Xoo and Xoc in routine diagnostic samples. The performance criteria analytical specificity (inclusivity and exclusivity) and robustness have been included in this validation.

3 Materials and methods

The MALDI-TOF MS test was performed according to I-BAC-079 (Identificatie van bacteriën d.m.v. MALDI-TOF). New MSPs were created according to I-BAC-080 (Opbouw bibliotheek voor MALDI-TOF). Additionally, new MSP's were created a second time following the same protocol, but with an elongated ethanol incubation step during extraction. Instead of centrifuging immediately after addition of ethanol to the bacterial suspension, the samples were incubated for 90 minutes after addition of ethanol at room temperature before centrifuging.

Table 1 shows the isolates that were used for the analytical specificity. Next to isolates of *X. oryzae* pv. *oryzae* (Xoo) and pv. *oryzicola* (Xoc), isolates of other Xanthomonads and

isolates of other species found in rice were selected. In Table 2 the isolates that were used for the robustness and the generation of MSP's are shown.

For robustness three isolates (Table 2) were purified on Wilbrink and YPG media and incubated for two, three or four days at 28°C before the MALDI-TOF MS analysis. The isolates used for the creation of the MSP's were incubated on Wilbrink medium at 28°C for two days.

Table 1. *Isolates used in this validation.*

PD nr.	Species	Host	Country	Year
PD 773	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Niger	1987
PD 774	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Niger	1987
PD 777	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Niger	1987
PD 848[#]; NCPPB 3002	*X. oryzae* pv. *oryzae*	*Oryza sativa*	India	1965
PD 7964[#]; CFBP 2532	*X. oryzae* pv. *oryzae*	*Oryza sativa*	India	1965
PD 7965; CFBP 7088	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Korea	-
PD 7966; CFBP 8172	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Benin	2013
PD 7999; CFBP 1946	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Phillipines	1979
PD 8000; CFBP 8320	*X. oryzae* pv. *oryzae*	*O. longistaminata*	Benin	2013
PD 8003; LMG 10839	*X. oryzae* pv. *oryzae*	*Oryza sativa*	China	-
PD 8004; LMG 25983	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Burkina Faso	2004
PD 8005; LMG 25984	*X. oryzae* pv. *oryzae*	*O. glaberrima*	Burkina Faso	2004
PD 8006; LMG 25985	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Mali	2004
PD 8007; LMG 25986	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Niger	2004
PD 8008; LMG 634	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Colombia	1982
PD 8009; LMG 806	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Phillipines	1980
PD 8010; LMG 9585	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Bolivia	-
PD 8011; LMG 9588	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Nepal	-
PD 8012; LMG 9589	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Indonesia	-
PD 996; NCPPB 1585	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Malaysia	1964
PD 7967[$]; CFBP 7331	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Mali	2004
PD 7968; CFBP 7338	*X. oryzae* pv. *oryzicola*	*O. longistaminata*	Mali	2009
PD 7969; CFBP 7342	*X. oryzae* pv. *oryzicola*	*Oryza* sp. (wild)	Burkina Faso	2010
PD 7970; CFBP 8316	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Madagascar	2013
PD 7971; CFBP 8318	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Burundi	2013
PD 8001; CFBP 7339	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Mali	2009
PD 8013; LMG 25977	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Burkina Faso	2010
PD 8014[$]; LMG 27222	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Mali	2004

PD 8015; LMG 656	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	India	-
PD 8016; LMG 9717	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	China	-
PD 8017; LMG 9723	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Phillipines	-
PD 995; NCPPB 633	*Xanthomonas citri* pv. *malvacearum*	*Gossypium*	Sudan	1959
PD 8299; LMG 736	*Xanthomonas vasicola* pv. *holcicola*	*Sorghum bicolor*	New Zealand	1969
PD 5426	*Xanthomonas axonopodis* pv. *begoniae*	*Begonia* sp.	The Netherlands	2007
PD 1119; LMG 586	*Xanthomonas cassavae*	*Manihot esculenta*	Colombia	1976
PD 8300; LMG 847	*Xanthomonas pisi*	*Pisum sativum*	Japan	1957
PD 990; NCPPB 409	*Xanthomonas citri* pv. *citri*	*Citrus limon*	New Zealand	-
PD 7981; CFBP 2426	*Acidovorax avenae* subsp. *avenae*	*Oryza sativa*	Japan	1963
PD 8291; LMG 18569	*Burkholderia gladioli*	*Oryza sativa*	Phillipines	1996
PD 8297; CFBP 4900	*Burkholderia glumae*	*Oryza sativa*	Japan	1967
PD 8293; CFBP 3997	*Burkholderia plantarii*	*Oryza sativa*	Japan	1982
PD 866; NCPPB 3090	*Dickeya chrysanthemi*	*Oryza sativa*	Japan	1977
PD 8292; LMG 20118	*Pantoea agglomerans*	*Oryza sativa*	Phillipines	-
PD 8295; CFBP 2065	*Pseudomonas fuscovaginae*	*Oryza sativa*	Japan	1976
PD 8294; CFBP 3228	*Pseudomonas syringae* pv. *oryzae*	*Oryza sativa*	Japan	1983
PD 8296; CFBP 1655	*Pseudomonas syringae* pv. *syringae*	*Oryza sativa*	Hungary	1956

[#]) these isolates are the same
[$]) these isolates are the same

Table 2. *Isolates used to create MSP's and isolates used for robustness.*

PD nr.	Species	Host	Country	Year
PD 7964*; CFBP 2532	*X. oryzae* pv. *oryzae*	*Oryza sativa*	India	1965
PD 8007*; LMG 25986	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Niger	2004
PD 996*; NCPPB 1585	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Malaysia	1964
PD 7969*; CFBP 7342	*X. oryzae* pv. *oryzicola*	*Oryza* sp. (wild)	Burkina Faso	2010
PD 8003; LMG 10839	*X. oryzae* pv. *oryzae*	*Oryza sativa*	China	-
PD 8008; LMG 634	*X. oryzae* pv. *oryzae*	*Oryza sativa*	Colombia	1982
PD 8017; LMG 9723	*X. oryzae* pv. *oryzicola*	*Oryza sativa*	Phillipines	-

*) these isolates were used to create MSP's. The three isolates without asterisk were used for robustness.

4 Results

Creation of MSP's

MSP's of Xoo isolates PD 7964 and PD 8007 and Xoc isolates PD 996 and PD 7969 were created twice: the first time using the standard extraction method described in I-BAC-079

and the second time with an elongated ethanol incubation step of 90 minutes at room temperature. The MSP's with the elongated ethanol incubation step have an extension '_2' added to their names. Both sets of MSP's were evaluated using the MALDI-TOF MS runs of the Xoo and Xoc isolates and the isolates of other Xanthomonads used for the analytical specificity. The data can be found in worksheet 'Exp.3 data per MSP set' in the labjournal located here: T:\PD\NIVIP\Bacteriologie\Onderzoek\MOVA BAC-2022-02 MALDI validatie X. oryzae\Labjournaal. To evaluate the MSP's only score values ≥2 were taken into account. For Xoo isolates it was expected that only MSP's of Xoo isolates had a score value ≥2, for Xoc isolates only Xoc MSP's were expected to have score value ≥2 and for the other Xanthomonad isolates no Xoo or Xoc MSP's should have a score value ≥2.

When considering only the MSP's created with the original extraction protocol, all Xoo and Xoc isolates had a best match with an MSP from their corresponding pathovar. However, for 18 isolates one or both MSP's of the other pathovar also had a score value ≥2 and/or one or more MSP's of other Xanthomonads had a score value ≥2. When considering only the MSP's made using the elongated ethanol incubation step, again all Xoo and Xoc isolates had a best match with an MSP from their corresponding pathovar. In this case, only 11 isolates had the problem with the deviating MSP's. Because of this, it was decided to do all further analyses with this second set of MSP's (elongated ethanol incubation step).

The spectra of all Xoo isolates were compared to the spectra of all Xoc isolates by visual inspection, following the method of subtyping (I-BAC-081). Two peaks, at around 4585 m/z and 9167 m/z, were found that were present in the spectra of all Xoc isolates, but that were not present in the spectra of the Xoo isolates (see Appendix A, Figure A1). For some isolates, these peaks are shifted to the right a little bit. The weight of the lighter peak is approximately half of the weight of the heavier peak. This means that they probably represent the same protein with a weight of approximately 9 kDa, that sometimes ends up having a +1 charge and sometimes a +2 charge during the ionization step. Both peaks were not present in the spectra of any of the other Xanthomonads either. This is clearly shown in Figure 1, where only the spectra of PD 7964, PD 8007, PD 996 and PD 7969 are shown, together with the spectra of the other Xanthomonads. The details of the *X. oryzae* subtyping protocol developed is described in R-BAC-081-001.

In the diagnostic workflow, when there is doubt about the pathovar of a *X. oryzae* isolate in a MALDI-TOF MS analysis, subtyping, using the peaks at around 4585 m/z and 9167 m/z, can be used to distinguish between the pathovars.

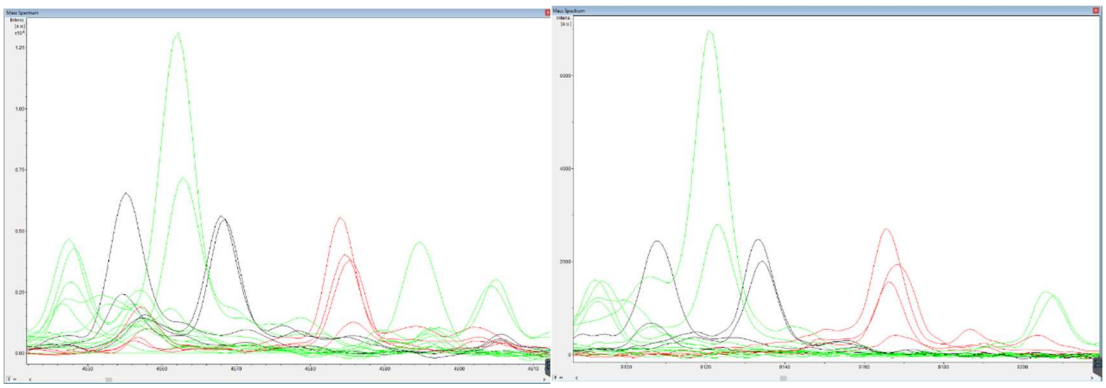

Figure 1. *Left panel: peak (in red) unique for Xoc isolates at around 4585 m/z; Right panel: peak (in red) unique for Xoc isolates at around 9167 m/z. Red: spectra from Xoc isolates PD 7969 and PD 996; black: spectra from Xoo isolates PD 7964 and PD 8007; green: spectra from other Xanthomonads.*

Robustness
For robustness, *X. oryzae* isolates (Table 2) were grown for two, three and four days on Wilbrink and YPG media at 28°C, in order to assess the effect of these parameters. When considering the highest score value for each isolate, the identification is correct for all isolates except for PD 8003 (Xoo) grown at YPG medium for two days (Table 3). In this case, the highest score value is a match with a MSP from a pv. *oryzicola* isolate. The two peaks (see previous paragraph and Appendix A, Figure A2) that can be used for subtyping are clearly missing in the spectra of this isolate on this day and medium, so it is clear that this isolate should have been identified as a pv. *oryzae* strain.

After four days of growth, the score values of PD 8017 on Wilbrink medium and PD 8008 on YPG medium are slightly below two. Next to this, the two peaks at around 4585 m/z and 9167 m/z that can be used for subtyping are decreasing in height over time when the isolates are grown on YPG medium (Appendix A, Figure A2).

Together these findings lead to the conclusion that Wilbrink medium should be used in the identification of *X. oryzae* using MALDI-TOF MS and not YPG medium. Furthermore, four days of growth is too long, two or three days is preferred and fits better in our diagnostic procedure.

Table 3. *Results acquired for the robustness: hits with highest score values.*

	Day 2		**Day 3**		**Day 4**	
Isolate no.	**Best hit with**	**Score value**	**Best hit with**	**Score value**	**Best hit with**	**Score value**
	Wilbrink medium					
PD 8003 Xoo	Xanthomonas oryzae pv. oryzae PD 7964_2	2,34	Xanthomonas oryzae pv. oryzae PD 7964_2	2,36	Xanthomonas oryzae pv. oryzae PD 7964_2	2,25
PD 8008 Xoo	Xanthomonas oryzae pv. oryzae PD 7964_2	2,24	Xanthomonas oryzae pv. oryzae PD 7964_2	2,28	Xanthomonas oryzae pv. oryzae PD 7964_2	2,25
PD 8017 Xoc	Xanthomonas oryzae pv. oryzicola PD 996_2	2,44	Xanthomonas oryzae pv. oryzicola PD 996_2	2,34	Xanthomonas oryzae pv. oryzicola PD 996_2	1,95
	YPG medium					
PD 8003 Xoo	Xanthomonas oryzae pv. oryzicola PD 996_2	2,32	Xanthomonas oryzae pv. oryzae PD 7964_2	2,30	Xanthomonas oryzae pv. oryzae PD 7964_2	2,25
PD 8008 Xoo	Xanthomonas oryzae pv. oryzae PD 7964_2	2,23	Xanthomonas oryzae pv. oryzae PD 7964_2	2,10	Xanthomonas oryzae pv. oryzae PD 7964_2	1,91
PD 8017 Xoc	Xanthomonas oryzae pv. oryzicola PD 996_2	2,49	Xanthomonas oryzae pv. oryzicola PD 996_2	2,36	Xanthomonas oryzae pv. oryzicola PD 996_2	2,13

Analytical specificity
All Xoo and Xoc isolates were identified correctly considering the best match (Table 4) after two days of growth on Wilbrink medium at 28°C. For eleven *X. oryzae* isolates a match with a score value ≥2 with an MSP from the other pathovar and/or another *Xanthomonas* species was found, see paragraph 'Creation of MSP's'. Since the pathovars of these isolates are known and the best match is correct, it is not necessary to do subtyping. Next to this, these isolates are part of the set that was used to develop the subtyping protocol and thus all do or do not have the xoc-specific peak according to what is expected.

Four out of six isolates belonging to other *Xanthomonas* species were identified correctly, while the remaining two were not (Table 4). This is most likely due to the absence of reference

spectra of these species. None of these isolates were identified as *X. oryzae*. The nine non-*Xanthomonas* isolates that were isolated from rice were not identified as *X. oryzae* either. Three of them were correctly identified on the species level, the others were only identified on the genus level, or not reliably identified at all.

Table 4. *Results acquired for the analytical specificity after two days of incubation on Wilbrink: hits with highest score values.*

PD nr.	Species	Best hit with MSP	Score value
PD 773	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.45
PD 774	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.37
PD 777	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.36
PD 848[#]	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.35
PD 7964[#]	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.26
PD 7965	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.27
PD 7966	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.38
PD 7999	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.34
PD 8000	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.35
PD 8003	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.28
PD 8004	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.38
PD 8005	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.32
PD 8006	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.17
PD 8007	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 8007_2	2.37
PD 8008	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.29
PD 8009	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.28
PD 8010	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.28
PD 8011	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.35
PD 8012	*X. oryzae* pv. *oryzae*	Xanthomonas oryzae pv. oryzae PD 7964_2	2.33
PD 996	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.49
PD 7967[$]	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.39
PD 7968	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.41
PD 7969	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 7969_2	2.30
PD 7970	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.35
PD 7971	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.37
PD 8001	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.42
PD 8013	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.32
PD 8014[$]	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.35
PD 8015	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.31
PD 8016	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.47
PD 8017	*X. oryzae* pv. *oryzicola*	Xanthomonas oryzae pv. oryzicola PD 996_2	2.46
PD 995	*Xanthomonas citri* pv. *malvacearum*	Xanthomonas citri pv malvacearum DSM 3849 DSM	2.30
PD 8299	*Xanthomonas vasicola* pv. *holcicola*	Xanthomonas vasicola DSM 16926T DSM_2	2.58
PD 5426	*Xanthomonas axonopodis* pv. *begoniae*	Xanthomonas axonopodis pv begoniae DSM 50850 DSM	2.36
PD 1119	*Xanthomonas cassavae*	Xanthomonas perforans DSM 18975T DSM	2.39

PD 8300	*Xanthomonas pisi*	Xanthomonas pisi DSM 18956T DSM_2	2.34
PD 990	*Xanthomonas citri* pv. *citri*	Xanthomonas axonopodis pv begoniae DSM 50850 DSM	2.21
PD 7981	*Acidovorax avenae* subsp. *avenae*	Acidovorax cattleyae DSM 17101T DSM	1.75
PD 8291	*Burkholderia gladioli*	Burkholderia gladioli LMG 6880 LMG	2.32
PD 8297	*Burkholderia glumae*	Burkholderia glumae DSM 9512T HAM	2.09
PD 8293	*Burkholderia plantarii*	Burkholderia plantarii DSM 9509T HAM	2.26
PD 866	*Dickeya chrysanthemi*	Dickeya dadantii ssp dieffenbachiae DSM 18013T HAM	1.76
PD 8292	*Pantoea agglomerans*	Pantoea eucrina DSM 24231T DSM	1.58
PD 8295	*Pseudomonas fuscovaginae*	Pseudomonas asplenii DSM 7231T HAM	2.41
PD 8294	*Pseudomonas syringae* pv. *oryzae*	Pseudomonas syringae ssp syringae DSM 6693 HAM	1.55
PD 8296	*Pseudomonas syringae* pv. *syringae*	Pseudomonas syringae ssp syringae DSM 6693 HAM	1.87

[#]) these isolates are the same
[$]) these isolates are the same

5 Discussion and conclusion

The analytical specificity of the identification of *X. oryzae* at the pathovar level *(*pv. *oryzae* and pv. *oryzicola)* using the MALDI-TOF MS test, including the additional subtyping step described here above, is good.

The robustness of the identification of *X. oryzae* pv. *oryzae* and pv. *oryzicola* using the MALDI-TOF MS test is good when isolates are incubated into Wilbrink medium for two or three days.

6 Recommendations

When the MALDI-TOF MS test results in a doubtful identification of *X. oryzae* on the pathovar level, use the peaks at 4585 m/z and 9167 m/z to distinguish between the pathovars. A doubtful identification can occur when the MALDI-TOF MS test results in more than one hit with a score value ≥2 when these hits belong to both *X. oryzae* pathovars and/or to other *Xanthomonas* species.

7 Decision

The MALDI-TOF MS test, including the additional subtyping step described here above, is fit for purpose for the identification of *X. oryzae* at the pathovar level and will be implemented in our diagnostic procedures.

8 References

1. EPPO, *PM 7/80 (1) Xanthomonas oryzae.* EPPO Bulletin, 2007. **37**: p. 543-553.

9 Approval

Approval by discipline head(s) or authorized employee

27/11/2023 Maria Bergsma-Vlami

Appendix A

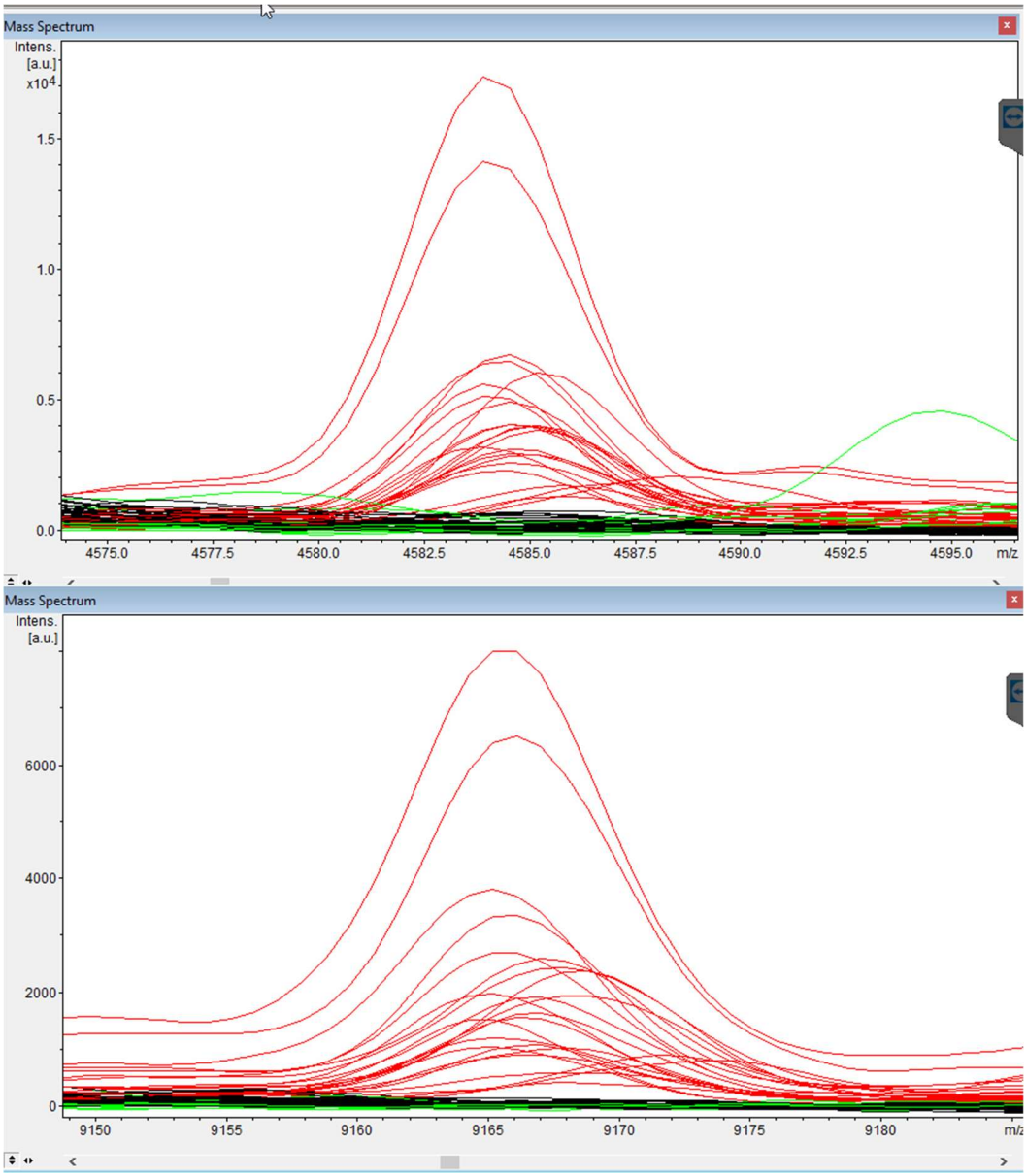

Figure A1. *Peaks unique for* X. oryzae *pv.* oryzicola (Xoc). *Upper panel: unique peak at around 4585 m/z; lower panel: unique peak at around 9167 m/z. Red: spectra from Xoc isolates; black: spectra from Xoo isolates; green: spectra from other Xanthomonads.*

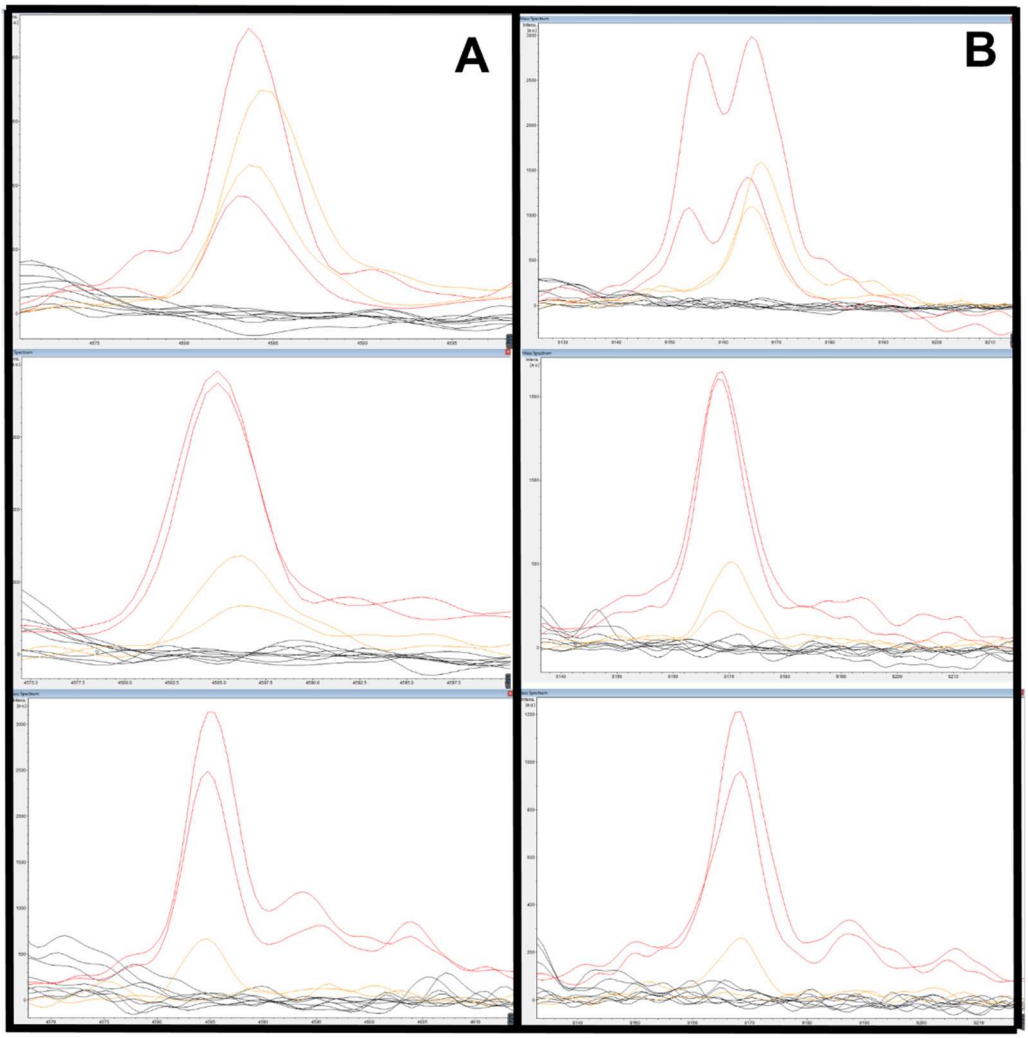

Figure A2. *Peaks at around 4585 m/z (panel A) and 9167 m/z (panel B) as seen in the robustness experiment. Both panels from top to bottom: two days of incubation, three days of incubation, four days of incubation. Black: spectra from PD 8003 and PD 8008 grown on YPG and Wilbrink media. Red: spectra from PD 8017 grown on Wilbrink medium. Orange: spectra from PD 8017 grown on YPG medium.*