Final Report (draft)

Project title (Acronym)
Consensus Detection and Identification Protocol for *Acidovorax citrulli* on cucurbit seeds (DIP-ACIT)

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Contents

1. Research consortium partners

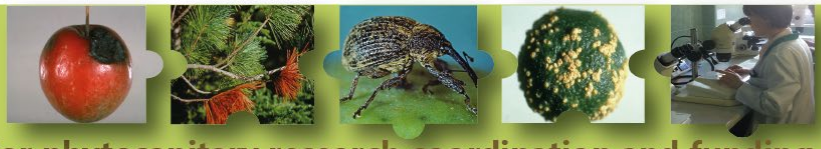
Applicant / Coordinator – Partner 1			
Organisation	University of Modena and Reggio Emilia (UNIMORE)		
Name of contact (incl. Title)	Prof. Dr. Emilio Stefani; Dr Davide Giovanardi	**Gender**:	M; M
Postal address	Dept. of Life Sciences, via Amendola 2 – Pad. Besta 42122 Reggio Emilia (Italy)		
E-mail	emilio.stefani@unimore.it		
Phone	+39 0522 522062		

Applicant – Partner 2			
Organisation	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES)		
Name of contact (incl. Title)	Dr. Amandine Cunty; Valérie Olivier	**Gender**:	F; F
Postal address	7 rue Jean Dixméras 49044 Angers Cedex 01		
E-mail	amandine.cunty@anses.fr; valerie.olivier@anses.fr		
Phone	+33 241207431		

Applicant – Partner 3			
Organisation	Groupe d'Etude et de contrôle des Variétés Et des Semences (GEVES)		
Name of contact (incl. Title)	Dr. Valerie Grimault	**Gender**:	F
Postal address	25 rue Georges Morel, 49071 Beaucouzé Cedex		
E-mail	valerie.grimault@geves.fr		
Phone	+33 2 41 22 58 50		

Applicant – Partner 4			
Organisation	Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA)		
Name of contact (incl. Title)	Dr. Nicoletta Pucci	**Gender**:	F
Postal address	Via C.G. Bertero 22, 00156 Rome (Italy)		
E-mail	nicoletta.pucci@crea.gov.it		
Phone	+39 06 82070356		

Applicant – Partner 5			
Organisation	Nederlandse Voedsel-en Warenautoriteit (NVWA)		
Name of contact (incl. Title)	Dr. Maria Bergsma-Vlami	**Gender**:	F
Postal address	Geertjesweg 15, 6706 EA Wageningen, The Netherlands		
E-mail	m.vlami@nvwa.nl		



Applicant – Partner 6			
Organisation	All-Russian Plant Quarantine Centre (ARPQC)		
Name of contact (incl. Title)	Dr. Konstantin Kornev	**Gender**:	M
Postal address	Pogranichnaya str. 32, Ramensky district, Moscow region, Bykovo, 140150, Russian Federation		
E-mail	konstantin.kornev@gmail.com		
Phone	+ 7 926 1866749		

Applicant – Partner 7			
Organisation	Benaki Phyotpathological Institute (BPI)		
Name of contact (incl. Title)	Dr. Maria Holeva	**Gender**:	F
Postal address	8, Stefanou Delta Street, 14561 Kifissia, Athens, Greece		
E-mail	m.holeva@bpi.gr		
Phone	+30 210 8180259 or +30 210 8180239		

Applicant – Partner 8			
Organisation	Biological Control Research Institute (BCRI)		
Name of contact (incl. Title)	Dr. Raziye Cetinkaya Yildiz	**Gender**:	F
E-mail	raziye.yildiz@tarim.gov.tr		

Applicant – Partner 9			
Organisation	Institute of Plant Protection and Environment (IPPE)		
Name of contact (incl. Title)	Dr. Tatjana Popovic	**Gender**:	F
Postal address	Teodora Drajzera 9, 11040 Belgrade, Serbia		
E-mail	tanjaizbis@gmail.com		

2. Short project report

2.1. Short executive summary

Acidovorax citrulli (*A. citrulli*) is the causal agent of bacterial fruit blotch (BFB) of cucurbits. Since there are no resistant commercial cultivars, successful management of BFB depends on exclusion of primary inoculum by using pathogen-free seeds and seedlings. Seed health testing reduces the risk of outbreaks. For this purpose, the Euphresco project had the goal to implement media and methods, validate them and, finally, develop a consensus protocol for the detection of *A. citrulli* on cucurbit seeds. Based on project's results, two validated agar media for direct isolation of the pathogen are recommended, together with an implemented protocol for the pathogenicity test. Additionally, MALDI-TOF mass spectrometry was proven to be a robust method for pathogen identification. A TaqMan real-time PCR protocol was also selected, validated and ring-tested. All together, the results of this project will allow the update of the EPPO Diagnostic Protocol PM7/127(1) with robust detection and identification procedures and validated data.

2.2. Project aims

The project aims and objectives were to provide validated methods, protocols and procedures for the detection and identification of *A. citrulli* in seed lots (melon and watermelon), as seed is the main pathway for entry and spread of the pathogen into new areas. Therefore, the following activities were planned:

a) The development of a consensus protocol for the detection of *A. citrulli* on cucurbit seeds in order to provide official laboratories with a validated procedure for seed analysis and certification;
b) The validation of a semi-selective agar medium for pathogen isolation from symptomatic plant material, in order to allow analysts to easily obtain a pure culture of the pathogen suitable for both identification, genotyping and pathogenicity tests;
c) The implementation of a pathogenicity test for confirmation of pathogen identity;
d) The organization of a test performance study on the following methods, in order to increase the robustness of the whole detection procedure, as described in the flow diagram of the EPPO Diagnostic Protocol:

    1) Pathogen isolation on a semi-selective medium;
    2) Identification of *A. citrulli* isolates, including grouping;
    3) Pathogenicity tests on melon and watermelon;
    4) Real-time PCR test on seed extracts;

2.3. Description of main activities

The following activities were undertaken in the framework of the DIP-ACIT project:

a) The choice of one or more semi-selective media for the isolation of *A. citrulli* from plant material. This was done by testing several available agar media, particularly mKB and NSA250.
b) The best performing agar media were validated in order to allow analysts to easily obtain a pure culture of the pathogen suitable for both identification, genotyping and pathogenicity tests.
c) The implementation of a pathogenicity test on melon and watermelon for the verification of pathogen identity and pathogenicity. In particular, two inoculation methods were applied: drop inoculation and injection.

d) A test performance study was organised. The test performance study included methods developed, implemented and/or validated during the first part of the project. Therefore, the test performance study included: 1) pathogen isolation on a validated semi-selective medium, identification and grouping; 2) the pathogenicity test to be done on both watermelon and melon seedlings using isolates belonging to group I and group II of *A. citrulli*; 3) the validated real-time PCR test for the detection of *A. citrulli* in seed extracts.

2.4. Main results

Isolation and Identification

Two media were tested and compared by partners UNIMORE, ANSES, GEVES, BPI, CREA, NVWA, ARPQC: mKB (an amended King's B medium) and NSA250 (an amended nutrient broth-sucrose-agar medium). Both media contained antibiotics to increase their selectivity towards *A. citrulli*. Both media proved to be suitable for the isolation of *A. citrulli*, although medium mKB proved to be slightly more selective than NSA250 in five laboratories: UNIMORE and GEVES were more satisfied using NSA250. Therefore, mKB was indicated for the ring test. Additional isolation media beyond mKB and NSA250 were tested by one partner (ARPQC). They were: YDC, NBY, NA, EBB, mEBBA. Again, mKB performed better than all the other media tested. mEBBA (Zhao *et al.*, 2009) also gave excellent results during the isolation of *A. citrulli* from plant material: its specificity is comparable to that of mKB. In other experiments, ANSES reported that isolation on mKB and/or NSA250 from older seedlings (3 weeks old), showing a bad phytosanitary aspect due to necrotrophic microorganisms (*e.g.*: from a seedling grow-out assay) resulted in the growth of a very high number of saprophytes: therefore, in such case, false negative isolation could not be ruled out. This was confirmed by GEVES: the isolation from older symptomatic seedlings increases the risk to have too many saprophytes growing on semi-selective media. In general, isolation on semi-selective media was far more satisfactory from seedlings used during a pathogenicity test than from seedlings coming from the seedling grow-out assay.

Pathogenicity assays of host plants (melon and watermelon) were implemented by three partners (UNIMORE, ANSES, GEVES). Partners were able to reproduce symptoms on plants. The best approach was to test putative *A. citrulli* colonies on the same plant species from which the isolates were obtained: therefore, isolates from melon are tested on melon and isolates from watermelon are tested on watermelon. Drop inoculation vs. infiltration were compared by ANSES and GEVES: partners reported that both drop inoculation and infiltration performed well during the implementation of the pathogenicity test, allowing the discrimination of *A. citrulli* from bacterial saprophytes. GEVES preferred infiltration than drop inoculation. In most cases, the inoculation of the pathogen resulted in the expression of symptoms on the test plants. In only two cases the result was a false negative. No case of a false positive was observed. GEVES observed that no significant difference was present for the two inoculation methods, but the infiltration method is able to develop symptoms that are more typical and easier to evaluate. In the case of a bioassay using plant macerates, same technique might be applied: in this case, the bioassay might be used as an enrichment assay to allow easier pathogen isolation from symptomatic seedlings (*e.g.*: from a seedling grow-out assay). The pathogenicity test was validated by GEVES. A final protocol for the pathogenicity test was implemented and proposed for the ring test: such protocol suggested the use of the drop inoculation with a pipette tip, allowing the tip to make a small wound on the stem to support pathogen penetration into the host tissue.

Identification and grouping of putative *A. citrulli* isolates was developed by partners UNIMORE and ANSES, using a set of target strains (12 for UNIMORE and 15 for ANSES) and non-target strains (20 for UNIMORE and 22 for ANSES). DNA extraction was done using the Blood and Tissue DNA extraction kit by Qiagen (UNIMORE) and the heat shock method (ANSES). DNA amplification followed the Schaad *et al.* (2000) method (UNIMORE) and the Bahar *et al.* method (ANSES). In all cases, inclusivity was 100% with a repeatability of 100% and exclusivity was also 100% with repeatability of 100%. Therefore, it was concluded that both Schaad *et al.* (2009) and Bahar *et al.* (2008) are two excellent and comparable methods for identifying pure cultures of putative *A. citrulli* isolates. Grouping of *A. citrulli* isolates was done by UNIMORE using a set of identified 20 strains of both groups I and II. The protocol of Zivanovic & Walcott (2017) was used: such PCR protocol allowed a precise group classification of all isolates and was selected for the TPS.

Alternatively, NVWA worked on the identification of *A. citrulli* by using the MALDI-TOF mass spectrometry technique. Two methods were used: the direct transfer method and the direct formic acid method. Ten *Acidovorax* spp. authentic strains were used for constructing an in-house database: among them, six *A. citrulli*, two *A. konjaci*, one *A. cattleyae* and one *A. avenae*. Results showed that *A. citrulli* isolates were always identified. Both methodologies (direct transfer and direct formic acid) performed equally well. Identification of *A. citrulli* by MALDI-TOF did not allow any group assignment: nonetheless, MALDI-TOF MS demonstrated its potential and simplicity for rapid and accurate pathogen identification.

One partner (ANSES) worked on the implementation of the seedling grow-out assay. Two sets of symptomatic seedlings from the seedling grow-out were analysed by direct isolation: one set of 2-week old and one set of 3-week old seedlings. Results were more satisfactory with the 2-week old seedlings, where the recovery rate was 100%, whereas the recovery rate from the 3-week old seedlings was only 13,3%. The high humidity in the sweat-box, together with the long time required for the incubation of seedlings induced a non-specific chlorosis and other symptoms on seedlings that made isolation/analysis quite cumbersome (false negative results were possible). The following recommendation is given to those labs performing the seedling grow-out assay: do not exceed to grow seedling more than 2 weeks, in order to prevent an excessive growth of plantlets and in order to avoid the growth of too many saprophytes on symptomatic tissue (that tissue where *A. citrulli* might develop necrotic lesions and that might be easily colonized by any possible saprophyte).

Validation

The EPPO standard PM7/98(2): "*Specific requirement for laboratories preparing accreditation for a plant pest diagnostic activity, annex 5*" was used as a reference document. The following partners participated to the validation of protocols: UNIMORE, ANSES, GEVES, NVWA validated the real-time PCR test according Woudt *et al*. (2009). ANSES validated the agar medium mKB: such medium proved to be very efficient in isolating *A. citrulli* from plant material in known concentration. The successful rate of isolation was 100%. UNIMORE validated the agar medium NSA250. Again, the successful rate of isolation was 100%. A few other bacteria grew on NSA250, but they were morphologically different. The rate of *A. citrulli* recovery was approximately 90%. Sensitivity threshold was approx. $4x10^3$ cfu/mL. Both media, mKB and NSA250, did not allow growth of fungi possibly present in the plant macerate. GEVES validated both media. Therefore, it is suggested to use mKB or NSA250 during the isolation of *A. citrulli* from symptomatic tissue, but not from seeds.

Symptomatic tissue might originate from field samples of from seedlings used during the seedling grow-out assay. Validation reports are available from participating partners.

A real-time PCR protocol published by Woudt *et al*. (2009) was validated. DNA extraction from plant material (seeds) was done according to two methods: 1) use of the DNeasy Plant MiniKit by Qiagen or 2) use of the QuickPick system by BioNobile. Both methods allowed obtaining DNA of the target organism in sufficient quantity and of good quality. Twenty target strains of both group I and II (from melon, watermelon and pumpkin) and thirty non-target strains were used. Two primer sets were used: IS1002 and Contig 22. Results obtained in all participating laboratories highlighted an inclusivity of 100% and exclusivity of 100% for both primer sets. Repeatability was again 100%. The detection threshold was between 10^1 (UNIMORE) and 10^2 (ANSES) cells/mL using the DNeasy Plant MiniKit by Qiagen. ANSES obtained a higher sensitivity by using the QuickPick system by BioNobile during the DNA extraction step.

The pathogenicity test was implemented by UNIMORE, ANSES and GEVES. Experiments were implemented taking into accounts the following parameters: i) the concentration of inoculum (UNIMORE tested different concentrations, from 10^7 down to 10^3 cells/mL); ii) the response of the host plants (melon vs. watermelon); iii) the inoculation technique (syringe stem injection vs. pipetting into a stem wound); iv) isolation from diseases tissue.
The results obtained highlighted the following suggestions/recommendations: a) the concentration of the bacterial suspension should be approx. 10^6 cells/mL to allow reproducibility of results; b) *A. citrulli* isolates from melon are to be tested on the same host plant; the same for watermelon isolates; that strategy ensured a faster plant response; c) both inoculation techniques performed the same way, but GEVES reported that symptom expression and pathogen re-isolation were better using the injection method; d) isolation from diseased tissue was in most cases successful (from 85 to 100%) using both mKB (it performed better at ANSES) and NSA250 (it performed better at UNIMORE and GEVES). Anyway, both media have been validated.

Test Performance Study
An inter-laboratory comparison has been organized among the following organisations: UNIMORE, ANSES, CREA, NVWA, ARPQC, BPI, BCRI and SFR-BO (the official lab of the RPPO, Emilia Romagna, Italy)to evaluate four different protocols that are recommended in the EPPO Diagnostic Protocol for the detection and identification of *A. citrulli*. Protocol n. 1 was: Isolation of *A. citrulli* on mKB and NSA250; Protocol n. 2 was: Identification of *A. citrulli* isolates; Protocol n. 3 was: Pathogenicity test on melon and watermelon; Protocol n. 4 was: Real-time PCR test on seed extracts according Woudt *et al*. (2009).

All material was prepared by UNIMORE and shipped in dry ice to ILT participating partners (seven in total + UNIMORE). The material consisted in: i) a set of plant extracts (melon and watermelon) with known *A. citrulli* contamination (0, 10^4; 10^5; 10^6 cells/mL); ii) a set of 10 cultures of look-alikes, two *A. citrulli* strains (belonging to Group I and Group II), one *A. cattleyae* strain; iii) a set of extracts from seed samples, spiked with 0, 10, 10^2, 10^3 cells/mL + one sample with natural infection.

Isolation of *A. citrulli* on semi-selective media. Eight partners participated to this ring test. The isolation of *A. citrulli* from symptomatic plant samples was done on both mKB and

NSA250. On mKB the percentage of agreement was 100% (therefore, partners were able to isolate *A. citrulli* from all contaminated samples). On NSA250, percentage of agreement varied from 85,7% (partner C7) to 100% (all the remaining partners): therefore, only one partner had some trouble with plant samples with a contamination rate below 10^5 cells/mL using NSA250. Therefore, the validated mKB revealed the most robust medium for *A. citrulli* isolation from plant tissue (melon and watermelon).

Identification of *A. citrulli*, including grouping. Eight partners participated in this test. Six partners were able to correctly identify the pathogen, including grouping in Group I or II: therefore, no false positives or negatives were detected in six labs. One partner (A1) wrongly identified 3 look-alikes and *A. cattleyae* as *A. citrulli* (four false positives); one partner (A6) misidentified the pathogen (two false negatives) and two look-alikes (two false positives). Notably, both partners (A1 and A6) had identification problems with same lookalikes. Partner A1 was not able to group the two false positive isolates: therefore, they realized that such strains were wrongly identified as *A. citrulli*. Partner A6 had more troubles, since they assigned to Group I the two lookalikes they wrongly identified as *A. citrulli*. Therefore, grouping of *A. citrulli* isolates posed more problems than strain identification: the reason of that might be related to a misinterpretation of possible, unspecific bands appearing in the gels.

Pathogenicity test. Five partners participated to this inter-laboratory test. Four of them were successful in performing the pathogenicity test, both on melon and on watermelon. Partner N3 only succeeded to perform such a test with the positive isolation control (PIC) and on watermelon only. It is not clear what happened in the performance of such test.

Real-Time PCR test on seed extracts. The real-time PCR was ring-tested by eight partners in total, on the same seed extracts provided by UNIMORE, but according two different protocols targeting: sequence IS 1002 (eight partners) and sequence Contig 22 (five partners). Additionally, partner A1 applied two protocols of extraction on the seed extracts they received. TaqMan PCR with the IS 1002 performed excellently, when the two highest concentrations were tested (10^2, 10^3 cells/mL) (analytical sensitivity 10^2 cells/mL); three partners out of eight were not able to detect the lowest concentration (10 cells/mL). Therefore, considering the results obtained, the ring-tested real-time PCR is globally considered very sensitive and reliable. The real-time PCR with Contig 22 performed well only for the highest contamination rate (10^3 cells/mL). Three partners were not able to detect the lowest pathogen concentration. Partner A1 improved the sensitivity of detection with an alternative extraction (using beads): such extraction protocol is the one routinely used in their laboratory. It can be concluded that the validated real-time PCR test is sufficiently reliable to ensure a robust (sensitive and specific) detection of *A. citrulli* infecting cucurbit seeds.

Results from the inter-laboratory comparison are summarized in following table:

	Assay 1: Direct isolation		Assay 2: Molecular identification including grouping	Assay 3: Pathogenicity on:		Assay 4: Real-Time PCR Detection threshold: 10^2 cells/mL	
	mKB	Nsa250		Melon	Watermelon	IS 1002	Contig22
Sensitivity %	100	90,5	85,71	100	100	78,6	75

Specificity %	100	100	90,91	100	100	85,71	75
Accuracy %	100	91,8	90,11	100	100	80	75
Accordance %	100	89,5	87,20	100	100	72,6	72
Concordance %	100	84,3	81,30	100	100	67,2	59,3

For Assay 1 (Direct isolation) results from the ILT show that mKB raised more problems during the isolation of *A. citrulli* from plant tissue: this might be due to laboratory experience, where the analyst is not sufficiently trained to recognize and select putative *A. citrulli* colonies for identification. Results of Assay 2 reflected, in part, the problem to correctly identify *A. citrulli* into a specific Group: this point might not be so important in phytosanitary analyses, since both groups are regulated in the same way. Assay 4 results showed that the use of IS1002 primer set may greatly improve specificity and accuracy of the PCR test. Although PIC, PAC and NAC were always correctly identified by all partners, problems raised in partner A6 laboratory using primer set IS1002 and in partner G4 laboratory using the primer set Contig 22. Additionally, in partner A1 laboratory the two different DNA extraction procedures produced different sensitivity. Therefore, it appears that the problems related to the performance of the real-time PCR test is more connected to the different laboratory experience, than to the test itself.

2.5. Conclusion and recommendations to policy makers

The Euphresco project DIP-ACIT was started to address a number of questions related to the EPPO standard PM7/127(1). All WPs provided results for the implementation of such standard. Two validated agar media are now available for the isolation of *A. citrulli* from symptomatic tissues: this appears particularly important, since *A. citrulli* is growing slower than other saprophytic bacteria possibly present in cucurbits tissues. The medium mKB performed somewhat better than NSA250 and should be preferentially used by laboratories with limited experience in analyzing plant material for the detection of *A. citrulli*. In experienced laboratories, both mKB and NSA250 (both validated) might be used according to the laboratory's preference. These recommendations will be considered in the next revision of the EPPO Diagnostic Protocol. The seedling grow-out test may give problems if seedlings are grown for more than 2 weeks: therefore, the laboratories performing detection and identification of *A. citrulli* should be aware of that. The inter-laboratory comparison indicated the robustness of direct isolation from plant material (not seed) and the good performance of the pathogenicity test.

2.6. Benefits to trans-national cooperation

Transnational cooperation will surely benefit from the Euphresco DIP-ACIT project: indeed, *A. citrulli* is a seed-borne and seed-transmitted pathogen. Cucurbit seed is an important commodity on the international trade. The availability of validated media, procedure, tests, and protocols will avoid possible controversies among trade partners on the phytosanitary quality of cucurbit seeds.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

- A success story was published on the use of MALDI-TOF in the identification of *A. citrulli*: Bergsma-Vlami M. (2018) MALDI-TOF mass spectrometry for plant pest diagnostics: the case of the accurate identification of Acidovorax citrulli isolates, including their grouping.
- A scientific note describing the results of the ring test will be proposed for the EPPO Bulletin: the short not is currently in preparation.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

None.

4. Open Euphresco data

None.