Draft

Pursuant to Article 12 paragraph 5 of the Law on Plant Health Protection ("Official Gazette of the Republic of Montenegro" 28/06), the Ministry of Agriculture, Forestry and Water Management adopted

RULEBOOK
ON PHYTOSANITARY MEASURES FOR DETECTION, PREVENTION OF SPREADING AND CONTROL OF POTATO RING ROT CAUSED BY BACTERIUM *Clavibacter michiganensis* (Smith) Davis et al. ssp. ***sepedonicus*** (Spieckermann et Kotthoff) Davis et al.*

(„Official Gazette of Montenegro" 66/10 from 19 Nov 2010)

I. BASIC PROVISIONS

Subject
Article 1

This rulebook prescribes phytosanitary measures for carrying out of systematic research, establishment of presence, dispersion, prevention of spreading, control and eradication of potato ring rot, caused by bacterium ***Clavibacter michiganensis*** (Smith) Davis et al. **ssp. *sepedonicus*** (Spieckermann et Kotthoff) Davis et al. (hereinafter: harmful organism) as well as procedures of laboratory testing for diagnosis, detection and identification of the harmful organism.

Meaning of terms
Article 2

Terms used in this rulebook shall have the following meanings:

- **probable presence or probable contamination** means established presence of harmful organism confirmed by primary testing, and final results are negative, in which case it is considered that the organism is still present or that contamination is probable due to biological i.e. clone relatedness or in case when mechanical means used on the plot can come in touch with host plants or water, because of the possibility of contamination;
- **contaminated plot** is the plot where presence of bacterium ***Clavibacter michiganensis*** (Smith) Davis et al. **ssp. *sepedonicus*** (Spieckermann et Kotthoff) Davis et al. is established by laboratory analysis of soil or plants*;*
- **safety zone** is an are which surrounds the contaminated plot, and it is established in accordance with standards on phytosanitary procedures of the European and Mediterranean Plant Protection Organization (EPPO) depending on assessment of risk from spreading of the harmful organism;
- **lot** is the quantity of plants which can be identified with regard to homogeneity of composition and source;
- **estate** means soil, facilities and means of transportation of the plant producer.
- .

II. SYSTEMATIC RESEARCH

Procedure of carrying out of the systematic research
Article 3

For the purpose of establishment of presence of potato ring rot, Phytosanitary Administration adopts an operational programme of special supervision through annual systematic research in accordance with annual programme.

Programme referred to in paragraph 1 of this Article establishes the number, origin, dynamics and time of samples taken, on the basis of clear scientific, statistical principles and harmful organism biology.

Within systematic research, health check of tubers is performed, and if necessary check of potato plants (*Solanum tuberosum* L.).

On the occasion of health check referred to in paragraph 3 of this Article, samples of seed potato and potato aimed at planting (hereinafter: mercantile potato) are taken form lots in storages and delivered to authorized laboratory for testing purposes.

In addition to sampling referred to in paragraph 4 of this article, in case of need, additional samples of tubers can be taken for visual check of cut tubers.

On the occasion, health check of potato plants, samples of plants are taken and delivered to the laboratory for testing purposes.

Laboratory testing referred to in paragraphs 4 and 6 of this Article is carried out in accordance with standard procedure for diagnosis of bacterium *Clavibacter michiganensis* (Smith) Davis et al. ssp. *sepedonicus* (Spieckermann et Kotthoff) Davis et al., of the European and Mediterranean Plant Protection Organization (EPPO) and the European Union (hereinafter: prescribed procedure), given in Annex 1 which is printed to this rulebook and makes its integral part.

European Union member states and European Commission are informed on results of systematic research referred to in paragraph 1 of this Article, at least once per year.

III. SUSPICION OF CONTAMINATION

Establishment of suspicion of contamination
Article 4

Suspicion of contamination with a harmful organism exists if:

- symptoms of disease have been perceived or
- result of immunofluorescence or other quick check test is positive.

In order to confirm or remove suspicion of contamination with harmful organism, further laboratory testing is carried out in accordance with the procedure prescribed.

Until obtaining of final results of laboratory testing referred to in paragraph 2 of this Article, the phytosanitary inspector shall:

1) prohibit transfer of all potato lots or consignments from which samples have been taken, except if that transfer is carried out under his supervision, on condition that there is no risk from spreading of harmful organism;
2) take all necessary measures for the purpose of detection of source of contamination with a harmful organism

3) Order corresponding additional measures for the purpose of prevention of spreading of harmful organism depending of assessed level of risk (official supervision over transfer of all tubers or plants inside and outside the estate, in case of existence of suspicion of contamination with harmful organism).

Keeping of proof material
Article 5

In case of suspicion of contamination with a harmful organism, until obtaining of the final laboratory testing result, the laboratory is obliged to keep and duly storage:

- all sampled tubers and, if possible, all sampled plants;
- entire remaining extract and additionally prepared material for quick check tests (e.g. glasses for immunofluorescence etc.);
- corresponding documentation.

On the basis of samples referred to in paragraph 1 indent 1 of this Article, in case of need, testing of varietal composition of potato tubers is carried out.

In case when contamination with harmful organism is confirmed by laboratory testing, laboratory is obliged, at least a month after informing the European Union members and European Commission on confirmed contamination with harmful organism, to keep and properly store:

- material referred to in paragraph 1 of this Article;
- a sample of artificially contaminated test aubergine plants and
- isolated culture of harmful organism.

IV. CONFIRMED CONTAMINATION

Procedure in case of confirmed contamination
Article 6

If contamination with harmful organism is confirmed by laboratory testing of samples of potatoes , plants or parts of potato plants, the phytosanitary inspector shall:

1) label as contaminated tubers and plants, plants, consignment or lot, means of transport, warehouses and their parts, all other facilities and objects, including packaging material from which sample was taken, and if needed, places of production and plots from which potato tubers and plants have been extracted;
2) establish the scope of probable contamination;
3) establish measures for safety zone on the basis of scope of confirmed contamination, scope of probable contamination and possible spreading of harmful organisms given in Annex 2 which is printed to this rulebook and makes it integral part.

Possible spreading of harmful organism referred to in paragraph 1 item 3 of this Article shall be established on the basis of::

- vicinity of other places of production of host plants;
- joint production and joint use of stocks of seed potato.

Phytosanitary inspector shall inform the Phytosanitary Administration on measures taken referred to in paragraph 1 of this Article, for the purpose of delimitation of area, i.e. declaration of the contaminated lot and establishment of limits of contaminated plot and safety zone..

Safety zone must be big enough to ensure protection of nearby areas.

In case of contamination of several lots, the source of primary contamination is determined, and the scope of probable contamination, in accordance with results of systematic research and risk assessment for spreading of harmful organism.

Notification on occurrence of bacterium *Clavibacter michiganensis* (Smith) Davis et al. ssp. *sepedonicus* (Spieckermann et Kotthoff) Davis et al., is published by the Phytosanitary Administration on its web page and in at least one printed media, which is distributed in the territory of Montenegro.

Scope of probable contamination
Article 7

Phytosanitary inspector shall establish the scope of probable contamination referred to in Article 6 paragraph 1 item 2 of this rulebook, on the basis of information and/or knowledge of possible contacts with established contamination before and after extraction of potato or contacts during production, taking into account:

1) tubers or plants grown in the place of production marked as contaminated place;
2) place or places of production which are anyhow connected to the production of tubers or plants which are marked as contaminated, including these places where same equipment and facilities are used for production;
3) tubers or plants are produced at places of production referred to in item 2 of this paragraph or which were located at these places of production in time while tubers and plants which were marked as contaminated were present in places of production marked as contaminated;
4) estates where potato originating form the place of production referred to in items 1-3 of this Article is located or treated;
5) all devices, vehicles, warehouses and their parts, as well as other facilities or objects, including packaging material, which could have come in touch with tubers or plants which are marked as contaminated;
6) all tubers and plants which were storaged or in touch with some of facilities or objects referred to in item 5 of this paragraph prior to their cleaning and disinfection;
7) tubers or plants which are considered probably contaminated due to clone relation with tubers and plants marked as contaminated, although results of testing carried out in accordance with prescribe procedures negative, and when necessary, test of varietal composition can be carried out in order to check identity of contaminated and clone-related potato tubers and plants and
8) places of production of potato tubers or plants referred to in item 7 of this paragraph.

Possible spreading of harmful organism shall be established on the basis of:

- vicinity of other places of production of potato and other host plants;
- joint production and joint use of stocks of seed potato.

Notification in case of confirmed contamination
Article 8

If contamination with harmful organism is confirmed by laboratory testing carried out in accordance with prescribed procedure, the European Union member states and the European Commission shall be informed contamination immediately after the procedure has been carried out.

Notification referred to in paragraph 1 of this Article must contain at least the following data on:

- name of variety and lot of potato;
- purpose of potato (mercantile or seed), namely the category of seed potato.

Phytosanitary Administration shall inform the body in charge of plant health protection of the other state which is threatened by the risk from spreading of contamination to potato, on containment with harmful organism, with the following data:

- name of potato or tomato variety and lot;

- name and address of sender and recipient;
- due date of potato lot;
- size of delivered potato lot;
- data from the plant passport, i.e. dispatch note (copy of plant passport, or copy of dispatch note), or the number of passport, register number of importer, producer, processor, distributor and warehouse keeper.

European Commission shall be reported on delivery of the notification referred to in paragraph 3 of this Article.

Along with notification referred to in paragraph 4 of this Article, after completion of all researches on establishment of contamination with harmful organism, the following data shall be submitted to the European Commission:

- date when contamination was confirmed;
- short description of research carried out for the purpose of identification of source and possible spreading of contamination, including data on sampling;
- on identified or possible sources of contamination;
- on scope of marked contamination, including the number of places of production, the number of lots with name of variety, and as regards seed potato, the category;
- on safety zone, including the number of places of production which are not marked as contaminated, but which are included in safety zone and
- and other data on confirmed sudden occurrence of harmful organism which may be requested by the European Commission.

In case of once confirmed contamination with harmful organism, Phytosanitary Administration prepares a report once a year, which is published on its web-site.

The report referred to in paragraph 6 of this Article shall contain the following data on:

- contamination and delimitation of contaminated lot and safety zone;
- taken measures;
- name of the potato variety and lot;
- purpose of potato (mercantile or seed), namely the category of seed potato;
- date when contamination was confirmed;
- research procedure (short description), carried out for the purpose of identification of source and possible spreading of contamination, including data on sampling;
- identified or possible sources of infection;
- on scope of marked contamination, including the number of places of production, the number of lots with name of variety, and as regards seed potato, the category;
- on safety zone, including the number of places of production which are not marked as contaminated, but which are included in safety zone and
- other data on harmful organism of significance for special supervision and planning thereof.

Phytosanitary Administration shall keep records on data referred to in paragraphs and 3.

Testing of clone-related potato
Article 9

Potato lots which are not clone-related with potato tubers or plants which are marked as contaminated shall be subject to obligatory laboratory testing in accordance with prescribed procedure.

Phytosanitary inspector shall, on the basis of Phytosanitary Administration data, determine testing of the number of samples of tubers or plants which is needed to establish probable primary source of contamination and scope of probable contamination, while sequence of testing depends on risk level.

After obtaining of laboratory testing results, referred to in paragraph 1 of this Article, phytosanitary inspector shall perform further establishment of containment, establish the scope of probable contamination and safety zone.

V. MEASURES

Measures and procedures with contaminated plants
Article 10

Tubers or plants marked as contaminated must not be planted, they must be subject, under supervision of the phytosanitary inspector, to one of the following measures and procedures, on condition there is no risk from spreading of harmful organism:

- destruction or
- use as animal feed after appropriate thermal processing, which leaves no possibility of survival of the harmful organism or
- disposal at a place approved for waste disposal, established not to have risk form uncontrolled spreading of harmful organism into surrounding (by filtering through soil pores to agricultural soil) or
- burning or
- industrial processing on condition that contaminated potato tubers or plants are dispatched immediately after establishment of contamination to the place of processing where there must be equipment for waste disposal, by use of which risk from spreading of harmful organism is eliminated and which contains the cleaning and disinfection system for vehicles which leave the place of processing or
- and other measures, for the purpose of control of spreading of harmful organism.

European Commission and European Union member states shall be informed on measures referred to in paragraph 1 of this Article and their justifiability.

Remaining waste occurring as a result of taken measures referred to in paragraph 1 of this Article shall be disposed in accordance with the procedure on waste disposal referred to in Article 13 of this rulebook.

Measures and procedures with probably contaminated potato
Article 11

Potato tubers or plants which are considered to be probably contaminated by the harmful organism in accordance with Article 7 of this rulebook, must not be planted; under supervision of the phytosanitary inspector they may be used:

- used as mercantile potato aimed at food, and it must be packed at places disposing with appropriate equipment for waste disposal and which is prepared for immediate delivery and use without subsequent re-packaging and it is allowed to handle the seed potato in the same places only if it is handled separately or after cleaning and disinfection or
- used as mercantile potato aimed at industrial processing, with direct and rapid delivery to the processing plant, which must dispose with appropriate equipment for waste disposal and system for cleaning and disinfection of vehicles leaving the place of processing or
- dispose in prescribed manner, on condition there is no risk from spreading of harmful organism.

Measures referred to in paragraph 1 of this Article shall be also applied to potato tubers or plants marked as contaminated, regardless of testing results referred to in Article 9 of this rulebook.

Cleaning and disinfection
Article 12

Devices, vehicles, storages and their parts or other facilities and objects, including packaging material, which are marked as contaminated or are considered to be probably contaminated, must be destroyed or cleaned or appropriately disinfected for the purpose of elimination of risk from spreading of harmful organism.

Facilities and objects, after performed disinfection referred to in paragraph 1 of this Article shall not be considered contaminated.

Waste disposal procedures
Article 13

Disposal of waste arising in the process of industrial processing referred to in Article 11 of this rulebook shall be carried out so as to avoid any risk from spreading of harmful organism, as follows:

1) potato waste (including discarded potato and peel) and other solid potato-related waste (including soil, rocks and other remains):
   - shall be disposed in a place designated for waste disposal, where there is no risk from uncontrolled spreading of harmful organism into surrounding (by filtering through pores in the ground to the agricultural soil), so that the waste is transported directly, in a closed vehicle or
   - it shall be burnt or
   - removed by application of other measures which do not entail risk from spreading of harmful organism, on which records are kept for the purpose of further reporting to the European Commission and European Union member states.
2) liquid waste arising from processing which contains solid particles in dispersed condition, must be filtrated or processed by the procedure of sedimentation prior to removal for the purpose of removal of solid particles, after which particles are removed in manner referred to in item 1 of this paragraph, and liquid part of waste must be:
   - fully heated at the temperature of at least 60° C prior to elimination, for at least 30 minutes or
   - eliminated under official supervision in another prescribed manner which makes it unable for the waste to come in touch with agricultural soil, on which records are kept for the purpose of further reporting to the European Commission and European Union member states..

Procedures referred to in paragraph 1 of this Article shall be applied to waste arising from handling, removal and processing of contaminated potato tubers.

Measures in safety zone
Article 14

Measures referred to in Annex 2 of this rulebook shall be applied in the safety zone.

Procedure with seed potato
Article 15

Seed potato must originate from material obtained in accordance with prescribed control procedure for which it has been established by performed laboratory testing that it is not contaminated by a harmful organism.

Testing of seed potato referred to in paragraph 1 of this Article shall be carried out:

1) in cases when contamination endangers seed potato production, on plants from initial phase of clone selection ;
2) in other cases:
   - or on any plant which is in the initial phase of clone selection;
   - on previous generations in vegetative reproduction chain.

Possession and use of harmful organism
Article 16

Harmful organism must not be possessed nor used in any manner.

Exceptions
Article 17

Notwithstanding Article 16 of this rulebook, harmful organism may be possessed or used solely for experimental, scientific and selection purposes, on condition that supervision over harmful organism is not thereby hampered and that there is no risk from its spreading.

Other measures
Article 18

For the purpose of control or prevention of spreading of harmful organism, in addition to measures prescribed by this rulebook other additional measures may be applied, on which European Union member countries and European Commission shall be informed..

By measures referred to in paragraph 1 of this Article, the following can be established:

- planting of only certified seeds potato or
- that seed potato which producer produced in their lot can be used as seed, only on their lot, on condition it meets prescribed health requirements and
- other measures in accordance with the Law.

VI FINAL PROVISION
Article 19

This rulebook shall enter into force on the eighth day from the day of its publication in the “Official Gazette of Montenegro”.

No:
Podgorica, 09th November 2010

MINISTER

Milutin Simović, MA

* Rulebook is harmonized with Council Directive ***93*/85/EC** from 4th October 1993 on Control of ***Clavibacter michiganensis*** (Smith) Davis et al. **ssp. *sepedonicus*** (Spieckermann et Kotthoff) Davis et al., cause of potato ring rot (*Council Directive 93/85EC of 4 October 1993 on control of Potato Ring Rot)*

ANNEX 1

TESTING SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION OF CAUSANT OF POTATO RING ROT, BACTERIUM *Clavibacter michiganensis* (Smith) Davis *et al*. ssp. *sepedonicus* (Spieckermann et Kotthoff) Davis et al.

Given testing scheme describes various procedures for:

- diagnosis of potato ring rot on potato tubers and plants;
- detection of bacterium *Clavibacter michiganensis ssp. sepedonicus* in samples of potato tubers and plants;
- identification of bacterium *Clavibacter michiganensis ssp. sepedonicus* (*C. m. subsp. sepedonicus*).

This annex contains optimal protocols for certain methods, valid (confirmed and approved) reagents and details regarding preparation of material for testing and control (reference) material. List of laboratories which participated in optimization and validation of protocols is given in Annex 1.

Since protocols include detection of quarantine organism and use of live cultures of C. m. subsp. sepedonicus the procedure must be carried out in prescribed quarantine conditions with corresponding facilities for waste disposal, storage and destruction in accordance with this rulebook.

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Testing parameters must ensure equalized and repeatable levels of detection of C. m. subsp. *sepedonicus* according to prescribed sensitivity thresholds for certain methods.

Precise preparation of positive controls is obligatory.

Testing in accordance with the required sensitivity threshold entails correct positioning, maintenance and calibration of equipment, careful storage and handling of reagents as well as undertaking of measures for prevention of contamination among samples, e.g. separation of positive controls from samples for testing. Standards of quality control must be applied in order to avoid administrative and other errors, especially on the occasion of marking samples and keeping of documentation.

Suspicion of presence of pathogens in a sample means positive result of the test of sample check, as demonstrated in chart-flow diagrams.

If result of the first check test (IF or PCR/FISH,) positive, is positive, then presence of bacterium C. m. subsp. *sepedonicus* is suspected and the other check test must be carried out. If result of the second check test is positive, suspicion is corroborated and testing must be continued according to the testing scheme. If result of the second check test is negative, then it is considered that sample is not contaminated with bacterium C. m. subsp. *sepedonicus*.

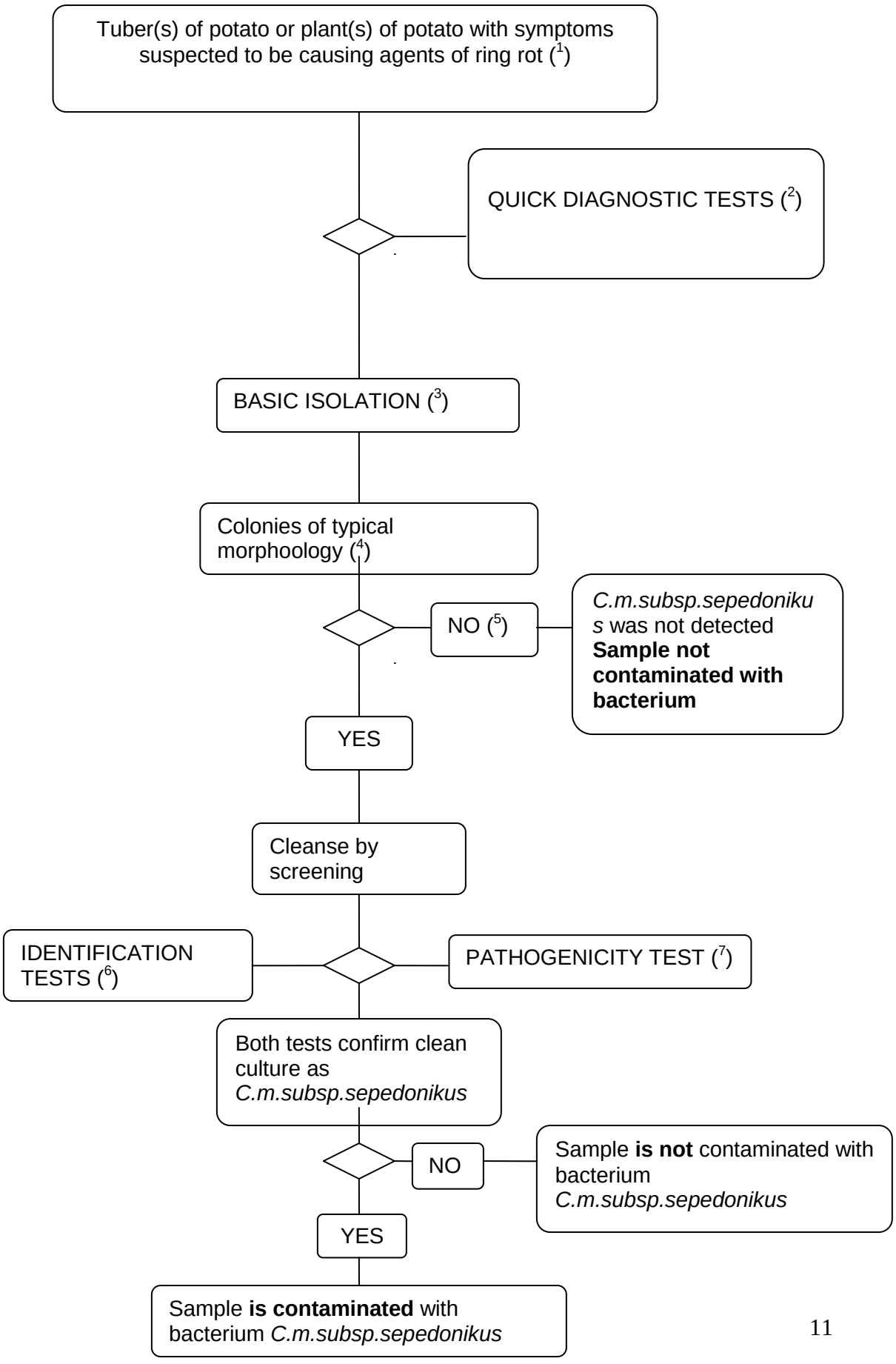
Positive result of the IF test is defined as positive reading of IF test corroborated by the second check test (PCR/FISH).

Confirmed presence of pathogen entails isolation and identification of clean culture of C. m. subsp. *sepedonicus* as well as confirmation of pathogenicity.

PART 1. CHART FLOW DIAGRAM

1.1. Scheme of detection of presence of causing agent of potato ring rot in potato tubers and plants with typical symptoms of ring rot

Testing procedure is aimed at tubers and plants of potato with symptoms typical for or indicating ring rot. Procedure included the quick check test, isolation of pathogen from contaminated conducting tissue on diagnostic nutritive medium and in case of a positive result, identification of clear culture of bacterium C. m. subsp. *sepedonicus*.


Tuber(s) of potato or plant(s) of potato with symptoms suspected to be causing agents of ring rot (1)
QUICK DIAGNOSTIC TESTS (2)
BASIC ISOLATION (3)
Colonies of typical morphoology (4)
NO (5)
C.m.subsp.sepedonikus was not detected Sample not contaminated with bacterium
YES
Cleanse by screening
IDENTIFICATION TESTS (6)
PATHOGENICITY TEST (7)
Both tests confirm clean culture as C.m.subsp.sepedonikus
NO
Sample is not contaminated with bacterium C.m.subsp.sepedonikus
YES
Sample is contaminated with bacterium C.m.subsp.sepedonikus

([1]) Description of symptoms is given in Section 2.

([2]) Corresponding tests are:
– IF test (Part 4);
– PCR test (Part 6);
– FISH test (Part 5).

([3]) Although isolation of pathogen from plant material with typical symptoms by method of dilution and screening on nutritive medium is simple, growing can be unsuccessful from sample in advanced stadium of infection. Saprophytic bacteria growing on diseased tissue can overgrow or inhibit pathogen on nutritive medium. Therefore, it is recommended to use non-selective and selective nutritive medium, MTNA best (Section 8) or biotest (Section 7).

([4]) Typical colony morphology is given in Part 8.

([5]) If isolation result is negative, but symptoms of disease are typical, then it is necessary to repeat the isolation procedure.

([6]) Reliable identification of clean cultures of C. m. subsp. *sepedonicus* is achieved by carrying out of tests described in Part 9.

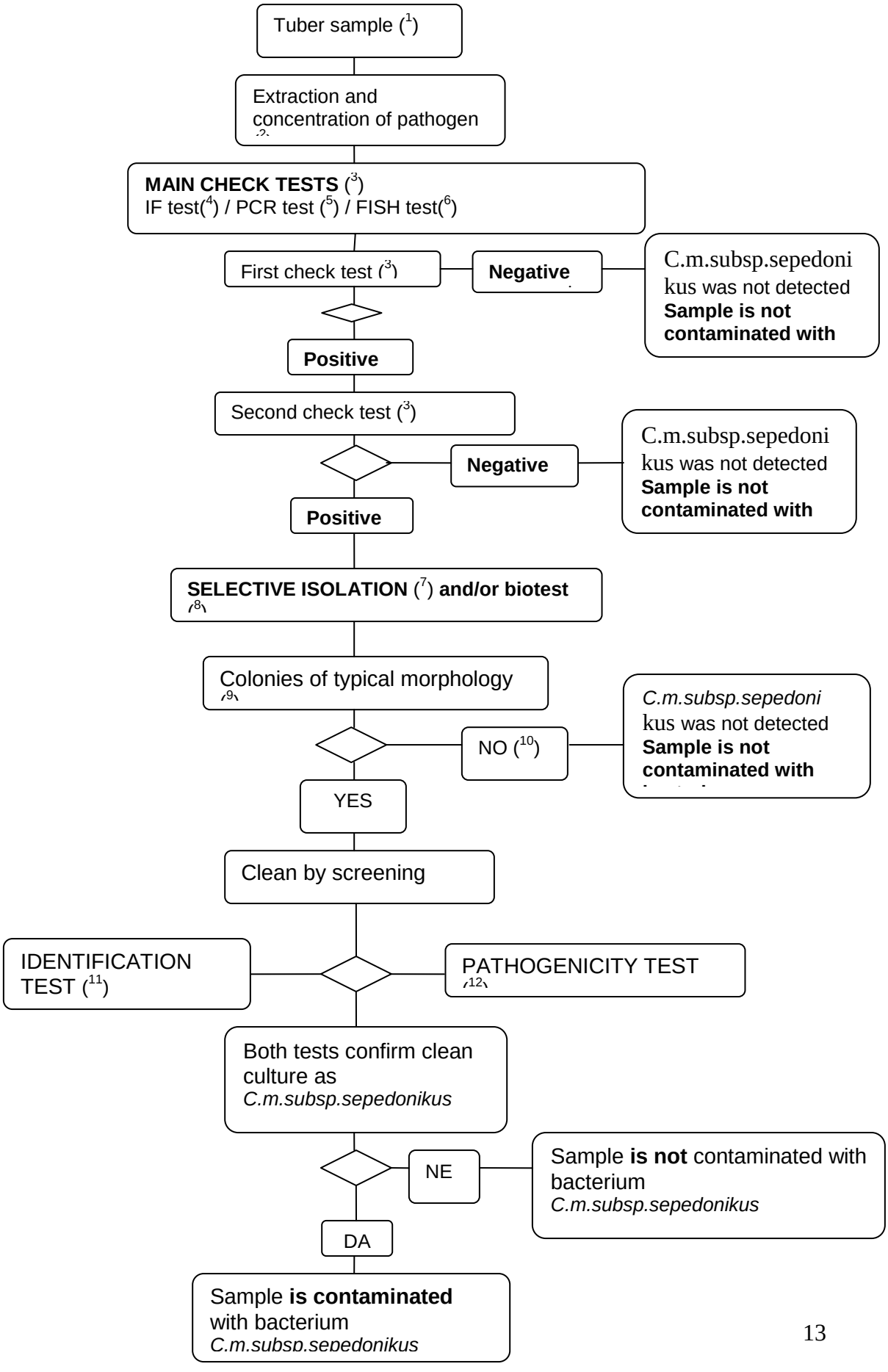
([7]) Pathogencity test is described in Part 10.

1.2. Scheme of detection and identification of bacterium *Clavibacter michiganensis* ssp.*sepedonicus* in samples of potato tubers without visible symptoms

Testing procedure aimed at detection of hidden contamination in potato tubers. Positive result of at least two check tests, which are based on various biological principles must be supplemented by isolation of pathogen after which, in case of isolation of typical colonies, ensues confirmation of clean culture of C. m. subsp. *sepedonicus*.

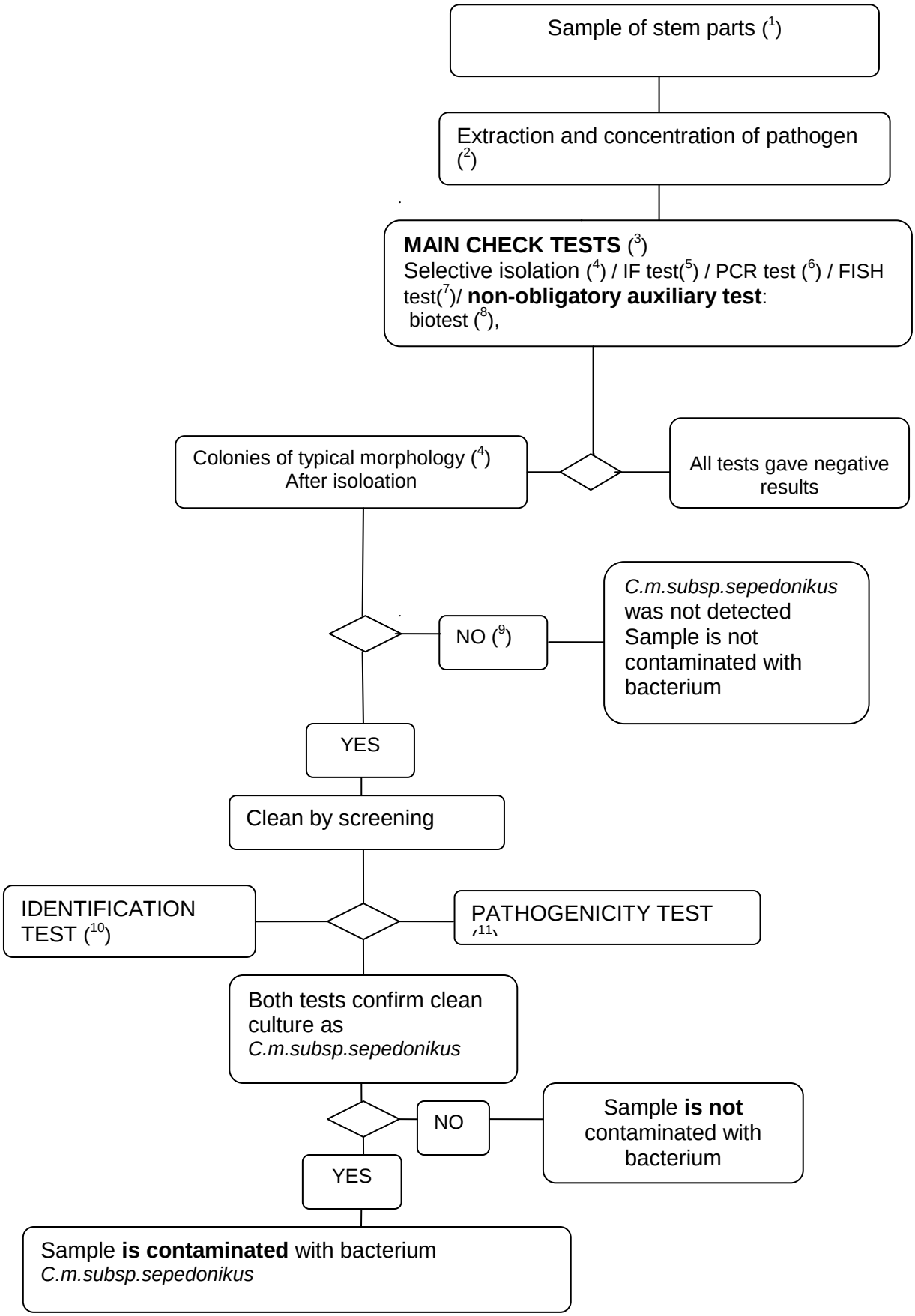
Positive result gained on the basis of only one of check tests is not enough to consider the sample contaminated.

Check tests and isolation must enable level of detection sensitivity from 10^3 to 10^4 cells/ml of resuspended sediment included as a positive control in every series of tests.


Tuber sample (1)
Extraction and concentration of pathogen (2)
MAIN CHECK TESTS (3)
IF test(4) / PCR test (5) / FISH test(6)
First check test (3)
Negative
C.m.subsp.sepedonikus was not detected Sample is not contaminated with
Positive
Second check test (3)
Negative
C.m.subsp.sepedonikus was not detected Sample is not contaminated with
Positive
SELECTIVE ISOLATION (7) and/or biotest (8)
Colonies of typical morphology (9)
NO (10)
C.m.subsp.sepedonikus was not detected Sample is not contaminated with
YES
Clean by screening
IDENTIFICATION TEST (11)
PATHOGENICITY TEST (12)
Both tests confirm clean culture as C.m.subsp.sepedonikus
NE
Sample is not contaminated with bacterium C.m.subsp.sepedonikus
DA
Sample is contaminated with bacterium C.m.subsp.sepedonikus

([1]) Standard size of a sample is 200 tubers, although the procedure can be carried out on smaller samples, if there are no 200 tubers available.
([2]) Methods of extraction and concentration of pathogens are described in Part 3.1.
([3]) If results of at least two tests, based on different biological principles are positive, it is necessary to perform isolation and confirmation of pathogen presence. To conduct at least one check test. When result of that test is negative, it is considered that such sample is negative. In case the result of the test is positive, it is necessary to carry out another or several check tests, based on various biological principles, in order to confirm the positive result. If results of other tests are negative, it is considered that the sample is negative. Further tests are not necessary.
([4]) Immunofluorescence test (IF).
Use polyclonal antibodies for IF check, additional monoclonal antibodies enable bigger specificity (Part 4).
([5]) PCR test.
Use confirmed and approved reagents and protocols for PCR (Part 6).
([6]) FISH test.
Use confirmed reagents and protocols (Part 5).
([7]) Selective isolation.
Use of MTNA or NCP-88 nutritive mediums and 1/100 solution of resuspended sediment is in many cases the corresponding method for direct isolation of C. m. subsp. *sepedonicus*. Typical colonies can be obtained 3 to 10 days after screening on nutritive medium. Pathogen can then be treated and identified. In order to fully use the test possibilities, it is necessary to carefully extract and prepare tuber sprouts cones of tubers in order to avoid saprophytic bacteria from potato tubers, which are competition, on the nutritive medium, to bacterium C. m. subsp. *sepedonicus* and can overgrow it. If pathogen can not be isolated on nutritive medium, it is necessary to repeat the isolation procedure by use of plants from biological test (Part 8).
([8]) Biotest is used for isolation of bacterium C. m. subsp. *sepedonicus* from extract of artificially inoculated aubergine (Solanum melongena). Test requires optimal conditions of incubation, as mentioned in this method. Bacteria which inhibit bacterium C. m. subsp. *sepedonicus* on MTNA or NCP-88 nutritive mediums will probably not pose an obstacle in this test (Part 7).
([9]) Typical morphology of a colony is described in Part 8.
([10]) Growing of bacterial culture or biotest can be unsuccessful because of competition or inhibition by saprophytic bacteria. If check tests provide clearly positive results, and isolation results are negative, repeat the isolation tests form the same resuspended sediment or repeat taking of conductive tissue in place of stolon connecting in a tuber i.e. from tuber eyes, from the same sample, and if necessary, test additional samples.
([11]) Reliable identification of clean cultures of C. m. subsp. *sepedonicus* is achieved by carrying out of tests described in Part 9.
([12]) Pathogenicity test is described in Part 10.

1.3. Scheme for detection and identification of bacterium *Clavibacter michiganensis* ssp. *sepedonicus* in samples of potato plants without symptoms


Sample of stem parts (1)
Extraction and concentration of pathogen (2)
MAIN CHECK TESTS (3)
Selective isolation (4) / IF test(5) / PCR test (6) / FISH test(7)/ non-obligatory auxiliary test:
biotest (8),
Colonies of typical morphology (4) After isoloation
All tests gave negative results
NO (9)
C.m.subsp.sepedonikus was not detected Sample is not contaminated with bacterium
YES
Clean by screening
IDENTIFICATION TEST (10)
PATHOGENICITY TEST (11)
Both tests confirm clean culture as C.m.subsp.sepedonikus
NO
Sample is not contaminated with bacterium
YES
Sample is contaminated with bacterium C.m.subsp.sepedonikus

([1]) Recommended sample sizes are given in Part 3.2.
([2]) Methods of extraction and concentration of pathogens are described in Part 3.2.
([3]) If results of at least two tests which are based on different biological principles are positive, it is necessary to carry out isolation and confirmation of pathogen presence. Conduct at least one check test. When result of that test is negative, it is considered that the sample is negative. In case the result of that test is positive, it is necessary to conduct another or several check tests, which are based on various biological principles, in order to confirm the positive result. If results of other tests are negative, it is considered that the sample is negative. Further tests are not necessary.
([4]) Selective isolation and typical colony morphology are described in Part 8.
([5]) IF test is described in Part 4.
([6]) PCR tests are described in Part 6.
([7]) FISH test is described in Part 5.
([8]) Biotest is described in Part 7.
([9]) Growing of a bacterial culture or biotest can be unsuccessful due to competition or inhibition by saprophytic bacteria. If check tests provide positive results, and isolation results are negative, repeat the isolation tests from the same resuspended sediment or repeat taking of conductive tissue in place of stolone connecting with tubers and if necessary, test additional samples.
([10]) Reliable identification of clean cultures of C. m. subsp. *sepedonicus* is achieved by carrying out of tests described in Part 9.
([11]) Pathogenicity test is described in Part 10.

PART 2. VISUAL CHECK FOR DETECITON OF RING ROT SYMPTOMS

2.1. Potato plants

In European climate conditions, symptoms are rarely found in the field, and often in the end of the season. In addition, symptoms are often covered or can be substituted with other diseases, ageing or mechanical impairments. Therefore, we can easily omit the symptoms on the occasion of field check. Symptoms of wilt are very different from those at brown rot; wilt is usually slow and limited to leaf edges, in the beginning. Young infected leaves often continue to grow, a lot less in infected parts, so leaves are of unusual shape. Due to blockage of conductive tissue, plants usually have chlorite yellow-orange parts between leave vessels . Infected leaves, even stems can decay in time. Often, leaves and tubers are small. Occasionally, plants are underdeveloped.

2.2. Potato tubers

Earliest symptoms are light glassiness or transparency of tissue without softening around conductive system, especially near sprout tuber part. Conductive ring on sprout part of tuber can be a little darker than usual. First easily perceived symptom is yellowish colour of conductive ring, and when tuber is lightly squeezed; small quantities of cheesy consistency come out from conductive vessels, containing millions of bacteria. Conductive tissue can turn brown and symptoms at tubers in this stadium are similar to brown rot symptoms caused by Ralstonia solanacearum. In the beginning, symptoms can be limited to one part of the conductive ring, not necessarily on sprout part of tuber and can be gradually extended to entire ring. As the infection advances, decay of conductive tissue occurs; outer and inner lining can be separated. In advanced stadiums of infection, cracks appear on surface of tubers, which are often reddish-brown on edges. Recently, several cases have been recorded in Europe in which centre of tuber was rotting at the same time as conductive ring which lead to secondary attack of pathogens and creation of internal holes and necroses. Secondary fungal or bacterial attack can cover symptoms and it can be hard, even impossible to distinguish advanced symptoms of ring rot from other ring rots. Occurrence of atypical symptoms of ring rot is possible.

PART 3. PREPARATION OF A SAMPLE

3.1. Potato tuber

Note:

- standard sample size is 200 tubers per test. More active sampling requires more tests on samples of this size. A larger number of tubers in a sample leads to inhibition or makes interpretation of results more difficult. However, the procedure can be applied to samples with less than 200 tubers, when less tubers are available.
- Validation of all pathogen detection methods, which are described in text below is based on testing of samples of 200 tubers.
- potato extract which is described in the text below can be used for establishment of presence of causant of brown potato rot, bacterium Ralstonia solanacearum.

Procedure before preparation of the sample:
Wash tubers. Use adequate disinfectants (while using PCR test, use compounds of chlorine for the purpose of removal of possibly present pathogen DNA) and detergents between every sample. Dry tubers in air. Washing procedures is especially useful (but not obligatory) for samples with too much soil, during carrying out of PCR test or direct isolation procedure.

3.1.1. With a clean and disinfected scalpel or knife for vegetables, remove crust from sprout part of tubers, so that conductive tissue is visible. Carefully cut a small cones part of conductive tissue on tuber sprouts part (hereinafter: cone) taking the less possible of surrounding, non-conducting tissue.

Note:

Separate all tubers with possible ring rot symptoms and test them separately.
If, on the occasion of removal of cones with potential symptoms of ring rot are perceived, then tubers should be visually checked. Every cut tuber with suspicious symptoms should be kept at least two days in the room temperature and keep in quarantine conditions (at 4 to 10 °C) until all tests are completed. All tubers (including these with suspicious symptoms) are storaged in accordance with Article 5 of this rulebook.

3.1.2. Put cone in a receptacle for single use which can be closed and/or hermetically closed (if receptacles have already been used, they must be thoroughly cleaned and disinfected with chlorine compound). They are preferably immediately processed. If that is not possible, keep them in receptacle without addition of buffer, 72 hours in a refrigerator or 24 hours in room temperature, at most. Drying and suberization of cone, as well as growth of saprophytes in the course of storage can hamper establishment of presence of bacteria causing agent of ring rot.

3.1.3. Process cones with one of the following procedures:

a) cover cones with sufficient quantity (around 40 ml) of extraction buffer (Annex 3) to cover cones i and ultracentrifuge (50-100 turns/min) four hours at the temperature below 24 °C or 16 to 24 hours with cooling;
or
b) homogenize cones with enough (around 40 ml) of extraction buffer (Annex 4), whether in a mixer (e.g. Waring or Ultra Thurax) or crunching in a well closed bag for maceration for single use (e.g.

bags Stomacher or Bioreba made of hard polythene, 150 mm x 250 mm, sterilized by radiation) using a rubber hammer or corresponding maceration apparatus (cruncher) (e.g. Homex).

Note:

If samples are homogenized in a blender, there is great danger from their cross-contamination. Take precaution measures in order to prevent appearance of aerosol or spilling in the extraction procedure. For every sample, use fresh sterilized knives and blender receptacles. If PCR test is conducted test procedure, prevent transfer of DNA to containers or apparatuses for maceration (cruncher). For PCR test, maceration in single-use bags is recommended and use of single-use tubes.

3.1.4. Decantate supernatant. If it is too opaque, clear it with slow centrifuging (at most 180 g 10 minutes in temperature from 4 to 10 °C) or by vacuum filtration (40 to 100 µm) and additionally wash filter with the extraction buffer (around 10 ml).

3.1.5. Concentrate bacterial fraction by centrifuging on 7000 g 15 minutes (or 10000 g 10 minutes) in temperature from 4 to 10 °C and pour supernatant taking care not to mix the sediment.

3.1.6. Resuspend sediment in 1.5 ml buffer for sediment (Annex 3). Use 500 µl for testing to C. m. subsp. *sepedonicus*, 500 µl for Ralstonia solanacearum and 500 µl as reference material. for keeping. Into last, reference part of 500 µl added sterile glycerol by final concentration from 10 to 25 % (v/v), mix and store at the temperature from –16 do –24 °C (days) or at –68 do –86 °C (months). Parts separated for establishment of presence of bacteria, keep at the temperature from 4 to 10 °C. Multiple freezing and de-freezing is not recommended.

If transportation of extract is necessary, ensure delivery in a transferable refrigerator in the course from 24 to 48 hours.

1.1.6

3.1.7. All positive controls and samples of C. m. subsp. *sepedonicus* must be separately prepared and processed in order to avoid contamination. That applies to immunofluorescence glasses and all other tests.

3.2. Potato plants

Note:

For detection of latent populations of bacterium *C.m.subsp.* testing of collective samples is recommended. Procedure can be suitably applied to collective samples from at most 200 parts of stems. (taking samples for carrying out of supervision must be based on a statistically representative sample of plant population which is being examined).

3.2.1. With a clear disinfected knife cut a part of 1 cm in size from lower part of every stem, just above the ground surface.

Shortly disinfect parts of stems with 70 % ethanol and immediately dry with absorbing paper. Put parts of stems in a closed sterile receptacle.

3.2.2. Process parts of stems with one of the following procedures:

a) cover them with sufficient quantity (around 40 ml) of extraction buffer (Annex 3) and ultracentrifuge at 50-100 turns/min four hours at the temperature below 24 °C or 16 to 24 hours with cooling, or
b) macerate parts of the stem (crunching) in a maceration bag (e.g. Stomacher or Bioreba) with corresponding quantity of extraction buffer (Annex 4) using a rubber hammer or appropriate maceration equipment (e.g. Homex). If that is not possible, keep the parts of stem in a refrigerator for 72 hours at the longest or in the room temperature 24 hours at the longest.

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3.2.3. After 15 minutes of sedimentation, decantate supernatant.

3.2.4. Additional clarification of extract or concentration of bacterial fraction is usually not necessary, but it can be achieved by filtration and/or centrifuging as described in Part 3.1.4 to 3.1.6.

3.2.5. Divide clean or concentrated extract of sample into two equal parts. Keep one half in the temperature from 4 to 10 °C during testing, and leave the other half for case of use in additional testing: 10-25 % (v/v) of sterile glycerol is added in extract and kept at the temperature from – 16 to – 24 °C(fro weeks)or in –68 do –86 °C (for months).

PART 4. IF TEST

Principle

Use of IF test as basic check test is recommended due to its proven consistency in achievement of required sensitivity thresholds of the method.

When IF test is used as the main check test and if IF reading is positive, PCR test or FISH test are additionally used as the other check test. When the IF test is used as the second check test and IF reading is positive, it is necessary to perform further testing by flow diagram in order to complete the analysis.

Note:

Always use polyclonal antibodies when IF test is used as the main check test. In case of a positive IF reading with polyclonal antibodies, further check by use of monoclonal antiboides can enable greater specificity, but it can also reduce sensitivity of the test.

It is recommended to determine the titre for every new series of antibodies. Titre is defined as greatest dilution by which optimal reaction is achieved in testing of suspension containing 10^5 to 10^6 cells per ml of corresponding strain of C. m. subsp. *sepedonicus* with use of fluorescein isothiocyanate (FITC) conjugated antibodies according to producer's instructions. Undiluted polyclonal and monoclonal antibodies should have the titre of at least 1:2000. During testing, use working diluted antibodies, which are near or equal to titre. Use confirmed and approved antibodies.

Test should be carried out on freshly prepared sample extracts. It can, if needed, be carried out successfully on extracts which were stored at the temperature from - 68 to - 86 °C with addition of glycerol. Glycerol can be removed by addition of 1 ml pellet buffer for sediment (Annex 4),

repeated 15 minute centrifuging at 7000 g and resuspending in equal volume of buffer for sediment. That is rarely necessary, especially if samples are fixed to glasses by flame.
(2.2).

For positive control, prepare separate glasses with homologous strain or some other defence strain of bacterium C. m. subsp. *sepedonicus*, suspended in potato extract, as given in Annex 2 and, as an option, in buffer.

As a similar control on the same glass, if possible, natural infected tissue should be used (sustained by lyophilization or freezing at -16 to - 24°C).

For negative control, parts of sample extract can be used which proved negative result in previous testing.

Use microscopic glasses with several openings, if possible with 10 opening with radius of at least 6 mm.

Test the control material in the same manner as samples.

4.1 Prepare IF glasses for testing according to one of the following procedures:

i) For suspensions of sediment with relatively little of starch:

Measure standard volume into the first opening with a pipette (15 µl is enough for openings with radius of 6 mm – for bigger openings, increase volume) of dilution of 1/100 of resuspended potato sediment. Then, measure with a pipette in the same row similar non/diluted suspensions (1/1) of sediment. The other row can be used as duplicate of the same or for another sample as demonstrated in figure 1.

ii) For other sediment suspensions:

Prepare decimal dilutions (1/10, 1/100) of resuspended sediment in sediment buffer. In one line of openings, measure with a pipette the standard volume (15 µl is enough for openings with radius of 6 mm – for bigger openings, increase volume) of resuspended sediment and every dilution. The second line can be used as the duplicate of the same or for another sample, as demonstrated in figure 2.

4.2. Leave the drops to dry at room temperature or heat them to the temperature of 40 to 45 °C. Fix bacterial cells to glass by heating (15 minutes at 60 °C), by pulling through flame, 95% ethanol or by special instructions of supplier of antibodies.

Before further testing, fixed glasses may, if needed, shortly (up to three months, at the longest) be storaged frozen in a desiccator.

4.3. IF procedure:

i) In accordance with the procedure for preparation of glasses for testing, which is described under 4.1(i):

Prepare a series of double dilutions of antibodies in IF buffer. First opening must have 1/2 titre (T/2), and remainder 1/4 titre (T/4), 1/2 titre (T/2), titre (T) and double titre (2T).

ii) In accordance with the procedure for preparation of glasses for testing which is described under 4.1(ii):

Prepare working dilution of antibodies in IF buffer. Working dilution affects specificity..

Figure 1. Preparation of glasses in accordance with items 4.1.(i) and 4.3.(i)

Dilutions of resuspended sediment

	1/100	1/1	1/1	1/1	1/1	▫ dillutions of resuspended sediment
(T=titar)	T/2	T/4	T/2	T	2T	▫ double dilutions of

serums/antibodies

Sample 1	● 1	● 2	● 3	● 4	● 5
Duplicate of sample 1 or sample 2	6	7	8	9	10

● ● ● ● ●

Figure 2. Preparation of glasses in accordance with items 5.1. (i) and 5.3.(ii)

Working dilutions of serums/antibodies

1/1	1/10	1/100	empty	empty	▫ decimal dilutions of resuspended sediment

Sample1	● 1	● 2	● 3	● 4	● 5
Duplicate of sample 1 or sample 2	● 6	● 7	● 8	● 9	● 10

4.3.1. Line up glasses on moist absorbing paper. Cover every opening completely with antibodies dilution. Volume of antibodies which is put in a single opening must be equal to volume of extract put.

Follow the following procedure if there are no special instructions of antibodies supplier:

4.3.2. Incubate glasses on wet paper, covered, 30 minutes at room temperature (18 to 25 °C).

4.3.3. Shake drops off every glass and carefully wash them out with IF buffer. Submerge them for 5 minutes in IF buffer-Tween (Annex 3) and after that in IF buffer for 5 minutes. Take care that aerosol is not created and that there is no transfer of drops because that could result in cross-contamination

4.3.4. Line up glasses on wet absorbing paper. Fill openings with diluted FITC conjugate used for titre establishment. Volume of conjugate applied to openings must be equal to volume of applied antibody. Carefully dry glasses with absorbing paper.

4.3.5. Incubate glasses on wet paper, covered, 30 minutes at room temperature (18 to 25 °C).

4.3.6. Shake off the conjugate drops from glasses. Wash out and wash as previously described (4.3.3). Carefully dry glasses.

4.3.7. Apply to every opening with a pipette 5-10 µl 0.1 M glycerol with phosphate buffer (Annex 3) or means against discoloration which is available in the market and put the cover glass.

4.4. Reading of IF test:

4.4.1. Check the prepared glasses under epifluorescent microscope with corresponding filters for FITC excitation, under oil or water immersion and augmentation from 500-1000 x. check every opening horizontally and vertically, under right corner and along outer edge from samples in which a small count of cells is visible or in there are not any, check at least 40 microscopic fields of view. Primarily, take a look at glass with positive control. Cells have to be expressed fluorescent and they have to be fully colored on the established titre of antibodies or working dilution. In case that there is no aberration with colouration, IF test must be repeated (Part 4).

4.4.2. Establish whether there are clearly visible fluorescing cells of morphology characteristic for bacterium C. m. subsp. *sepedonicus*. Intensity of fluorescence has to be equal or better as with positive control strain on equal antibodies dilution. Cells which are incompletely coloured or with weak fluorescence have to be neglected.

Test must be repeated if there is suspicion of contamination. Suspicion can arise if all glasses in the series show positive cells due to buffer contamination or if positive cells have been found (out of openings) on glass surface.

4.4.3. There are several problems regarding specificity of the immunofluorescence test. In concentrated extract of potato cones or parts of stems, there can be populations of fluorescent cells of atypical morphology and saprophytic bacteria with which cross-reaction occurs and which are similar by size and morphology to bacterium C. m. subsp. *sepedonicus*.
4.4.4. Take into account only the fluorescent cells of typical size and morphology in titre or working dilution of antibodies as described in item in 4.3.

4.4.5. Interpretation of IF test reading:

(i) If clearly fluorescent cells of characteristic morphology are found, determine the average count of typical cells by microscopic field of view and calculate the number of typical cells per ml of resuspended sediment (Annex 4).

Reading of IF test is positive for samples which have at least 5 x 10^3 of typical cells per ml of resuspended sediment. Sample is considered potentially contaminated and further testing is necessary;

(ii) Reading of IF test is negative for samples which have less than 5 x 10^3 cells per ml of resuspended sediment and sample is considered negative. No further testing is necessary.

PART 5. FISH TEST

Principle

When FISH test is used as the first check test and if positive result is obtained by its use, the IF test must be carried out as the second obligatory check test. If the FISH test is used as the other check test and if it gives a positive result, further testing by diagram should be carried out for establishing of a final diagnosis.

Note:

Use valid olygo-trials specific for bacterium C. m. subsp. *sepedonicus* (Annex 7). Preliminary testing with this method should enable a repeatable detection of at least 10^3 to 10^4 cells of bacterium C. m. subsp. *sepedonicus* per ml which are added to sample extracts which proved negative in previous testing.

The procedure should be carried out in fresh prepared sample extract, but it can also be successfully applied to sample extract which was preserved with glycerol at the temperatures from – 16 to – 24 °C or from – 68 to – 86 °C.

For negative control, use parts of sample extract which were negative in the previous testing to C. m. subsp. *sepedonicus* were negative.

For positive control, prepare suspensions containing 10^5 to 10^6 cells/ml 0,01 M phosphate buffer (PB) of bacterium C. m. subsp. *sepedonicus* (e.g. strain NCPPB 4053, or PD 406) from a culture which is 3 – 5 days old (for preparation, see Annex 2). Prepare separated glasses for positive control with a homologues strain or some other reference strain (isolate) of bacterium C. m. subsp. *sepedonicus*, solved in potato extract, as given in Annex 2.

Use of ecubacteria olygo-check market with FITC enables control of hybridization process because all ecubacteria which are present in the sample will be coloured.

Test control materials by application of the same procedure as for samples.

5.1**.** Fixation of potato extract
The following protocol is based on Wullings et al. procedure (1998):

5.1.1. Prepare solution for fixation (Annex 7).

5.1.2. Measure with a pipette 100 µl of every sample extract into Eppendorf tube and centrifuge for 7 minutes at 7000 g.

5.1.3. Remove the supernatant and solve sediment in 500 µl of fixative prepared 24 hours earlier, at most. Stir on centrifugal mixer and incubate overnight at 4°C. Alternative fixative is 96 % ethanol. On that occasion it is necessary to solve sediment in 50 µl 0,01M PB and 50 µl 96 % ethanol. Stir on centrifugal mixer and incubate at 4 °C for 30 to 60 minutes.

5.1.4. Centrifuge for 8 minutes at 7000g, remove the supernatant and resuspend the sediment in 75 µl 0,01 PB (Annex 3).

5.1.5. Apply 16 µl of fixed suspensions in openings of a clean glass as demonstrated in figure 3. Apply to every glass, two undiluted different samples and use 10 µl for preparation of solution 1:100 (in 0.01 M PB). Remaining fixed solution of the sample (49 µl) can be kept at - 20 °C, after adding one volume of 96 % ethanol. If FISH test is to be repeated, remove ethanol by centrifuging and add equal volume of 0.01 PB (stir on a centrifugal mixer).

Figure 3. Glasses for FISH test

<table>
<tr><td>Sample 1</td><td>Empty</td><td>Empty</td><td>Empty</td><td>Empty</td></tr>
<tr><td>◯</td><td>◯</td><td>◯</td><td>◯</td><td>◯</td></tr>
<tr><td>Opening 1</td><td>Opening 2</td><td>Opening 3</td><td>Opening 4</td><td>Opening 5</td></tr>
<tr><td>Sample 1</td><td>Empty</td><td>Empty</td><td>Empty</td><td>Sample 2</td></tr>
<tr><td>◯</td><td>◯</td><td>◯</td><td>◯</td><td>◯</td></tr>
<tr><td>Opening 6</td><td>Opening 7</td><td>Opening 8</td><td>Opening 9</td><td>Opening 10</td></tr>
<tr><td colspan="2">Cover glass 1</td><td></td><td>Cover glass 2</td><td></td></tr>
</table>

5.1.6. Dry glasses in air (or in a dryer at 37 °C) and fix them by moving through flame. At this step, procedure can be stopped and hybridization can be continued the following day. Glasses should be kept at room temperature in a dry dust-free space.

5.2. **Pre-hybridization and hybridization**

5.2.1. Prepare lyzozyme dilution containing 10 mg lyzozyme (Sigma L–6876) in 10 ml buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8,0). That dilution can be kept, but it can be defrozen and melted only once. Cover every sample well with approximately 50 µl of lyzozyme dilution and incubate for 10 minutes at room temperature. Then, immerse glasses only once in demineralised water and carefully dry with filter paper.

Alternatively, instead of lyzozyme 50 µl 40 to 400µg ml–1 proteinase K in buffer (20 mM Tris-HCl, 2 mM CaCl2, pH 7,4) on every opening and incubate at 37 °C 30 minutes.

5.2.2. Perform dehydration of cells by consecutive immersing of glasses into 50 %, 80 % and 90 % ethanol. Place the glasses on holder and dry in air.

5.2.3. Prepare wet chamber for incubation, by covering the bottom of the hermetic box with absorbing or filter paper soaked in 1x hybmix (Annex 7). Firstly, incubate the box in the apparatus for hybridization of nucleic acids at 55 °C for 10 minutes at least.

5.2.4. Prepare 45 µl per glass of solution for hybridization (Annex 7) and previously incubate for five minutes at 55 °C.

5.2.5. Place glasses on heating medium (thermo block) at 45 °C and apply 10 µl of hybridized solution on each of four openings.

5.2.6. Place two cover glasses (24 × 24 mm) taking care not to leave any air in openings. Leave the glasses in heated wet chamber and hybridize in the dark (overnight) in hybridization device at 55 °C.

5.2.7. Prepare three receptacles with 1 L of ultra clean water, 1 L 1x hybmix (334 ml 3x hybmix and 666 ml of ultra clean water) and 1 L 1/2x hybmix (167 ml 3x hybmix and 833 ml of ultra clean water). Primarily, incubate every receptacle in a water bath at 55 °C.

5.2.8. Remove cover glasses, and place the microscopic glasses on a holder.

5.2.9. Remove check excess by incubation for 15 minutes at 55 °C in a receptacle with 1x hybmix.

5.2.10. Transfer glass holder in washing solution (1/2 x hybmix) and incubate for 15 minutes more.

5.2.11. Shortly immerse glasses in ultra clean water and place them on filter paper. Remove excessive moist by filter paper. Apply to every opening 5 to 10 µl of protective solution against discoloration with a pipette (e.g. Vectashield, Vecta Laboratories, CA, USA or equivalent), and cover the whole microscopic glass with a big cover glass (24 x 60 mm).

5.3. **FISH test reading**

5.3.1. Check the glasses immediately under epifluorescent microscope with oil immersion, and augmentation of 630 or 1000 x. With a filter appropriate for fluorescein-isothiocyanate (FITC), the ecubacteria cells (including most of the gramm negative cells) in the sample, show as fluorescent green. By use of filter for Tetramethylrhodamine-5-isothiocyanate, cells of bacterium C. m. subsp. *sepedonicus*, with Cy3, show as fluorescent red. Compare

cell morphology with positive controls morphology. Cells must be clearly fluorescent and entirely colored. If there is divergence in coloration, FISH test (Part 9.4) must be repeated. Check every opening vertically and horizontally under normal angle and along the outer edge. For samples in which a small count of cells is visible or there are not any, check at lest 40 microscopic fields of vision.

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5.3.2. Check whether there are clearly visible fluorescent cells of morphology characteristic of bacterium C. m. subsp. *Sepedonicus*.

Fluorescence intensity must be equal or stronger then with positive control. Cells which are not fully colored or which are of weak fluorescence must not be taken into account.

5.3.3. Test must be repeated if there is suspicion of contamination. Suspicion can arise if all glasses in a series show positive cells due to buffer contamination or if positive cells have been found out of openings on glass surface.

5.3.4. There are several problems connected to specificity of the FISH test. In concentrated extract of extracted cones of sprout parts of tubers or stem parts, populations of fluorescent cells of atypical morphology and saprophytic bacteria which are similar to bacterium C. m. subsp. *Sepedonicus,* by size and morphology, although more rarely than with an IF test.

5.3.5. Take into account only the fluorescent cells of typical size and morphology (see 5.3.2).

5.3.6. Interpretation of the FISH test results:

(i) FISH test results are considered valid if, by application of a FITC filter green fluorescent cells are perceived, size and morphology of which is typical of bacterium C. m. subsp. *sepedonicus* and if by application of rhodamine filter, red fluorescent cells are perceived. If clearly fluorescent cells of characteristic morphology are found, establish the average count of typical cells per microscopic field of view and calculate the count of typical cells per ml of resuspended sediment (Annex 4). Samples with at lest 5 x 10^3 of typical cells per ml of resuspended sediment are considered potentially contaminated. Further tests are necessary. Samples with less than 5 x 10^3 of typical cells per ml of resuspended sediment are considered negative.

(ii) FISH Test is negative if, by application of rhodamine filter, no prominently fluorescent red cells with size and morphology typical of bacterium C. m. subsp. *sepedonicus*, on condition that typical prominently fluorescent red cells in positive control preparations are perceived.

PART 6. PCR TEST

Principles

When PCR test is used as main check test and result is positive, IF test must be carried out for isolation as the other obligatory check test. When PCR test is used as the second check test and result is positive, further testing by diagram is needed for establishment of final diagnosis.

Use of this method as main check test is recommended solely if specialized expertise is available.

Note:

Preliminary testing by application of this method should enable repeatable detection of 10^3 to 10^4 of cells of bacterium C. m. subsp. *sepedonicus* per ml, which are added to sample extract which gave negative result in previous testing. In order to achieve the highest level of sensitivity and specificity in all laboratories, it is necessary to carry out an experiment for method optimization.

Use valid (confirmed and approved) reagents and protocols for PCR. It is desirable to choose a method with internal control.

Undertake appropriate precaution measures in order to avoid contamination of the sample with targeted DNA. In order to present contamination with targeted DNA, PCR tests should be carried out by experienced experts, in specialized laboratories for molecular biology.

Negative controls (for extraction of DNA and PCR procedure) should always be processed as last samples in the procedure in order to establish whether there was DNA transmission.

Following negative controls should be included in the PCR test:

- sample extract which was negative in previous testing to C. m. subsp. *sepedonicus*;
- buffer used for extraction of bacteria and DNA from the sample;
- reaction mix of PCR (PCR-reaction mix).

The following positive controls should be included in the PCR test:

- aliquots of resuspended sediments to which bacterium C. m. subsp. *sepedonicus* is added (for preparation, see Annex 2);
- suspension in water of 10^6 cells per ml C. m. subsp. *sepedonicus* virulent isolate (e.g. NCPPB 2140 or NCPPB 4053);
- If possible, use in PCR test the DNA extracted from positive control samples.

In order to avoid possible contamination, prepare positive controls spatially separately from samples for testing.

S Since application of PCR protocol requires use of sample extract with less soil possible prior to initiation of this test performance procedure, it is desirable to wash potato samples well.

6.1. **Methods of DNA cleansing**

Use samples for positive and negative control as previously described. Prepare control material in the same manner as samples.

There are various methods for cleansing of targeted DNA from complex substrates of samples for the purpose of removal of PCR inhibitors and other enzyme reactions and method of concentration of targeted DNA.

The following method is optimal (standardized) for use with valid (confirmed and approved) PCR methods (Annex 6).

6.1.(a)Method by Pastric (2000)

1. Measure with a pipette 220 µl for buffer lysis [100 mM NaCl, 10 mM Tris-HCl (pH 8,0), 1 mM EDTA (pH 8,0)] in Eppendorf tube of 1,5 ml.

2. Add 100 µl of sample extract and put in a thermo block or water bath on 95 °C for 10 minutes.

3. Put tube on ice for 5 minutes.

4. Add 80 µl of basic stock solution Lysozyme (50 mg of Lysozyme
per ml in 10 mM Tris-HCl, pH 8,0) and incubate fro 30 minutes at 37 °C.

5. Add 220 µl Easy DNA® solution A (Invitrogen), mix well on vortex i.e. centrifugal mixer and incubate for 30 minutes at 65 °C.

6. Add 100 µl Easy DNA® solution B (Invitrogen), mix roughly on centrifugal mixer (vortex) until achievement of free flow of precipitate in the tube and of uniform viscosity of the sample

7. Add 500 µl of chloroform and mix on centrifugal mixer (vortex) until viscosity is reduced and mixture becomes homogenous.

8. Centrifuge 15000 g for 20 minutes at 4 °C do divide phases and create interphase.

9. Transfer the upper phase to the new Eppendorf tube.

10. Add 1 ml 100 % ethanol (- 20 °C), shortly stir on centrifugal mixer (vortex) and incubate on ice for 10 minutes.

11. Centrifuge at 15000 g for 20 minutes at 4 °C and remove ethanol from sediment.

12. Add 500 µl 80 % ethanol (- 20 °C) and stir by turning of the tube.

13. Centrifuge at 15000 g for 10 minutes at 4 °C, retain sediment, remove ethanol.

14. Leave the sediment to dry on air or in vacuum centrifuge (DNA speed vac).

15. Resuspend sediment in 100 µl of sterile ultra clean water and leave at room temperature for at least 20 minutes.

16. Keep at - 20 °C until it is needed for PCR.

17. Separate possible white precipitate (sediment) by centrifuging and use for PCR 5 µl of supernatant containing DNA.

6.1.(b) Other methods

Other methods of DNA extraction can be applied, e.g. Qiagen DNeasy Plant Kit, if equal effectiveness is proven in cleansing of DNA from control samples containing 10^3 to 10^4 pathogenous cells per ml.

6.2. **PCR**

6.2.1. Prepare moulds (samples) for testing and control to PCR according to approved protocol (Annex 6). Prepare one decimal dilution of the DNA extract from sample (1:10 in ultra clean water).

6.2.2. In a non-contaminated area, prepare the corresponding mixture for PCR according to published protocols (Annex 6). Approved PCR protocol is a multiplex reaction which includes also the internal PCR control.

6.2.3. Add 5 µl of DNA extract on 25 µl of reaction mixture in sterile tubes for PCR..

6.2.4. Include the sample for negative control which contains only reaction mixture for PCR and, instead of a sample, add the same source of extra clear water which is used for preparation of reaction mixture for PCR.

6.2.5. Put tubes in device for PCR (thermal cycler) which is used in preliminary testing and initiate the optimal (standardized) PCR programme (Annex 6).

6.3. **Analysis of PCR products**

6.3.1. By electrophorese in agarose gel split the multiplied PCR products. At least 12 µl of reaction mixture of multiplied DNA from every sample, mixed with 3 µl of buffer for application (Annex 6), apply in 2.0 % (w/v) agarose gel in u tris-acetat EDTA buffer (TAE) (Annex 6), and subject to voltage from 5 to 8 V per cm. Use corresponding DNA standard, e.g. 100 bp scale

6.3.2. Color electrophoretic lines of DNA in gel by immersion of gel in ethidium bromide (0,5 mg/l) 30 do 45 minutes taking adequate precaution measures for work with this mutagen.

6.3.3. Check coloured gel on short-wave UV transilluminator (λ = 302 nm) and search for multiplied fragments of expected length (Annex 6) and document them.

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6.3.4. For every new finding/case, check the authenticity of multiplied PCR of the product, by restricitve enzyme analysis of the remaining multiplied DNA of the sample, by sample incubation at the optimal temperature and in optimal time, with appropriate enzyme and buffer (Annex 6). Separate the fragments which appear by electrophorese in agarous gel, as previously mentioned, and after colouring with ethidium bromide on an UV transilluminator, observe the characteristic UV pattern (matrix) and compare it to the positive control before and after separation.

Interpretation of the PCR test results:

PCR test is negative if there is no visible PCR product of expected length, which is characteristic of bacterium C. m. subsp. *sepedonicus*, in the tested sample, but it is visible in all positive control samples (with multiplex PCR with primers for internal control which are specific for the host plant: in tested sample, the other product of PCR of expected size must be multiplied).

PCR test is positive if the PCR product specific of bacterium C. m. subsp. *sepedonicus* of expected length and expected pattern and restriction pattern is visible, on condition that it is not multiplied in any of the negative control samples. Reliable confirmation of positive result can be achieved by repetition of this wit another pair of PCR primers (Part 9.3).

Note:
It can be suspected that inhibition of PCR reaction occurred if from the positive control sample

containing C. m. subsp. *sepedonicus* in water, the expected product is obtained, and from positive controls from C. m. subsp. *sepedonicus* in potato extract, negative results are obtained. In multiplex PCR complex which are carried out by use of internal PCR controls, it is considered that reaction inhibition occurred if none of the two products has been obtained.

If expected product is obtained from one or several negative controls, one can suspect that contamination has occurred.

PART 7. BIOTEST

Note:

Preliminary testing by this method should enable repeatable detection (establishment of presence) from 10^3 to 10^4 colony forming units (CFU) of bacterium C. m. subsp. *sepedonicus* per ml, which are added to sample extracts which were negative in previous testing (preparation in Annex 2).

Greatest detection sensitivity can be expected if fresh prepared sample extracts and optimal growth conditions are applied. However, this method can be successfully applied with extracts which have been kept with glycerol at the temperature from – 68 to – 86 °C.

Some varieties of aubergine represent a great selective medium for enriching, for growth of aC. m. subsp. *sepedonicus*, even when there are no symptoms, and they are excellent as hosts for confirmation tests.

Growth conditions should be optimal in order to reduce risk from obtaining of falsely negative results.

Details regarding plant growing are given in Annex 8.

7.1. Distribute all remaining quantity of resuspended sediment left for testing referred to in 3.1.6 or 3.2.5 on aubergines by one of the methods – inoculatin by section or injection inoculation (in more detail in 7.3 or 7.4). UsE only plants of aubergine plants in stadium of two to three real leaves, until full development of the third real leaf. It takes 15 to 25 aubergine plants per one sample, in order to fully utilize the resuspended sediment and ensure efficient inoculation.

7.2. Do not water aubergine plants one to two days prior to inoculation in order to reduce turgor.

7.3. Inoculation by section

7.3.1. Holding plant between fingers, apply a drop (round 5-10 µl) with a pipette of resuspended sediment to the plant between cotyledon and the first leaf.

7.3.2. With a sterile scalpel, make a diagonal cut, around 1 cm long and around 2/3 of plant width deep, starting form the resuspended sediment drop.

7.3.3. Firmly close the cut with sterile vaseline from the syringe.

7.4. Injection inoculation

Inoculate aubergine plants right above the cotyledon with an injection with a thin needle (at least 23G). Distribute the sample to aubergine test plants.

7.5. For positive control, inoculate 5 plants with suspension form 10^5 to 10^6 cells per ml of water of the known culture of C. m. subsp. *sepedonicus* and, when possible, with extract of naturally contaminated tuber tissue (Part 4) with the same inoculation method (7.3 or 7.4).

7.6. For negative control, inoculate 5 plants with sterile buffer for sediment solution (sterile pellet buffer) with same inoculation method (7.3 or 7.4).

7.7. Grow plants in quarantine conditions up to four weeks at 25 to 30°C, with enough of light and high humidity (70 do 80 %) adequately water them, taking care not to cause water saturation or wrinkling due to lack of water. Cells of C. m. subsp. *sepedonicus* do not develop (they die) at temperatures exceeding 30 °C, and optimal temperature is 21 °C. . In order to avoid contamination, grow plants for positive and negative control on clearly separated tables in a greenhouse or growing chamber, or, if the space ifs limited, make sure that plants are clearly separated between certain processes in the course of carrying out of the procedure. It is of key importance what there are no any insects in greenhouses or growing chambers because they can transfer bacteria from one sample to another.

7.8. Check occurrence of symptoms regularly after seven days. Count plants which show symptoms of C. m. subsp. *Sepedonicus*, causing agent of leaf wilt in aubergine, which can begin as wilt of edges or between leaf nerves. Withered tissue can at firs look dark green or spotted and later onit becomes pale and necrotic. Withered surface between leaf nerves looks greasy, as if moistened by water. Necrotic tissue has light yellow edge. Plants do not always die; the longer the period preceding developing of symptoms, the greater possibility of survival. Plants can overgrow contamination. Young aubergine plants are much more sensitive to low populations of C. m. subsp. *sepedonicus* than older plants; that is why plants are used in or immediately before the three right leaves phase.

Wilt can be caused by populations of other bacteria of fungi which are present in extract of tuber tissue. These are: *Ralstonia solanacearum, Erwinia carotovora subsp. carotovora and E. carotovora subsp. atroseptica, Erwinia chrysanthemi, Phoma exigua var. foveata*, as well as populations of a large number of saprophytic bacteria. Especially, *Erwinia chrysanthemi* can cause symptoms on leaves and wilt which is very similar to symptoms of C. m. subsp. *sepedonicus*. The only difference is occurrence of darkening-blackening of stems in case of infection with bacterium *Erwinia chrysanthemi*. Other wilts differ from that caused by C. m. subsp. *sepedonicus* because entire leaves or plants wither very quickly. Also, colouring by Gram can be prepared; that is how C. m. subsp. *sepedonicus* will be distinguished from *Erwinia* spp.

7.9. As soon as symptoms are perceived in aubergine, it is necessary to perform re-isolation, using parts of withered leaf tissue and stem tissue (3.1.3 tissue maceration). Carry out surface disinfection of leaves and plants of aubergine, by rinsing them with 70 % ethanol. Carry out IF or PCR test on aubergine juice and isolate on corresponding (selective) nutritive medium (Part 8). Colouring by Gram can be prepared (Annex 9). Identify treated cultures of possible C. m. subsp. *sepedonicus* and confirm pathogenicity (Parts 9 and 10).

7.10. In certain circumstances, especially when growth conditions are not optimal, it is possible that C. m. subsp. *sepedonicus* exists as latent infection in aubergines, even after incubation period up to 4 weeks. If symptoms are not perceived after 4 days, carry out the IF/PCR test on a prepared

sample of parts of stems – 1 cm of every test plant, taken above the place of inoculation. If test is positive, it is necessary to carry out isolation anew on corresponding (selective) nutritive medium, according to procedure referred to in Part 8. Identify treated cultures of possible C. m. subsp. *sepedonicus* and confirm pathogenicity (Part 9 and 10).

Interpretation of biotest results.

Biotest results are valid if positive control plants show typical symptoms, if bacterium can be isolated anew form these plants, and if negative controls do not result in any symptoms.

Biotest is negative if test plants are not infected with bacterium C. m. subsp. *sepedonicus*, on condition that bacterium C. m. subsp. *sepedonicus* is detected in positive controls.

biotest is positive if test plants are infected with bacterium *C. m. subsp. sepedonicus.*

PART 8. ISOLATION OF *C. m. subsp. sepedonicus*

Note:
Diagnosis can be confirmed only if C. m. subsp. *sepedonicus* is isolated and identified (Part 9) and if pathogenicity is confirmed (Part 10). Although C. m. subsp. *sepedonicus* is a demanding organism, it can be isolated form symptomatic tissue.

However, it can be overgrown by fast growing saprophytic bacteria and that is why isolations directly from tissue of tuber or stem are difficult (Part 3.1.6 or 3.2.5). Direct isolation of C. m. subsp. *sepedonicus* is possible by selective nutritive medium and corresponding solved suspension of resuspended sediment from cones or stems of potato.

It is necessary to perform isolation from all tubers or parts of potato stem with symptoms from test aubergines in which no symptoms were not noticed but the IF/PCR tests from prepared sample was positive. (Part 7.10). Maceration of aubergine stems should, if needed, be carried out as given in Part 3.1.3.

For positive controls, prepare decimal solutions of suspension 10^6 cfu per ml C. m. subsp. *sepedonicus* (e.g. NCPPB 4053 or PD 406). In order to avoid every possibility of contamination, prepare positive controls completely separately from samples which are tested.

For every fresh prepared series of selective nutritive medium, it is necessary to check whether it corresponds for growth of pathogens before it is used for testing of routine samples.

Test control material in the same manner as sample or samples.

8.1. **Growing on a selective nutritive medium**

8.1.1. From 100 µl of aliquot form sample of resuspended potato sediment or aubergine juice, prepare decimal dilutions in sediment buffer (Annex 3).

8.1.2. Isolation from non-diluted resuspended sediment of potato usually does not succeed due to difficult growing of C. m. subsp. *sepedonicus* and competition of saprophytes. Since the bacterium is usually present in high populations in contaminated tissue, saprophytes can usually be removed by dilution, while pathogen remains. Therefore, it is recommended to smear 100 µl from every

sample (when using Petri cups with diameter 90 mm – adjust the quantity for Petri cups of other sizes), 1/100 to 1/10 000 of diluted suspension on nutritive medium MTNA or on NCP-88 (Annex 5), by smearing technique.

Note:
Alternative strategy is to smear the initial aliquot per 100 µl of resuspended sediment of potato on first medium, and then make smear on another medium with a stick, while you apply remains of extract from the first medium onto the other one.

In the end, repeat it with the third medium, by which you obtain the similar dilution effect.

8.1.3. Incubate the medium in the dark at 21 to 23 °C.

8.1.4. First check of mediums and assessment of C. m. subsp. *Sepedonicus*-like colonies, in comparison to control mediums is after 3 days, and further assessments after 5, 7 and possible 10 days.

8.2. **Treatment of suspicious colonies**

Note:
C. m. subsp. *Sepedonicus*-like colonies should be screened on nutritive medium YGM if they will be used for inoculation of aubergine and/or further identification; this should be done before the mediums become too contaminated, i.e. three to five days after, preferably.

8.2.1. By smearing, apply C. m. subsp. *Sepedonicus*-like colonies on one of the following nutritive mediums: (compositions are given in Annex 5) :

- nutritive agar with addition of dextrose (used only for screening)
- agar with addition of yeast, peptone and glucose,
- agar with addition of yeast extract and mineral salts.

Incubate the screened medium at 21 °C to 24 °C up to 10 days.

C. m. subsp. *sepedonicus* grows slowly, it usually creates creamy, dome-like colonies with spiky top in the course of 10 days.

8.2.2. Smear again in order to achieve cleanliness.
Growth rate is enhanced by screening. Typical colonies are creamy white or ivory-like, sometimes yellow, rounded, smooth, convex – dome-like, slimy-liquid, with entire edges and usually 1 to 3 mm in diameter.
Simple colouring by Gram (Annex 9) can help in selection of colonies for further testing.

8.2.3. Identify possible cultures (Part 9) and carry out the test of isolate pathogenicity (Part 10) .

PART 9. IDENTIFICATION

Identify clean cultures of probable isolates of C. m. subsp. *sepedonicus* by use of at least two of the following tests which are based on various biological principles.

If needed, include the familiar reference strains for every test.

9.1. **Nutritive (growing) and enzyme tests for identification**

Establish phenotype properties which are universally present or absent in bacterium C. m. subsp. *sepedonicus*, by methods by Lelliott and Stead (1987), Klement et al. (1990), Schaad (2001), unknown author (1987).

Incubate all nutritive mediums at 21 °C and check after six days. If there is no growth of colonies, incubate up to 20 days.

Familiar control of C. m. subsp. *Sepedonicus* should be included in all tests. Nutritive and physiological tests should be carried out by use of cultures from nutritive agar. Conduct morphological comparisons using cultures form nutritive agar with addition of dextrose .

Tests	*Expected results*
Oxidation/fermentation (O/F) test	Inert or week oxidative
Oxidase activity	–
Growth at 37 °C	–
Urease activity	–
Esculin hydrolysis	+
Starch hydrolysis	– or weak
Tolerance of 7% solution of NaCl	–
Indole creation	–

Catalase activity	+
Creation of H_2S	–
Use of citrates	–
Solving of gelatine	–
Creation of acids from glycerol	–
Creation of acids from lactose	– or weak
Creation of acids from rhamnose	–
Creation of acids from salicine	–
Colouring by Gram (Annex 9)	+

9.2. **IF test**

(a) Prepare suspension of approximately 10^6 cells per ml in IF buffer (Annex 3).
(b) Prepare double dilutions of solution of corresponding serum.
(c) Apply IF procedure (Part 4).
(d) IF test is positive if IF titre of culture is equal to titre of positive control.

9.3**. PCR test**

(a) Prepare suspension of approximately 10^6 cells per ml in ultra clean water.

(b) Heat 100 µl of cell suspension in closed tubes in a thermo block or hot water bath for four minutes at 100 °C. If needed, adding of fresh prepared NaOH by final concentration of 0,05M can accelerate cell decomposition. Samples can be used at– 16 do – 24 °C until use.

(c) Use the corresponding PCR procedures for multiplication of specific fragments of C. m.

subsp. *sepedonicus* (e.g. Pastrik, 2000; Annex 4; Li and de Boer, 1995; Mills et al., 1997; Pastrik and Rainey, 1999; Schaad et al., 1999.)

(d) Identification of C. m. subsp. *sepedonicus* is positive if the PCR products are of same size and have the same polymorphism of length of restriction fragments as positive control strain.

9.4. **Test FISH**

(a) Prepare suspension of approximately 10^6 cells per ml in ultra clean water.
(b) Apply FISH procedure (Part 5).
(c) FISH test is positive if reactions from the culture and positive control are equal.

9.5. **Fat acids profiling (FAP)**

(a) Grow culture on trypticase-soy-agar (Oxoid)72 hours at 21 °C (+/– 1 °C).
(b) Apply appropriate FAP procedure (Janse, 1991; Stead, 1992).
(c) FAP test is positive if profile of possible (tested) culture is identical to positive control profile. Presence of characteristic fat acids: 15:1 Anteiso A, 15:0 Iso, 15:0 Anteiso, 16:0 Iso, 16:0 and 17:0 Anteiso, to a large extent points to C. m. subsp. *sepedonicus*. Other genus such as *Curtobacterium*, *Arthrobacter* and *Micrococcus* also have some of these acids, but 15:1 Anteiso A is a rare acid in these bacteria, but it appears in all bacteria *Clavibacter spp*. Between 1 to 5 %. At C. m. subsp. *sepedonicus* value is usually around 5 %.

9.6**. BOX-PCR**

(a) Prepare suspension of approximately 10^6 cells per ml of ultra clean water.
(b) Apply the test in accordance with the procedure (Smith et al., 2001).

Part 10. PATHOGENICITY CHECK TEST

Pathogenicity check test must be carried out for final confirmation of presence of C. m. subsp. *sepedonicus* and for assessment of virulence of cultures identified as C. m. subsp. *sepedonicus*:

10.1. Prepare inoculums of approximately 10^6 cells per ml from cultures of isolate which is tested three days old and corresponding strain of positive control of C. m. subsp. *sepedonicus*.

10.2. Inoculate plants of 5 to 10 young seedlings of aubergine in phase of 3 real leaves (part 7.3 or 7.4).

10.3. Incubate the inoculated seedlings at 18 to 24 °C with sufficient light and relatively high humidity and adequately plant in order to avoid over humidity or stress from wilt (Part 7.7). In case of inoculation with clean cultures, typical wilt appears in the course of two weeks, but plants do not show symptoms (Part 7.8) after that time, incubation should proceed for one week more at temperatures which are favourable for aubergine growth, but not higher than 25 °C (Annex 8). If

symptoms are not present after three weeks, the culture can be confirmed as pathogenous form of C. m. subsp. *sepedonicus*.

10.4. Conduct isolation from plants with symptoms by removal of the part of stem 2 cm long above the place of inoculation which is powdered sand suspended in a small quantity of sterile distilled water or 50 mM phosphate buffer (Annex 3). Isolate the pathogen from suspension with spread on MTNA and YPGA (Annex 5),incubate three to five days at 21 to 23°C and check whether typical colonies of bacterium C. m. subsp. *Sepedonicus* have been formed.

Annex 1

Laboratories included in optimization and validation of protocols

Laboratory	Location	state
Agentur für Gesundheit und Ernährungssicherheit	Vienna and Lintz	Austria
Departement Gewasbescherming	Merelbeke	Belgium
Plantedirektoratet	Lyngby	Denmark
Central Science Laboratory	York	England
Scottish Agricultural Science Agency	Edinburgh	Scotland
Laboratoire National de la Protection des Végétaux, Unité de Bactériologie	Angers	France
Laboratoire National de la Protection des Végétaux, Station de Quarantaine de la Pomme de Terre	Le Rheu	France
Biologische Bundesanstalt	Kleinmachnow	Germany
Pflanzenschutzamt Hannover	Hannover	Germany
State Laboratory	Dublin	Ireland
Plantenziektenkundige Dienst	Wageningen	Netherlands
Norwegian Crop Research Institute, Plant Protection Centre	Aas	Norway
Direcção-Geral de Protecção das Culturas	Lisbon	Portugal
Nacionalni institut za biologijo	Ljubljana	Slovenia
Centro de Diagnostico de Aldearrubia	Salamanca	Spain

Annex 2

Preparation of positive and negative controls for basic check tests, PCR/IF and FISH

- Make a suspension of culture of virulent strain of bacterium *C. m*. subsp. *sepedonicus* (NCPPB 4053 or PD 406) grown for 72 hours at MTNA basic nutritive medium in 10 mM phosphate buffer, to obtain cell density of approximately 1 to 2 × 10^8 cfu per ml. That is usually a feebly opaque suspension with optical density from 0.20 at 600 nm.
- Extract sprout cones from 200 potato tubers of white peel variety which are known not to be infected with *C. m*. subsp. *sepedonicus.*
- Process cones with usual method and resuspend sediment in 10 ml.

- Prepare 10 sterile micro tubes of 1.5 ml with 900 µl of resuspended sediment.
- Add in first micro tube 100 µl of suspension of *C. m.* subsp. *Sepedonicus* and stir on centrifugal mixer (vortex).
- Prepare decimal solutions of bacterial suspensions in five next micro-tubes..
- Use these six micro-tubes with contaminated extract for positive control. Use four micro-tubes with non-contaminated extract for negative control. In accordance with that, mark the micro-tubes.
- Prepare aliquots of 100 µl in micro-tubes of 1.5 ml so that nine copies of every control sample are obtained. Keep them until use at – 16 do – 24 °C.
- Confirm presence and quantity of C. m. subsp. *sepedonicus* in control samples by an IF test, primarily.
- For the PCR test, perform extraction of DNA on positive and negative control samples for every series of samples for testing.
- For IF and FISH tests, carry out testing on positive and negative control samples for every series of samples for testing.
- In IF, FISH and PCR tests, bacterium C. m. subsp. *sepedonicus* must be detected in at least 10^6 and 10^4 cells/ml of positive controls and in none of the negative controls.

Annex 3
Buffers for testing procedures

Note: unopened sterile buffers can be kept up to one year.

1. Buffers for extraction procedure

1.1. Extraction buffer (50 mM phosphate buffer, pH 7.0)

This buffer is used for extraction of bacterium from plant tissue by homogenization or shaking.

- Na_2HPO_4 (waterless) 4.26 g
- KH_2PO_4 2.72 g

Distilled water 1 L

Solve the ingredients, check pH and sterilize in an autoclave for 15 minutes at 121 °C. next additional steps may be useful:

	Purpose	Quantity (per L)
Lubrol flakes	Deflocculant*	0.5 g
DC silicone against foaming	anti-foaming agent *	1.0 ml
Tetrasodium pyrophosphate	antioxidant	1.0 g
Polyvinylpyrrolidone -40000 (PVP-	Binding of PCR inhibitors	50 g

40)		
Use for the method of extraction by homogenization		

1.2. Buffer for sediment-pellet buffer (10 mM phosphate buffer, pH 7.2)

This buffer is used for resuspension and dilution of extracts of cones taken out from tuber sprouts cones after concentration into sediment by centrifuging.

- $Na_2HPO_4x12H_2O$ 2.7 g
- $NaH_2PO_4x2H_2O$ 0.4 g
- Distilled water 1 L

Dissolve ingredients, check pH and sterilize in an autoclave for 15 minutes at 121 °C.

2. Buffers for IF test

2.1. IF buffer (10 mM phosphate buffer with addition of salt (PBS), pH 7.2)

This buffer is used for solving of antibodies.

- $Na_2HPO_4x12H_2O$ 2.7 g
- $NaH_2PO_4x2H_2O$ 0.4 g
- NaCl 8.0 g
- distilled water 1 L

Dissolve ingredients, check pH and sterilize in an autoclave for 15 minutes at 121 °C.

2.2. IF buffer – Tween

This buffer is used for washing of glasses.
Add 0.1 % Tween 20 to buffer.

2.3. Phosphate buffer with glycerol, pH 7.6

This buffer is used as liquid for coverage of openings in IF test in order to enhance fluorescence.

- $Na_2HPO_4x12H_2O$ 3.2 g
- $NaH_2PO_4x2H_2O$ 0.15 g
- Glycerol 50 ml
- Distilled water 100 ml

Covered solutions against discoloration are available in the market, e.g. Vectashield® (Vector Laboratories) or Citifluor® (Leica).

Annex 4
Establishment of contamination level if IF and FISH tests

1. Establish average count of typical fluorescent cells per field of view (c).
2. Calculate the number of typical fluorescent cells per opening of microscopic glass (C).

   $C = c \times S/s$

   where
   S = area of opening of glass with several openings, and
   s = area of objective field.
   $s = \pi i2/4G2K2$

   where
   i = field coefficient (depends on the of ocular and amounts 8 to 24)
   K = tubus coefficient (1 or 1.25)
   G = objective augmentation (100x, 40 x etc.)
3. Calculate the count of typical fluorescent cells per ml of resuspended sediment (N)

   $N = C \times 1000/y \times F$
   where
   y = volume of resuspended sediment in every opening and
   F = factor of dilution of resuspended sediment.

Annex 5
Nutritive mediums for isolation and growing of *C. m. subsp. sepedonicus*

(a) Basic nutritive mediums

Nutritive agar (NA)
Nutritive agar (Difco) 23.0 g
Distilled water 1.0 L
Dilute ingredients and sterilize in an autoclave at 121 °C for 15 minutes.
Nutritive dextrose agar (NDA)
Difco bacto nutritive agar containing 1 % D(+) of glucose (monohydrate). Sterilize in autoclave at 115 °C for 20 minutes.
Yeast, peptone and glucose agar (YPGA)
Yeast extract (Difco) 5.0 g
Bacto peptone (Difco) 5.0 g
D(+) glucose (monohydrate) 10.0 g
Agar Bacto (Difco) 15.0 g
Distilled water 1.0 L
Dilute ingredients and sterilize in an autoclave at 121 °C for 15 minutes.
Yeast and mineral salts agar (YGM)

Bacto yeast extract (Difco) 2.0 g
D(+) glucose (monohydrate) 2.5 g
- K_2HPO_4 0.25 g
- KH_2HPO_4 0.25 g
- $MgSO_4x7H_2O$ 0.1 g
- $MnSO_4xH_2O$ 0.015 g
- NaCl 0.05 g
- $FeSO_4x7H_2O$ 0.005 g
- Bacto agar (Difco) 18 g
- Distilled water 1 L

Dissolve ingredients and sterilize 0.5 l volume of nutritive medium in an autoclave at 121 °C at 15 minutes.

(b) Valid selective nutritive mediums

MTNA
Unless otherwise stated, all ingredients are from BDH.
- Yeast extract (Difco) 2.0 g
- Manitol 2.5 g
- K_2HPO_4 0.25 g
- KH_2PO_4 0.25 g
- NaCl 0.05 g
- $MgSO_x7H_2O$ 0.1 g
- $MnSO_4xH_2O$ 0.015 g
- $FeSO_4x7H_2O$ 0.005 g
- Agar (Oxoid no. 1) 16.0 g
- Distilled water 1.0 L

Dissolve ingredients, set pH to 7.2. After sterilization in an autoclave (at 121 °C for 15 minutes) and cooling at 50 °C, add antibiotics: trimetoprim 0.06 g, nadalyxyl acid 0.002 g, amfotericin B 0.01 g.

Basic antibiotics solutions: trimetoprim (Sigma) and nalydixyl acid (Sigma) (both 5 mg/ml), in 96 % methanol, amphotericin B (Sigma) (1 mg/ml) in dymethyl sulfoxide. Basic solutions are sterilized by filtrating.

Note:

Period of duration of basic nutritive medium is three months. After antibiotics are added, the period is prolonged for one month, when it is kept in a cooling device.

NCP-88
- Nutritive agar (Difco) 23 g
- Yeast extract (Difco) 2 g
- D-manitol 5 g
- K_2HPO_4 2 g
- KH_2PO_4 0.5 g
- $MgSO_4.7H_2O$ 0.25 g
- distilled water 1 L

Dissolve ingredients, set pH at 7.2. After sterilization in an autoclave (at 121 °C for 15 minutes) and cooling at 50 °C, add the following antibiotics: Polymyxin B sulphate (Sigma) 0.003 g, nalydixyl acid (Sigma) 0.008 g, Cyuclohexymid (Sigma) 0.2 g.

Prepare basic solutions of antibiotics: nalydyxyl acid in 0.01 M NaOH, cyclohexymide in 50 % ethanol, polymyxin B sulphate in distilled water. Basic solutions are sterilized by filtration.

Note:

Period of duration of basic nutritive medium is three months. After antibiotics are added, the period is prolonged for one month, when it is kept in a cooling device.

Annex 6

Valid (confirmed and approved) protocols and reagents for PCR

Note:

Preliminary testing should enable repeatable detection of 10^3 to 10^4 cells of bacterium C. m. subsp. *sepedonicus* per ml of sample extract.

Preliminary testing must not give falsely positive results at selected bacterial strains.

1. Protocol for multiplex PCR with internal PCR control (Pastrik, 2000)

1.1. Oligonucleotide primers
Upstream primer PSA-1 5´- ctc ctt gtg ggg tgg gaa aa -3´
Downstream primer PSA-R 5´- tac tga gat gtt tca ctt ccc c -3´
Upstream primer NS-7-F 5´- gag gca ata aca ggt ctg tga tgc -3´
Downstream primer NS-8-R 5'- tcc gca ggt tca cct acg ga -3'
Expected length of multiplied product from DNA mould of *C. m. subsp. sepedonicus* = 502 pb (a couple of PSA primers).
Expected length of multiplied product from 18S rRNA internal control = 377 pb (a couple of NS primers).

1.2. PCR reaction mixture (reaction mix for PCR)

Reagent	Quantity per reaction	Final concentration
Sterile ultra clean water	15.725 µl	
10x PCR buffer[1] (15 mM $MgCl_2$)	2.5 µl	1x (1.5 mM $MgCl_2$)
BSA (fraction V) (10 %)	0.25 µl	0.1 %

mix d-NTP (20 mM)	0.125 µl	0.1 mM
Primer PSA-1 (10 µM)	0.5 µl	0.2 µM
Primer PSA-R (10 µM)	0.5 µl	0.2 µM
Primer NS-7-F (10 µM)[2]	0.1 µl	0.04 µM
Primer NS-8-R (10 µM)[2]	0.1 µl	0.04 µM
Taq polymerase (5 U/µl)[1]	0.2 µl	1.0 U
Sample volume	5.0 µl	
Total volume	25.0 µl	

[1] Method was validated by use of Taq polymerase Perkin Elmer (AmpliTaq or Gold) and Gibco BRL.
[c] Concentration of primers NS-7 F and NS-8-R was optimal for extraction from sprout cones of potato by homogenization method and cleansing of DNA by Pastrik (2000) (Part 6.1.a and 6.2).
If extraction by shaking is used or some other method of DNA isolation, it is necessary to perform optimization of reagent concentration again.

1.3. PCR reaction conditions

Conduct the following programme:

- 1 cycle: (i) 3 minutes at 95 °C (denaturation of DNA chain)
- 10 cycles: (ii) 1 minute at 95 °C (denaturation of DNA chain)
- (iii) 1 minute at 64 °C (binding of primer)
- (iv) 1 minute at 72 °C (copy elongation)
- 25 cycles: (v) 30 seconds at 95 °C (denaturation of DNA chain)
- (vi) 30 seconds at 62 °C (binding of primer)
- (vii) 1 minute at 72 °C (copy elongation)
- 1 cycle: (viii) 5 minutes at 72 °C (final elongation)
- (ix) keep at 4 °C.

Note:

Optimal conditions of use of this programme are defined for use on PCR MJ Research PTC 200 thermal cycler. Modification of duration of cycle steps (ii), (iii) (iv), (v), (vi) and (vii) je is probably necessary in case of use of other models of thermal cyclers.

1.4. Analysis of products of multiplication by restriction enzyme

PCR produces multiplied DNA of bacterium *C. m.* subsp. *sepedonicus* show characteristic polymorphisms in length of restriction fragments with enzyme Bgl II after incubation at 37 °C for 30 minutes. Restriction fragments obtained from fragment specific for *C. m.* subsp. *sepedonicus* have size 282 bp and 220 bp.

2. Preparation of buffer for application

2.1. Bromphenol blue (10 % basic solution)
Bromphenol blue 5 g
Distilled water (bidistilled) 50 ml
2.2. buffer for application
Glycerol (86 %) 3.5 ml
Bromphenol blue (5.1) 300 µl
Distilled water (bidistilled) 6.2 ml
3. 10x Tris acetate EDTA buffer (TAE), pH 8.0
Tris buffer 48.4 g
cold vinegar acid 11.42 ml
EDTA (disodium salt) 3.2 g
Distilled water 1.00 L
Dissolve by 1× prior to use.
Available in the market (e.g. Invitrogen or replacement).

Annex 7
Valid (confirmed and approved) reagents for FISH test

1. Olygo-checks

Specific check for Cms CMS-CY3-01: 5´- ttg cgg ggc gca cat ctc tgc acg -3´
Non-specific ecubacteria check EUB-338-FITC: 5´- gct gcc tcc cgt agg agt -3´

2. Solution for fixing

(CAUTION! SOLUTION FOR FIXING CONTAINS PARAFORMALDECHYDE WHICH IS TOXIC. WEAR GLOVES AND DO NOT INHALE IT. WORK IN DIGESTOR IS RECOMMENDED)

(i) Heat 9 ml of water for molecular biology (e.g. ultra clean water) at around 60 °C and add 0.4 g of paraformaldehyde. Paraformaldehyde is solved after adding of 5 drops of 1N NaOH and mixing on a magnetic mixer.

(ii) Set pH to 7.0 by adding 1 ml 0.1M of phosphate buffer (PB; pH 7.0) and 5 drops of 1N HCl.

Check pH value with indicator paper and if it is necessary set it HCl or NaOH. (CAUTION! IN SOLUTIONS WITH PARAFORMALDECHYDE, DO NOT USE pH-METER.))

(iii) Filtrate solution through membrane filter of 0.22 µm and keep at 4 °C until use and protect from dust.

(iv) Note:

Alternative solution for fixation: 96 % ethanol

3**.** 3x Hybmix

NaCl 2.7 M
Tris-HCl 60 mM (pH 7.4)
EDTA (sterilized by filtration in an autoclave) 15 mM
Dilute by 1x, as needed.

4. Hybridization solution

1x Hybmix
Sodium dodecil sulphate (SDS) 0.01 %
Check EUB 338 5 ng/µl
Check CMSCY301 5 ng/µl
Prepare quantities of hybridization solution by calculations in Table 1. For every glass (with 2 different samples in duplicate) it takes 90 µl of hybridization solution.

Proposed quantities for preparation of hybridization mixture

	2 glasses	8 glasses
Sterile ultra clean water	50.1	200.4
3x hybmix	30.0	120.0
1 % SDS	0.9	3.6
Check EUB 338 (100 ng/µl)	4.5	18.0
Check CMSCY301 (100 ng/µl)	4.5	18.0

Total volume (µl)	90.0	360.0

Note:

Keep all solutions containing olygo-checks sensitive to light at the temperature of –20 °C.
Protect from direct sunlight or electric light during use.

5**.** 0.1M of phosphate buffer, pH 7.0

- Na_2HPO_4 8.52 g
- KH_2PO_4 5.44 g
- Distilled water 1.00 L

Dilute ingredients, check pH and sterilize in autoclave at 121 °C ,15 minutes.

Annex 8
Aubergine growing

Plant seed of aubergine (*Solanum melongena*) in pasteurized compost for seeds.
Plant seedlings in pasteurized compost in pots after cotyledons are fully developed (10 to 14 days)..
Aubergine should be grown in a greenhouse in following conditions:

Day length: 14 hours or natural daylight, if it is more than 14 hours;

Temperature:
- day: 21 do 24 °C,
- night: 15 °C.

Sensitive aubergine varieties:
- »Black Beauty«;
- »Long Tom«;
- »Rima«;
- »Balsas«

Annex 9
Procedure of coloration by Gram (Hucker's modification) (Doetsch, 1981.)

Crystal violet solution

- Dissolve 2 g of crystal violet in 20 ml of 95 % ethanol.
- Dissolve 0.8 g of ammonium-oxalate in 80 ml of distilled water.

Mix the two solutions.

Lugol's solution

- Iodine 1 g

- Calium-iodide 2 g
- Distilled water 300 ml

Powder ingredients by use of pestle and mortar. Add ingredients to water and stir i.e. mix in order to dissolve them in a closed receptacle.

Solution of safranin for contrast
Basic solution:

- Safranin O 2.5 g
- 95 % ethanol 100 ml

Mix and store.

Dissolve: 1:10 in order to obtain the working solution.

Colouration procedure:

1) Prepare the smears, dry in air and fixate by heating.;
2) Cover the glass with crystal violet solution for one minute;
3) Shortly wash out with trickling water;
4) Cover with Lugol's solution for one minute;
5) Wash out with trickling water and dry with filter paper;
6) Remove colour with 95 % ethanol, which is added drop by drop until all colour is removed or immerse smear for 30 seconds and lightly shake;
7) Wash out with trickling water and dry with filter paper;
8) Cover with solution of safranin for 10 seconds and
9) Wash out with trickling water and dry with filter paper.

Gramm positive bacteria are coloured violet-blue, and Gramm negative bacteria are coloured pink-red.

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ANNEX 2

MEASURES TAKEN IN SAFETY ZONE

1. Measures at places of production marked as contaminated:

1.1 On a lot marked as contaminated:

1.1.1 in the course of at least four growing years, after the year in which contamination was established:

- obligatory removal of wild-growing plants of potato and other host plants to harmful organism; and
- it is prohibited to plant potato tubers or plants, as well as to sow seeds in botanical sense, as well as sowing and planting of other plants hosts to harmful organisms or crops for which it is established that they enable spreading of harmful organism;

a) in the first season of potato production ensuing after the period from sub-item 1.1.1., on condition that on the plot for at least two consecutive years before planting, on occasion of systematic research, no wild-growing plant of potato nor other host plants, it is allowed to produce exclusively mercantile potato, with tuber testing in accordance with prescribed procedure;

b) ensuring after that form sub-item a), with corresponding crop rotation, which must be at least bi-annual, if one wants to produce seeds potato, production of seeds or mercantile potato is allowed, with conducting of systematic research is carried out in accordance with Article 3 of this rulebook or

1.1.2 in the course of four growing years ensuing after the year in which contamination was established:

- the measures of removal of wild-growing potato are carried out, as well as of all other host plants to harmful organism and
- plot is maintained as idle land or permanent pasture with intensive pasture or frequent low mowing,

In the first season of production of potato, ensuing after period from sub-item 1.1.2., on condition that during carrying out of systematic research, in at least two growing years prior to sowing, there were no wild-growing plants of potato found on the plot, nor other wild-growing host plants, it is allowed to produce seeds or mercantile potato, with testing of extracted tubers in accordance with the prescribed procedure;

1.2 At all other plots within the contaminated place of production, on condition that phytosanitary inspector established that danger from wild-growing plants of potato, as well as other harmful organism host plants:

1.2.1 in growing year ensuing after the year in which contamination is established:
- it is prohibited to plant potato tubers or plants and to saw potato seeds in botanical sense as well as to saw or plant other host plants to harmful organisms; or
- it is allowed to plant certified seeds potato aimed exclusively at production of mercantile potato;

1.2.2 it is allowed to plant only certified seeds potato or seeds potato established by official testing that it is not contaminated by a harmful organism and that it is produced under technical supervision at places of production which are not marked as contaminated, for seeds or mercantile production;

1.2.3 at least, still in the third year ensuing after established contamination it is allowed to plant only certified seeds potato or seeds potato produced under technical supervision from certified seeds potato, for seeds or mercantile production;

1.2.4 in every growing year from sub-items 1.2.1, 1.2.2 and 1.2.3 measures are taken to remove the wild-growing plants of potato and other host plants to harmful organism if they are present and official check of extracted potato form every lot is carried out in accordance with prescribed procedure.

1.3 Immediately after establishment of contamination with a harmful organism and after the first next growing year, all devices, equipment and storage premises in the place of production which were used in production of potato must be cleaned, disinfected in accordance with Article 11 of this rulebook.

1.4 In a greenhouse aimed at plant production where it is possible to fully replace the substitute for growing:
- it is prohibited to plant potato tubers or plants and to sow potato seeds in botanical sense, until in that greenhouse, under supervision of the phytosanitary inspector, measures are taken to destroy harmful organism and remove all plant material from host plants , while, as a minimum measure, complete replacement of substrate for growing is performed, as well as cleaning and disinfection of production unit and entire equipment and until the phytosanitary inspector approves production of potato; and
- it is allowed to plant exclusively certified seeds potato or mini-tubers or mini plants from culture of plant tissue, originating from tested sources.

2. Measures in entire safety zone:

2.1 Immediately after establishment of contamination, the phytosanitary inspector orders, if needed, cleaning and disinfection of all devices, equipment and storage premises on estates which were used in production of potato or tomato with application of corresponding procedures, as stated in Article 12 of this Rulebook.

2.2 Phytosanitary inspector shall, immediately and at least in the course of three growing years after the year in which contamination was established:
- supervise estates where potato tubers are grown, stored, found, or treated, including estates from which and at which devices were used for performing of activities related to the mentioned activities;
- order planting of certified seeds potato or seeds potato produced under technical supervision within entire safety zone, and testing of seeds potato after extraction, produced

in places of production which are considered probably contaminated, in accordance with Article 7 of this rulebook;

- order to handle at all estates within that area, the produced seeds potato separately from mercantile potato, or to perform cleaning, and if needed, disinfection between handling seeds ad mercantile potato;
- carry out systematic research referred to in Article 3 of this rulebook.

2.3 In case of need, the Phytosanitary Inspectorate can prepare programme of replacement of all stocks of seeds potato in appropriate time period.

2.4 2