EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION
ORGANISATION EUROPEENNE ET MEDITERRANEENNE POUR LA PROTECTION DES PLANTES
Summary sheet of validation data for a diagnostic test

The EPPO Standard PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* describes how validation should be conducted. It also includes definitions of performance criteria.

Laboratory contact details	Naktuinbouw Sotaweg 22, 2371 GD Roelofarendsveen, Netherlands
Short description of the test	Detection of Curtobacterium flaccumfaciens pv. flaccumfaciens from bean (Phaseolus spp.) seeds by Isolation on semi selective media
Date, reference of the validation report	2023-08-17 - Validation report Cff SPN-B005 v3.0
Validation process according to EPPO Standard PM7/98?	yes
Is the lab accredited for this test?	yes
Was the validated data generated in the framework of a project?	no
Description of the test	
Organism(s)	Curtobacterium flaccumfaciens pv. flaccumfaciens (CORBFL)
Detection / identification	detection
Method(s)	Isolation
Method: Isolation	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	yes
New test being considered for inclusion in the next version of the EPPO diagnostic protocol?	yes
EPPO Diagnostic Protocol name	PM 7/102 Curtobacterium flaccumfaciens pv. flaccumfaciens (version 1)
Name of the test	Direct isolation with NBY and SSM
As or adapted from an IPPC diagnostic protocol	no
Is the test modified compared to the reference test	yes Instead of using semi- selective media NBY and SSM, mMSCFF and mMSCFF+b Next to D2ANX media are described in the testing method validated
Other information	
Other details on the test	1.000 seed subsamples are tested by static

	incubation of the seed in 0,15M NaCL @4C for 12-18hrs. the w:v ration of the seed and buffer are the (TSW * 1,9) +50mL. After incubation the seed are crushed 20x by hand before Dilution plating execution. Per medium 100µl pure,10x and 100x diluted extract are spread and incubated @28C for 6days. Morphological scoring if suspects is done after 6 days
Performance Criteria :	
Organism 1.:	**Curtobacterium flaccumfaciens pv. flaccumfaciens(CORBFL)**
Analytical sensitivity	
What is smallest amount of target that can be detected reliably?	Executed experiment 1 for analytical sensitivity using 4 morphologically different Cff isolates (red, yellow, purple and orange) when spiked in a seed extract background of common bean (ZZB-476) showed for all four isolates a LOD of <90 CFU on mMSCFF, mMSCFF+b as well as on D2ANX media. A stock solution (OD600=’.1) of the 4 Cff isolates was prepared by O/N growth in Triptic soy broth (TBS). DNA of the stocks was isolated using the Qiagen Blood and tissue extraction kit. With the concentration of the isolated DNA the amount of CFU/mL was calculated. Next to this per isolate, making use of the same stock solution, three independant 10x dilution series were prepared and were each plated in triplicate on the three media. a qualitative scoring of the 9 plates per dilution, per media was done until the dilution where no growth in any of the 9 plates was found. using this qualitative data in a statistical analysis resulted in a LOD <90 CFU for all four isolates on all three media. Experiment 2 focused on the ability of detecting 1 Cff infested seed in a sample of 1000 seed. 8 samples consisting of 999 seed of a Cff free seed batch was spiked with 1 seed of an artificially innoculated with Cff seed batch. All samples were testedaccording the proposed protocol and plated on mMSCFF, mMSCFF+b and D2ANX medium. In all 8 samples growth was observed on all three media demonstrating the ability to detect one infested seed in a sample of 1000 seed.
Diagnostic sensitivity	
Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98	not demonstrated in this validation
Analytical specificity - inclusivity	
Number of strains/populations of target organisms tested	22 Cff isolates collected from different sources, years and origins were selected and in a standard dilution spread plated in triplicate on the three semi selective media, mMSCFF, mMSCFF+b and D2ANX. CFU were counted and results were compared.
Specificity value	100%, All 22 isolates showed growth on all three

	media in a compairable fashion over the media.
Analytical specificity - exclusivity	
Number of non-target organisms tested	26 bacterial isolates were selected as non-target isolates based on them belonginging to the curtobacterium Spp. (but not Cff), none to be a seedborne pathogen in bean seed (Xap, Pss, Psp, Xff) and common found saprohytes from bean seed samples on non-selective media (KB). all isolates were in a standard dilution spread plated on the three semi selective media, mMSCFF, mMSCFF+b and D2ANX. morphologically, only Curtobacterium Spp. showed suspect growth on all three media. for Xap and Xff isolates growth was observed on only one of the media but were not morphologically selected as Cff suspect colonies.
Specificity value	the 2 Curtobacterium Spp. showed equal growth as the Cff isolates in the inclusivity experiment so are falsely scored positive for Cff but would be excluded to be Cff by the suspect confirmation PCR also present in our protocol.
Cross reacts with	Curtobacterium sp.
Diagnostic Specificity	
Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test	not demonstrated in this validation
Reproducibility	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	Reproducibility was demonstrated to be 100%. To demonstrate reproducibility the Cff spike control prepared by different analists and plated on different batches of media was monitored for two months.
Repeatability	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	Five, 1000 seed samples, 1 Cff negative, 3 Cff medium infected and 1Cff highly infected, were prepared and tested at three different days by the same analist using the same equipment and batches of media. all samples tested at the three different timepoints showed a 100% score in regards to the expected.
Test performance study	
Test performance study?	no
Other information	
Any other information considered useful	mMSCFF medium/Liter pepton 5 g meat extract 3 g sucrose 5 g agar 15 g Set pH 6,8 and autoclave (15 min, 121°C) After autoclaving and cooling (45°C) add: Nystatin 200.000 U Skimmed milk 5 g Congo red 50 mg Naladixic acid 10 mg Nitrofurantoin 10 mg Oxacillin 1 mg Sodium azide 1 mg mMSCFF+b medium/Liter pepton 5 g meat extract 3 g sucrose 5 g agar 15 g Set pH 6,8 and autoclave (15 min,

	121°C) After autoclaving and cooling (45°C) add: Nystatin 200.000 U Skimmed milk 5 g Congo red 50 mg Naladixic acid 10 mg Nitrofurantoin 10 mg Oxacillin 1 mg Polymixine B sulphate 39.000 U D2ANX medium/liter Tris-HCl 1,2 g Caseïne hydrolysate 4 g MgSO4 anhydrous 0,15 g NH4Cl 1 g H3BO3 1 g Yeast extract 2 g Glucose 10 g Agar 18 g Set pH 7,4 and autoclave (15 min, 121°C) After autoclaving an cooling (45°C) add: Nalidixic acid 28 mg Cycloheximide 100 mg Polymixine B sulphate 78.000 U
The following complementary files are available online:	• Validation report Cff SPN-B005 v3.0 EPPO

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