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 BROWN ROT CAUSED BY BACTERIUM *Ralstonia solanacearum* (Smith) Yabuuchi et al.*

**(„Official Gazette of Montenegro" 67/10 from 24 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 brown rot, caused by bacterium ***Ralstonia solanacearum*** (Smith) Yabuuchi et al. (hereinafter: harmful organism), previously known as *Pseudomonas solanacearum* (Smith) Smith, 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 *Ralstonia solanacearum* 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 number of units of one variety of a plant, plant products and establishments under supervision, which can be identified by homogeneity of its contents and origin;
- **estate** means soil, facilities and means of transportation of the plant producer.

## II. SYSTEMATIC RESEARCH

### Systematic research
### Article 3

For the purpose of establishment of presence of the cause of potato brown rot, the 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 is adopted on the basis of clear scientific, statistical principles and harmful organism biology.

Systematic research referred to in Article of this Article shall be carried out on the following host plants (hereinafter: main host plants):

- on potato (*Solanum tuberosum* L.), including tubers, not including seeds in botanical sense;
- on potato (*Lycopersicon lycopersicum* (L.) Karsten ex Farw), not including fruits and seed.

For the purpose of establishment of other sources of contamination which can jeopardize production of main host plants, the Phytosanitary Administration performs assessment of risk from harmful organism.

If it is estimated that there is risk from spreading of harmful organism, in production areas of the main host plants, targeted and systemic researches are carried out:

- on other host plants, including wild-growing plants from family *Solanaceae;*
- in surface waters which are used for irrigation or sprinkling of main host plants;
- in liquid waste from industrial processing or plants for packaging of main host plants and it is used for irrigation or sprinkling of main host plants; i
- as needed, on substrates used for plant growth, soil, solid waste from industrial processing or plants for packaging of main host plants.

### Procedure of carrying out systematic research
### Article 4

In the course of carrying out the annual systematic research, or in case of suspected presence of harmful organism:

1) in case of potato:
    - in appropriate time periods, visual check is performed (by cutting a tuber) of the seed potato crops and potato which is not aimed at planting, i.e. mercantile potato, and if necessary, samples are taken for the purpose of laboratory testing, during vegetation period or from a storage;
2) in case of tomato:
    - in appropriate time periods during vegetation period, visual checks of plants aimed at planting are carried out (hereinafter: nursery plant) aimed at market production;
3) on other host plants, in surface waters, including liquid waste, as well as in other material, especially in substrates, soil and solid waste from industrial plants for processing and packaging:
    - samples are taken and submitted to the laboratory for testing.

Laboratory testing referred to in this article shall be carried out in accordance with standard procedures for diagnosis, detection and identification of bacterium *Ralstonia solanacearum* (Smith) Yabuuchi et al., given in Annex 1 which is printed to this Rulebook and makes its integral part.

Systematic research records
Article 5

Annual records are kept for procedures of carrying out and results of systematic research, for:

1) potato:
   - estimation of total area where potato is grown (seeds and mercantile) in hectares;
   - area, demonstrated separately for seeds (by category) and mercantile potato, and as needed, by areas;
   - number and time of taking samples aimed at testing;
   - number of visual checks on a plot during growing;
   - number of visual checks of tubers and size of a sample;
2) tomato:
   - estimation of total number of nursery plants aimed at market production of tomato;
   - number of visual checks and samples ;
3) Other host plants including wild-growing plants from family *Solanaceae*:
   - plant variety;
   - number of samples taken and time of sampling;
   - name of area or water from which samples are taken;
   - testing procedure;
4) surface waters and liquid waste from industrial processing and plants for packaging of main host plants:
   - number of samples taken and time of sampling;
   - name of area or water or location of estate from which samples are taken;
   - testing procedure.

For the purpose of further informing of European Union member states and the European Commission, information on details and results of systematic research referred to in paragraph 1 of this Article, carried out in the last production year are gathered and archived by 1st June of the current year, except for the potato kept for planting in own household, for which data must be gathered by 1st September of the current year.

III. SUSPICION OF CONTAMINATION

Establishment of suspicion of contamination
Article 6

Suspicion of contamination with a harmful organism exists if:

- on the occasion of visual checks symptoms of disease have been perceived, and result of screening tests is positive or
- by testing to latent contamination, a positive result was obtained by screening test

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

In case of suspicion of contamination with harmful organism referred to in paragraph 1 of this Article, laboratory is obliged to keep and store in a prescribed manner until obtaining of the final result of laboratory testing the following:

- all sampled tubers, if it is possible to sample all plants;
- all remaining extract and additionally prepared material for tests (e.g. imunofluorescence glasses etc.) and
- corresponding documentation.

On the basis of samples referred to in paragraph 3, indent 1 of this Article, in case of need, testing of varietal composition is carried out, in order to check identity of contaminated and clone related tubers and plants of potato.

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 3 of this Article;
- a sample of artificially contaminated potato and aubergine test plants and
- isolated culture of harmful organism.

Measures in case of suspicion of contamination
Article 7

Until gaining of final results of laboratory testing referred to in Article 6 paragraph 2 of this Rulebook, the phytosanitary inspector shall:

1) prohibit transfer of plants and tubers from all crops, lots and 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, and measures are applied especially in case of production of main host plants and transfer of lots of seed potato produced in the place of production from which samples referred to in item 1 of this paragraph have been taken

Notification in case of suspicion of contamination
Article 8

In case of suspicion of contamination with harmful organism and when there is risk from transfer of contamination form main host plants or by surface waters from Montenegro to other states, the Phytosanitary administration shall notify and establish cooperation with the body in charge of plant health protection of the respective state.

## IV. CONFIRMED CONTAMINATION

Procedure in case of confirmed contamination of main host plants
Article 9

If contamination with harmful organism is confirmed by laboratory testing of samples of main host plants, the phytosanitary inspector shall:

1) carry out research for the purpose of establishing the scope and primary source of contamination (one or several);
2) label as contaminated:
   - main host plants, consignment or lot from which sample has been taken;
   - devices, equipment, means of transport, warehouses and their parts, all other facilities and objects, including packaging material, which were in touch with main host plants from which sample has been taken and
   - places of production, lots, and, if necessary, greenhouses aimed at plant protection whether the sample was taken in time of growing, after extraction or picking of main host plants;
3) establish the scope of probable contamination;
4) establish safety zone on the basis of scope of confirmed contamination, scope of probable contamination and possible spreading of organisms, in which measures given in Annex 2 which is printed to this rulebook and makes it integral part.

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 f the contaminated lot and establishment of limits of contaminated plot and safety zone, for the production places, a lot, and when necessary, greenhouses aimed at plant production determined by the inspector to be contaminated.

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 *Ralstonia solanacearum* (Smith) Yabuuchi 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 and primary source of contamination of main host plants
Article 10

Phytosanitary inspector shall, on the occasion of carrying out research referred to in Article 9 paragraph 1 item 1 of this rulebook, for the purpose of establishing the scope of contamination and one or several primary sources of contamination of main host plants, take into account the following:

1) places of production:
   - where potato which is clone related with potato which is determined to be contaminated with harmful organism is produced or was produced;
   - where tomato which originates from the same source as tomato determined to be contaminated with harmful organism is produced or was produced;
   - where potato or tomato which are under supervision of the phytosanitary inspector because of suspicion of contamination with harmful organism are or were produced;
   - where potato which is clone related is or was produced with potato produced in the place of production where contamination with harmful organism was established;
   - where potato or tomato are produced, in the vicinity of contaminated places of production including places where same facilities and equipment for production were used;
   - where surface waters are used for irrigation or sprinkling from the source which is suspected of contamination or it has been established that it is contaminated by the harmful organism;

- where surface waters are used for irrigation or sprinkling from the same source which is used in the places of production where contamination was established or there is suspicion of contamination with harmful organism;
- which are or were flooded by surface waters in which contamination was established or there is suspicion of contamination with harmful organism

2) surface waters which are used for irrigation, sprinkling or which have flooded the lots or places of production at which contamination with harmful organism was established.

Research referred to in paragraph 1 of this Article includes further laboratory testing in accordance with procedures prescribed from Annex 2 of this rulebook, at least of all seed potato lots, which is clone related with contaminated potato.

Scope of probable contamination of main host plants
Article 11

Phytosanitary inspector shall establish for main host plants the scope of probable contamination referred to in Article 9 paragraph 1 item 3 of this rulebook, on the basis of possible contacts before, after and during production, irrigation or sprinkling or on the basis of clone relatedness with plants or tubers which are marked as contaminated, taking into account:

1) main host 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 main host plants which are marked as contaminated, including these places where same equipment and facilities are used for production;
3) main host plants which 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 main host plants which were marked as contaminated were present in places of production marked as contaminated;
4) estates where main host plants originating form the place of production referred to in items 1-3 of this Article are located or processed;
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 main host plants which are marked as contaminated;
6) all main host plants which were storage or in touch with some of facilities or objects referred to in item 5 of this paragraph prior to their cleaning and disinfection;
7) tomato plants originating form the same source as tomato which is marked as contaminated, i.e. tubers and plants of potato which are sister of parent clone-related to tubers and/or plants of potato which is marked as contaminated with harmful organism, and which , although results of testing carried out in accordance with prescribe procedures referred to in Annex 2 are negative, are considered probably contaminated, and as for tomato, because of the same source as tomato marked as contaminated. When necessary, test of varietal composition can be carried out in order to check identity of contaminated and clone-related tubers or plants of potato;
8) places of production of main host plants referred to in item 3 of this paragraph;
9) places of production of main host plants for which water marked as contaminated is used for irrigation or springing;
10) main host plants produced in lots which were flooded by surface water established to be contaminated with harmful organism.

Safety zone
Article 12

Phytosanitary inspector establishes safety zone referred to in Article 9 paragraph 1 item 4 of this rulebook, at proposal of the Phytosanitary Administration, on the basis of scope and source of primary contamination, scope of probable contamination and possible spreading of harmful organism.

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

- vicinity of other places of production of main host plants;
- joint production and joint use of stocks of seed potato;
- places of production at which surface water is used for irrigation or sprinkling of main host plants, where there is or was possibility of surface inflow of water from the places of production marked as contamination or the possibility of flood with water from the places of production which are marked as contaminated.

Procedure in case of contamination of other host plants
Article 13

If laboratory testing carried out in accordance with prescribed procedures referred to in Annex 2 to this rulebook confirm contamination with harmful organism in corps of other host plants in areas where production of main host plants is jeopardized, the phytosanitary inspector shall:

- perform research of main host plants in accordance with Article 10 of this rulebook;
- mark the contaminated host plants from which sample was taken;
- establish probable contamination and safety zone in accordance with Articles 11 and 12 of this rulebook

Procedure in case of contamination of surface waters
Article 14

If the laboratory testing carried out in procedures referred to in Annex 2 to this rulebook confirm presence of harmful organism in surface water (including liquid waste form industrial processing of main host plants or in plants for their packaging) or in wild-growing plants form family *Solanaceae* growing therein, and production of main host plants is jeopardized due to use of water for irrigation, sprinkling or due to floods, the phytosanitary inspector shall:

- take sample of surface waters and present wild-growing plants from family *Solanaceae* in appropriate time periods, for the purpose of establishment of the scope of contamination ;
- mark, in an extent which is large enough, as contaminated the surface water from which sample was taken, on the basis of analysis of sample referred to in indent 1 of this paragraph;
- establish probable contamination and safety zone on the basis of marked contamination referred to in indent 2 of this paragraph and possible spreading of harmful organism in accordance with Article 11 of this rulebook.

Possible spreading of harmful organism is established on the basis of:

- place of production of main host plants which are jeopardized by flood or which are bordering with surface water marked as contaminated;
- every individual area of irrigation which is connected to surface water which is marked as contaminated;

- water courses connected to surface water which is marked as contaminated, taking into account:
  a) direction and speed of the course of water marked as contaminated;
  b) presence of wild-growing host plants from family *Solanaceae.*

Notification in case of confirmed contamination
Article 15

In case of confirmed contamination with harmful organism, European Union member states and the European Commission are informed on the data regarding:

1) potato:
   - name of variety and lot,
   - purpose (mercantile or seed), namely the category of seed potato,
2) for tomato plant
   - name of variety and lot, namely category.

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 the main host plants, on the following:

- name of potato or tomato variety and lot,
- name and address of sender and recipient,
- due date of potato or tomato lot,
- size of delivered potato or tomato 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, distributor and warehouse keeper.

European Commission shall be reported on notification referred to in paragraph 2 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 and possible sources of contamination;
- on scope of marked contamination, including the number of places of production, and for potato, 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;
  - on contaminated water, including name and location of water and area where irrigation is prohibited;
  - phyto-cetificate and number of plant passport for all consignments or lots of tomato plants marked as contaminated,
  - and other data on confirmed sudden occurrence of harmful organism which may be requested by the European Commission.

V. MEASURES

Measures and procedures with contaminated main host plants
Article 16

Main host plants which are considered contaminated in accordance with Article 9 paragraph 1 item 2 indent 1 of this rulebook 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:

- use as animal food 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 filtration through soil pores to agricultural soil or by contact with water which could be used for irrigation or agricultural soil; or
- burning or
- industrial processing on condition that they are directly and immediately dispatched to the place of processing, where there must be officially approved 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 at least these vehicles which leave the place of processing; or
- other measures on condition there is no risk form 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 19 of this rulebook.

Measures and procedures with probably contaminated main host plants
Article 17

Main host plants which are considered to be probably contaminated by the harmful organism in accordance with Articles 11 and 14 paragraph 1 indent 3 of this rulebook, including the main host plants which are grown in places of production which are considered to be probably contaminated in accordance with Article 11 of this rulebook must not be planted; under supervision of the phytosanitary inspector they may:

1) in case of potato tubers:
   - 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
   - store appropriately, on condition there is no risk from harmful organism.
2) in case of other parts of main host plants, including stem and leaf remains:
   - destroy or
   - dispose in a prescribed manner, on condition there is no risk from spreading of harmful organism.

Cleaning and disinfection
Article 18

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 19

Disposal of waste arising in the process of industrial processing referred to in Article 14 of this rulebook shall be carried out so as to avoid any risk from spreading of harmful organism, as follows:

1) potato and tomato waste (including discarded potato, peel and tomato) and other solid potato and tomato-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 or by contact with water which might be used for irrigation of agricultural soil), also, 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.
2) liquid waste arising from processing containing 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 officially approved manner which makes it unable for the waste to come in touch with agricultural soil or water which may be used for irrigation of agricultural soil.

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

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

Measures in safety zone
Article 20

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

After completion of all researches, data on taken measures referred to in paragraph 1 of this Article shall be collected and archived, once a year, for the purpose of further reporting of the member states of the European Union and European Commission.

Procedure with seed potato
Article 21

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 of confirmed contamination in own production of seed potato:
   - on previous generations in the vegetative reproduction chain, including the initial phase of clone selection and systematic testing of clones of basic seed;
   - on all clones of basic seed or previous generations in vegetative reproduction chain, including plants from the initial phase of clone selection, when it is determined that there is no clone relatedness;
2) in other cases:
   - or on any plant which is in the initial phase of clone selection;
   - or on representative samples of basic seed;
   - on previous generations in vegetative reproduction chain.

Possession and use of harmful organism
Article 22

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

Exceptions
Article 23

Notwithstanding Article 22 of this rulebook, harmful organism may be possessed nor used solely for experiments, 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 24

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

VI. FINAL PROVISION
Article 25

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

No:
Podgorica, 16th November 2010

MINISTER

* Rulebook is harmonized with the Council Directive ***98/57/EC*** from 20th July 1998 on control of ***Ralstonia solanacearum*** (Smith) Yabuuchi et al.

**ANNEX I**

## TESTING SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION OF BACTERIUM *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*

### TESTING SCHEME APPLICATION AREA

Given testing scheme describes various procedures for:

- diagnosis of potato, tomato and other host plants brown rot;
- detection of bacterium *Ralstonia solanacearum* in samples of potato tubers,, potato plants, tomato and other host plants as well as in water and in soil;
- identification of bacterium *Ralstonia solanacearum (R. solanacearum).*

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 *R. Solanacearum* as control (reference) materials, the procedure must be carried out in prescribed quarantine conditions with corresponding facilities for waste disposal in accordance with this rulebook.

Testing parameters must ensure equalized and repeatable levels of detection of *R. solanacearum* 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.

Positive results obtained in the first quick check test (IF test, PCR/FISH, selective isolation) must be confirmed by another check test which is based on another biological principle.

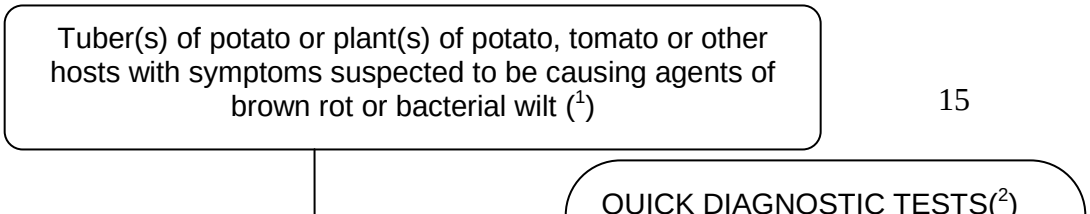
If result of the first check test (IF test, PCR/FISH) is positive, then presence of bacterium *R. Solanacearum* is suspected and the other check test must be carried out. If result of the second check test is positive, suspicion is corroborated and testing must be continued according to the testing scheme. If result of the second check test is negative, then it is considered that there is no bacterium *R. solanacearum* in the sample. 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 *R. solanacearum* as well as confirmation of pathogenicity.

## SECTION I
## APPLICATION OF TESTING SCHEME

**1) Scheme for detection and diagnosis of cause of potato brown rot (*R. solanacearum)* in potato tubers and plants of potato, tomato and other host plants with symptoms of potato brown rot.**

Testing procedure us aimed at tubers and plants of potato with symptoms typical for or indicating potato brown rot. Procedure included the quick check test, isolation of pathogen from contaminated conducting tissue to (selective) medium and in case of a positive result, identification of clear culture as bacterium *R. solanacearum*.


Tuber(s) of potato or plant(s) of potato, tomato or other hosts with symptoms suspected to be causing agents of brown rot or bacterial wilt ([1])
QUICK DIAGNOSTIC TESTS([2])

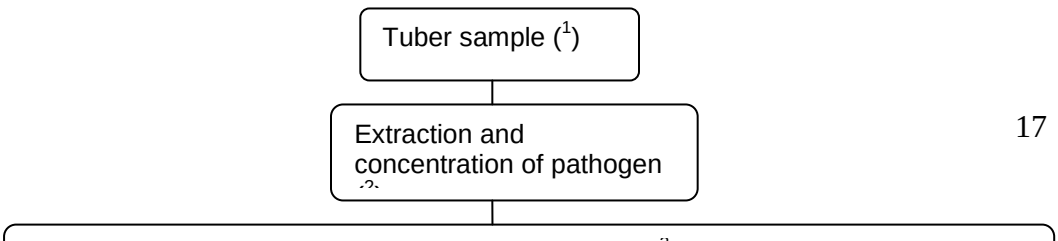
([1]) Description of symptoms is given in Section II. 1.
([2]) Quick diagnostic tests make easier establishment of probable diagnosis, but they are not necessary. Negative result does not always guarantee absence of pathogen
([3]) Bacterial exudate test from connective tissue of a plant is described in Section VI. A. 1.
([4]) Test for detection of poly-β-hydroxybutyrate in bacterial cells is described in Section VI.A.2.
([5]) Serological agglutination tests on bacterial exudate or exudate or extract from tissue with symptoms are described in Section VI.A.3.
([6]) IF test on bacteria exudate suspended in water or on extract from tissue with symptoms is described in Section VI.A.5.
([7]) FISH test on bacterial exudate suspended in water or on extract from tissue with symptoms is described in Section VI.A.7.
([8]) ELISA test on bacterial exudate suspended in water or on extract from tissue with symptoms is described in Section VI.A.8.
([9]) PCR test on bacterial exudate suspended in water or on extract from tissue with symptoms is described in Section VI.A.6.
([10]) Pathogen can usually be easily isolated from plant material with symptoms by method of diluting and isolation to nutritive medium (Section II.3.)
([11]) Typical colony morphology is described in Section II.3.d.
([12]) Growing of bacterial culture can be unsuccessful in an advanced stadium of infection because of competition or over-reproduction of saprophytic bacteria. If symptoms of illness are typical, and isolation test is negative, isolation test must be repeated, on a selective nutritive medium preferably.
([13]) Reliable identification of clean cultures of probable isolates of bacterium *R. Solanacearum* is achieved by carrying out of tests described in Section VI.B. Non-specific characterization is not obligatory but it is recommended for every new case.
([14]) Pathogencity test is described in section VI.C.

### 2). Scheme of detection and identification of bacterium *R. Solanacearum* in samples of potato tubers without symptoms

Principle:

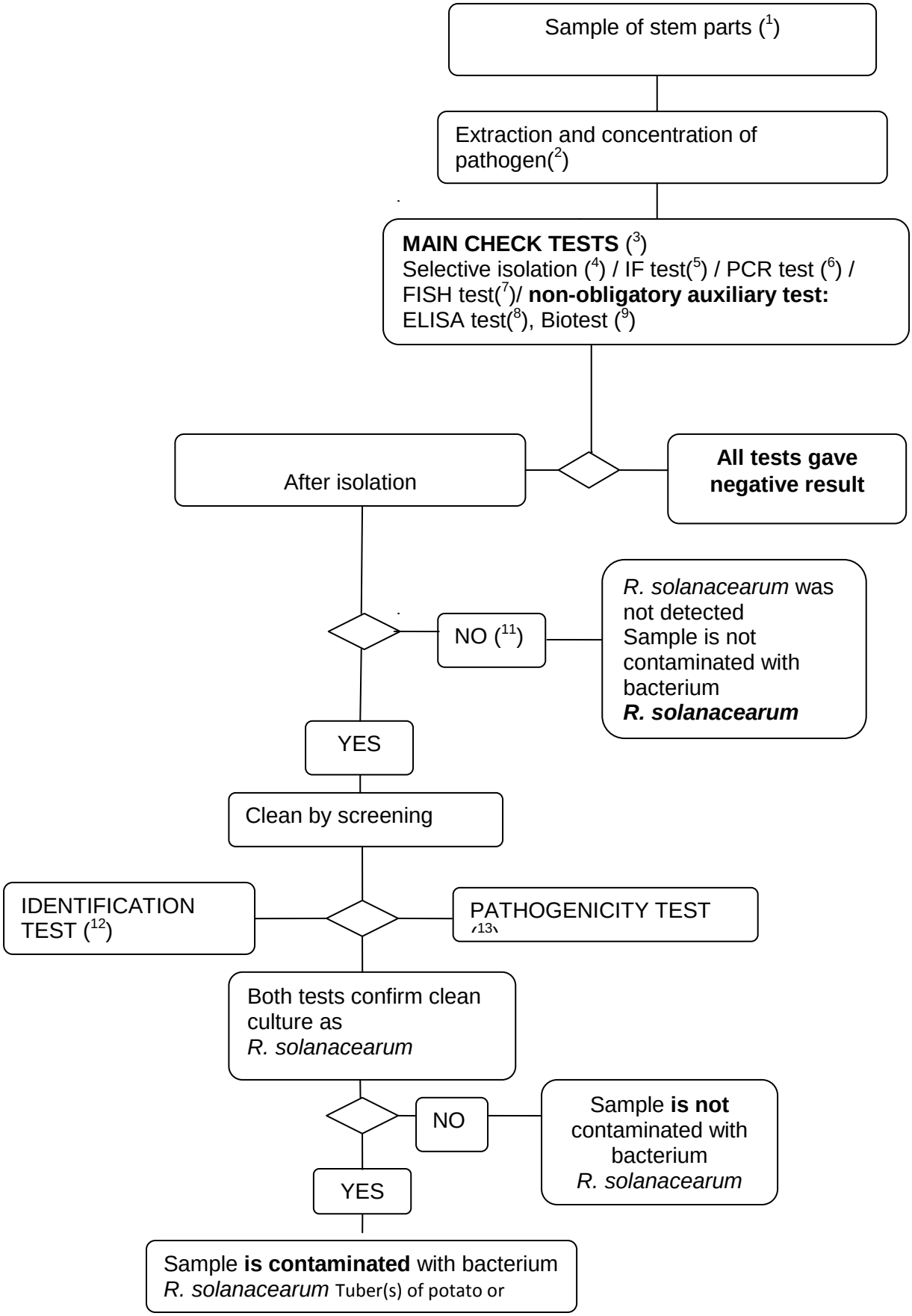
Testing procedure aimed at detection of hidden contamination in potato tubers. Positive result of at least three check tests ([3]), which are based on various biological principles must be supplemented by isolation of pathogen after which, in case of isolation of typical colonies, ensues confirmation of clean culture as *R. solanacearum.*

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 detection threshold 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

([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 Section III. 1.1.
([3]) If results of at least two tests, based on different biological principles are positive, it is necessary to perform isolation and confirmation. 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]) IF test is described in Section VI.A.5.
([5]) Selective isolation is described in Section VI.A.4.
([6]) PCR tests are described in Section VI.A.6.
([7]) FISH test is described in Section VI.A.7.
([8]) ELISA tests are described in Section VI.A.8.
([9]) Biotest is described in Section VI.A.9.
([10]) Typical morphology of a colony is described in Section II.3.d.
([11]) 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.
([12]) Reliable identification of clean cultures of probable isolates of bacterium *R. solanacearum i* is achieved by carrying out of tests described in Section VI.B.
([13]) Pathogenicity test is described in Section V

### 3). Scheme for detection and identification of bacterium *R. solanacearum* in samples of plants of potato, tomato or other host 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:
ELISA test(8), Biotest (9)
After isolation
All tests gave negative result
R. solanacearum was not detected
Sample is not contaminated with bacterium
R. solanacearum
NO (11)
YES
Clean by screening
IDENTIFICATION TEST (12)
PATHOGENICITY TEST
Both tests confirm clean culture as
R. solanacearum
NO
Sample is not contaminated with bacterium
R. solanacearum
YES
Sample is contaminated with bacterium
R. solanacearum Tuber(s) of potato or

([1]) For recommended size of samples Section III.2.1.
([2]) Methods of extraction and concentration of pathogens are described in Section III.2.1.
([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. 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 is described in Section VI.A.4.
([5]) IF test is described in Section VI.A.5.
([6]) PCR tests are described in Section VI.A.6.
([7]) FISH test is described in Section VI.A.7.
([8]) ELISA test is described in Section VI.A.8.
([9]) Biotest is described in Section VI.A.9.
([10]) Typical morphology of a colony is described in Section II.3.d.
([11]) 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.
([12]) Reliable identification of clear cultures of probable isolates of bacterium *R. solanacearum* is achieved by carrying out of tests described in Section VI.B.
([13]) Pathogenicity test is described in Section VI.C.

## SECTION II
## DETAILED METHODS FOR DETECTION OF BACTERIUM *R. SOLANACEARUM* IN POTATO TUBERS, PLANTS OF POTATO, TOMATO OR OTHER HOST PLANTS WITH SYMPTOMS OF POTATO BROWN ROT

### 1. Symptoms

1.1. Symptoms on potato

Potato plant

Initial phase of infection on a lot is recognized by withering leaves on the top of the plant at high temperatures during the day, from which it recovers during night. In early phases of withering, leaves remain green, but later on, it begins to become yellow and brown necrosis is developed. Epinasty occurs. Soon, irrecoverable withering of shoot or whole plant occurs, which leads to decay and dying away. Conductive tissue o horizontally cut stem of withered plant is usually brown and milky bacterial exudate drops or can be squeezed out from the cut. When the cut plant is put vertically in water, bacterial exudate pours out of conductive vessel.

Potato tuber

Potato tubers should be cut horizontally or vertically near tuber eyes. Initial phase of infection is recognized by change of colour of the conductive ring from glassy yellow to light brown, from which, after several minutes, pale creamy bacterial exudate appears. Later on, conductive tissue becomes browner and necrosis can be spread to parenchymal tissue. In advanced stages of illness, bacterial exudate can pour from sprout part and form eyes to which soil particles are stuck. On the peel, reddish-brown dimples can appear due to decay of conductive tissue on the inside. In advanced stages of illness, secondary development of fungal and bacterial soft rot is usual.

1.2. Symptoms on potato

Potato plant

First visible symptom is withering of the youngest leaves. In conditions which are favourable for pathogens (soil temperature around 25 °C, humidity saturation) in the course of several days, epinasty occurs as well as withering of only one side or entire plant, which leads to complete decay. In less favourable conditions (soil temperature under 21 °C) withering occurs more rarely, but a lot of adventive roots can be developed on the stem. It is possible to perceive water moist-like lines along the stem, form its basis, which are proof of necrosis in conductive system. If plant is cross-cut, from the conductive tissue the colour of which turned into brown, white and yellowish bacterial exudate drips.

1.3. Symptoms on other host plants

Plants *Solanum dulcamara* and *S. Nigrum*

In natural conditions symptoms of withering at these host plants are rarely perceived, except if soil temperature exceeds 25 °C or if levels of inoculum are extremely high (e.g. *S. nigrum* growing in the vicinity of ill plant of potato or tomato). If withering occurs, symptoms are the same as with tomato. Plants *S. Dulcamara* grow with stems and roots and waters and withering

is not visible on them, but on a horizontal section of the stem basis or parts of stem which are under water, light brown colour of connective tissue is visible. Bacteria can come out form cut connective tissue, in form of slimy exudate or fibres if we put the cut stem vertically in water, even if it does not demonstrate symptoms of withering.

**2. Quick check tests**

Quick check tests make easier establishment of probable diagnosis, but they are not crucial. Apply one or several following valid (approved) tests:

2.1. Stem exudate test ( Section VI.A.1.)

2.2. Detection of poly-β –hydroxybutyrate grains (PHB)

Characteristic PHB grains in cells of *R. solanacearum* become visible by coloration of thermally fixed spread of bacterial exudate from contaminated tissue of microscope glass by Nile blue A and Sudan black B . ( Section VI.A.2.).

2.3. Serological agglutination tests ( Section VI.A.3.)

2.4. Other tests

Other appropriate quick check tests include IF test (Section VI.A.5.), FISH test (Section VI.A.7.), ELISA tests (Section VI.A.8.) and PCR tests (Section VI.A.6.).

## 3. Isolation procedure

1) Take bacterial exudate or parts of discoloured tissue from the conductive ring of potato tuber or conductive vessels of a potato stem, tomato stem or stem of other host plants with withering symptoms. Suspend in a smaller quantity of distilled water or 50 mM phosphate buffer (Annex 4) and leave it 5 – 10 minutes.
2) Prepare a set of decimal suspension dissolutions.

3) Transfer 50-100 µl of suspension and dissolution to usual nutritive medium (NA, YPGA or SPA; explanation in Annex 2.) and/or on Kelman's tetrazolium medium (Annex 2.) and/or on a validated (approved) selective medium (e.g. SMS A; see Annex 2.) Spread, by application of appropriate technique of application of dissolution on medium. If it is necessary, prepare separate mediums with dissolved cell suspension of bacterium *R. solanacearum* biovar 2 for positive control.
4) Incubate mediums form two to six days on 28 °C.

   On usual nutritive medium, virulent isolates of bacterium *R. Solanacearum* create flat, irregular and liquid colonies of pearly creamy-white colour, often with characteristic spirals in the centre. Non-virulent forms of bacterium *R. Solanacearum* create small, round non-fluid (not liquid), greasy colonies which are entirely creamy-white.
   On Kelman's tetrazolium medium and on SMSA medium, spirals are blood red.
   Non-virulent forms of bacterium *R. Solanacearum* create small, round, non-fluid, greasy colonies which are entirely dark red.

## 4. Identification tests for bacterium *R. solanacearum*

Tests for confirmation of idenfiticaiton of *R. solanacearum* probable isolates are given in Section VI.B.

## SECTION III

### 1. Detailed methods for detection and identification of bacterium *R. solanacearum* in samples of potato tubers without symptoms

1.1. Preparation of a sample

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 correspondingly applied to samples with less than 200 tubers, when less tubers are available;
- validation of all detection methods, which are described in text below is based on testing of samples of 200 tubers;
- potato extract which is described in text below can be used for detection of bacterium *Clavibacter michiganensis subsp. sepedonicus* which causes potato tuber ring rot.

Non-obligatory processing before preparation of the sample:

(a) Incubate sample on 25-30 °C in the period up to two weeks prior to carrying out of tests, in order to stimulate reproduction of populations of bacterium *R. solanacearum.*

(b) 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.

1.1.1. With a clean and disinfected scalpel or knife for vegetables, remove crust form sprout part of tubers, so that conductive tissue is visible. Carefully cut a small cones part of conductive tissue (core) taking the less possible of surrounding, non-conducting tissue.

Note:

Separate all (rotten) tubers with possible brown rot symptoms and test them separately. If, on the occasion of removal of cones from sprout part (core of sprout) potential symptoms of brown rot are perceived, then tuber must be visually checked and cut near sprout. Every cut tuber with suspicious symptoms should be kept at least two days in the room temperature in order to suberize and keep at the temperature from 4 to 10 °C in appropriate quarantine conditions. All tubers, including these with suspicious symptoms are storaged in accordance with corresponding provisions of this rulebook.

1.1.2. Put sprout cores 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.

Process sprout cores with one of the following procedures:

a) add enough (around 40 ml) of extraction buffer (Annex 4) to cover sprout cores and ultracentrifuge at 50-100 turns/min, 4 hours at the temperature below 24 °C or 16 to 24 hours with cooling; or

b) homogenize sprout cores 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 adequate 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 smearing in the extraction procedure. For every sample, use fresh sterilized knives and blender receptacles. During the PCR 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.

1.1.3. Pour 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 buy vacuum filtration (40 to 100 µm) and additionally wash filter with the extraction buffer (around 10 ml).

1.1.4. 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.

1.1.5. Resuspend sediment in 1.5 ml buffer for sediment (Annex 4). Use 500 µl for testing *R. solanacearum,* 500 µl for *Clavibacter michiganensis subsp. sepedonicus* and 500 µl as reference material. Into reference aliquot (part) of 500 µl and remaining aliquots which are tested, added sterile glycerol by final concentration from 10 to 25 % (v/v), mix strongly in centrifugal blender and store at the temperature from –16 to –24 °C (weeks) or from –68 to –86 °C (months). During testing of aliquot (parts) which are used for establishment of presence of bacteria, keep a 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 All positive controls and samples of *R. solanacearum* must be separately prepared and processed in order to avoid contamination. That applies to immunofluorescence glasses and all other tests.

1.2. Testing

See the diagram, description of tests and optimal protocols in corresponding Annexes:
Selective isolation (See Section VI.A.4.)
IF test (See Section VI.A.5.)
PCR tests (See Section VI.A.6.)
FISH test (See Section VI.A.7.)
ELISA tests (See Section VI.A.8.)
Biotest (See Section VI.A.9.)

## 2. Detailed methods for detection and identification of bacterium *R. solanacearum* in samples of potato, tomato plants or other host plants without symptoms

2.1. Preparation of sample

Note:

For detection of latent populations of bacterium *R. solanacearum* testing of collective samples is recommended. Procedure can be suitably applied to collective samples from at most 200 parts of stems. If systematic researches are carried out, they must be based on a statistically representative sample of plant population which is being examined.

2.1.1. Put parts of stems of 1 to 2 cm in size in a closed sterile receptacle according to the following sampling procedures:

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

Tomato plants form a lot or greenhouse. With a clean disinfected knife cut the lowest side side shoots on every plant, cutting right above its conjunction with the stem. From every side shoot cut the lower part of 1 cm in size.

Other host plants. With a clean, disinfected knife or shears cut a part of 1 cm in size from lower part of every stem, just above the ground level. If *S. dulcamara* is sampled or other host plants growing in water, cut a part of 1-2 cm in size from the underwater part of plant or from the stolone with water roots.

When sampling at a certain location, it is recommended to test the statistical representative sample form at least 10 plants by sampling place for every variety of wild-growing plants which are potential hosts. Detection of pathogens is most reliable by the end of spring and during summer and autumn, although natural infections can be detected all year round at perennial plant *Solanum dulcamara* which grows in waterways. Well-known hosts are wild-growing potato plants (tubers remained in the ground), *Solanum dulcamara, S. nigrum, Datura stramonium* and other varieties of family *Solanaceae.* Other host plants are *Pelargonium* spp. and *Portulaca oleracea*. European varieties of wild-growing plants which can, in specific environmental conditions, be potential hosts of populations of *R. solanacearum* biovar 2 / strain 3 in roots and/or in rhizosphere are *Atriplex hastata, Bidens pilosa, Cerastium glomeratum, Chenopodium album, Eupatorium cannabinum, Galinsoga parviflora, Ranunculus sceleratus, Rorippa* spp, *Rumex* spp., *Silene alba, S. nutans., Tussilago farfara* and *Urtica dioica.*

Note:

At this phase, visual check of plants can be performed (coloration of fibres of bacterial exudate). Separate all parts of stems with symptoms and test them separately (See Section II.)

2.1.2. Shortly disinfect parts of stems with 70% ethanol and dry with absorbing paper. Process parts of stems with one of the following procedures:

a) cover them with sufficient quantity (around 40 ml) of extraction buffer (Annex 4) i 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.

2.1.3. After 15 minutes of sedimentation, decantate supernatant.

2.1.4. 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 Section III. 1.1.3. - 1.1.5.

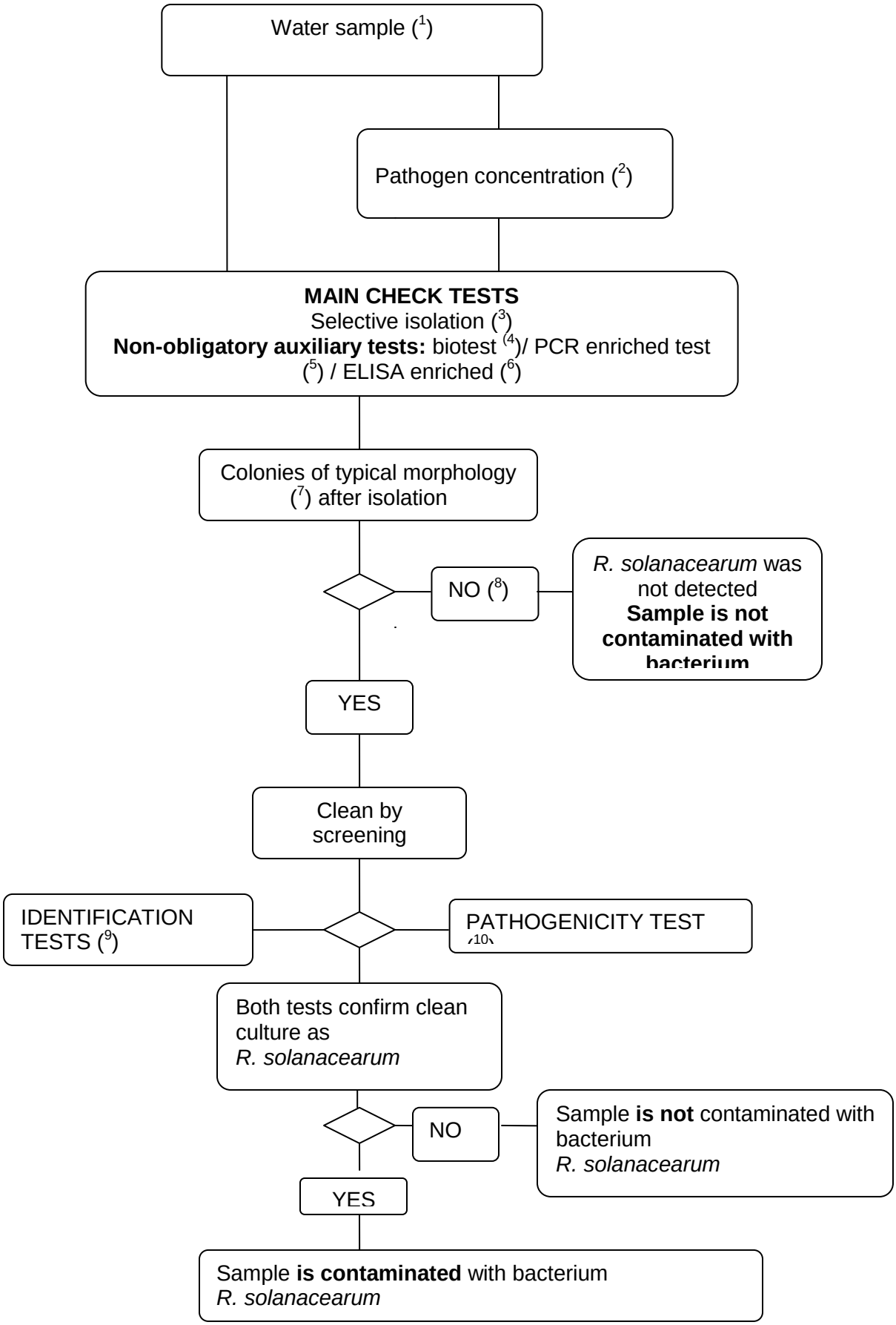
2.1.5. Divide clean or concentrated extract of sample into two equal parts. Keep one half in the temperature from 4 to 10 °C, and the other half with 10-25 % (v/v) of sterile glycerol in the temperature from -16 to - 25 °C (for weeks) or from - 68 to -86 °C (for months) in case further testing is necessary.

2.2. Testing
See diagram and description of tests and optimal protocols in corresponding annexes:

Selective isolation (See Section VI.A.4.)
IF test (See Section VI.A.5.)
PCR tests (See Section VI.A.6.)
*FISH test (*See *Section VI.A.7.)*
*ELISA tests (*See *Section VI.A.8.)*
*Biotest (*See *Section VI.A.9*.)

## SECTION IV

### 1) Scheme for detection and identification of bacterium *R. solanacearum* in water


Water sample ([1])
Pathogen concentration ([2])
**MAIN CHECK TESTS**
Selective isolation ([3])
**Non-obligatory auxiliary tests:** biotest ([4])/ PCR enriched test ([5]) / ELISA enriched ([6])
Colonies of typical morphology ([7]) after isolation
NO ([8])
*R. solanacearum* was not detected **Sample is not contaminated with bacterium**
YES
Clean by screening
IDENTIFICATION TESTS ([9])
PATHOGENICITY TEST ([10])
Both tests confirm clean culture as *R. solanacearum*
NO
Sample **is not** contaminated with bacterium *R. solanacearum*
YES
Sample **is contaminated** with bacterium *R. solanacearum*

([1]) For recommended procedures of sampling see Section IV.2.1.
([2]) Methods of concentration of pathogen are described in Section IV.2.1. By concentration, populations of both pathogens and competitive saprophytic bacteria are increased, and that procedure is recommended only if it will not inhibit isolation.
([3]) Selective isolation is described in Section VI.A.4.
([4]) Biotest is described in Section VI.A.9.
([5]) Enriching methods for PCR test are described in Section VI.A.4.2. and Section VI.A.6.
([6]) Enriching methods for ELISA test are described in Section VI.A.4.2. and Section VI.A.8.
([7]) Typical morphology of a colony is described in Section II.3.d.
([8]) Growing of bacterial culture can be unsuccessful because of competition or inhibition by saprophytic bacteria. If it is suspected that large populations of saprophytic bacteria will influence reliability of isolation, repeat isolation after sample is diluted in sterile water.
Reliable identification of clean cultures of probable isolates of bacterium *R. solanacearum* is achieved by application of tests described in Section VI.B.
([9]) Pathogenicity test is described in Section VI.C.

## 2. Methods for detection and identification of bacterium *R. solanacearum* in water

Principle

Valid (approved) procedure described in this section can be applied for detection of pathogens in samples of surface water as well as for examination of samples of liquid waste arising from processing of potatoes or waste waters. However, it is important to mention that expected sensitivity of detection will depend on substrate. Sensitivity of isolation is influenced by populations of competitive saprophytic bacteria which are mainly larger in waste waters arising from treatment and processing of potato than in surface waters.

Although it can be expected that by further described procedure only $10^3$ cells per litre of surface water will be detected, sensitivity of detection in liquid waste arising from processing of potato and in waste waters will be significantly lower. Therefore, testing of waste waters is recommended after every cleansing procedure (e.g. sedimentation or filtration) in the course of which populations of saprophytic bacteria are diminished. When reliability of obtained negative results is evaluated, limitations in sensitivity of testing procedure should be taken into account. Although this procedure is successfully applied in researches, the aim of which is to establish presence or absence of pathogens in surface water, one should be aware of its limitation if it is used in researches of liquid waste arising from processing of potato or waste waters.

2.1. Preparation of sample

Note:

- Detection of bacterium *R. solanacearum* in surface waters is most reliable in late spring and during summer and autumn when water temperature is above °C.
- Repeated sampling at various times during the above mentioned periods at certain sampling places increases reliability of detection because it impact of weather changes will be smaller.
- Impact of abundant precipitation and geographic properties of running waters should be taken into account in order to avoid significant impact of dilution which can hide presence of pathogens.
- Samples of surface water should be taken near host plants if there are any.

2.1.1. At chosen sampling locations, water samples should be taken in sterile tubes or single-use bottles, if possible at the depth bigger than 30 cm and at the distance of 2 m from the bank, at most. In case of liquid waste arising from processing of potatoes and waste waters, take samples at the place of their discharge. Recommended sample amount is 500 ml per sampling location. If smaller samples are wanted, it is recommended to take samples at least three times at every sampling location, while every sample consists of two sub-samples of at least 30 ml. Choose at least three sampling locations for intensive research, at every 3 km of running waters and ensure taking of samples from tributary streams of running waters.

2.1.2. Transport samples in dark (containers) at low temperatures (4 to 10 °C) and test them in the course of 24 hours from sampling.

2.1.3. Bacterial fraction may, if needed, be concentrated by one of the following methods:

(a) centrifuge 30 to 50 ml of sub-samples at 10 000 g 10 minutes (or 7 000 g for 15 minutes) at the temperature from 4 to 10 °C if possible, decantate supernatant and resuspend sediment (pellet) in 1 ml of sediment buffer (Annex 4.);

(b) perform membrane filtration (smallest pore size 0.45 µm), after which filter is washed out with 5 to 10 ml of sediment buffer, and after what is washed out. This method is convenient for larger quantities (volumes) of water containing a small number of saprophytes.

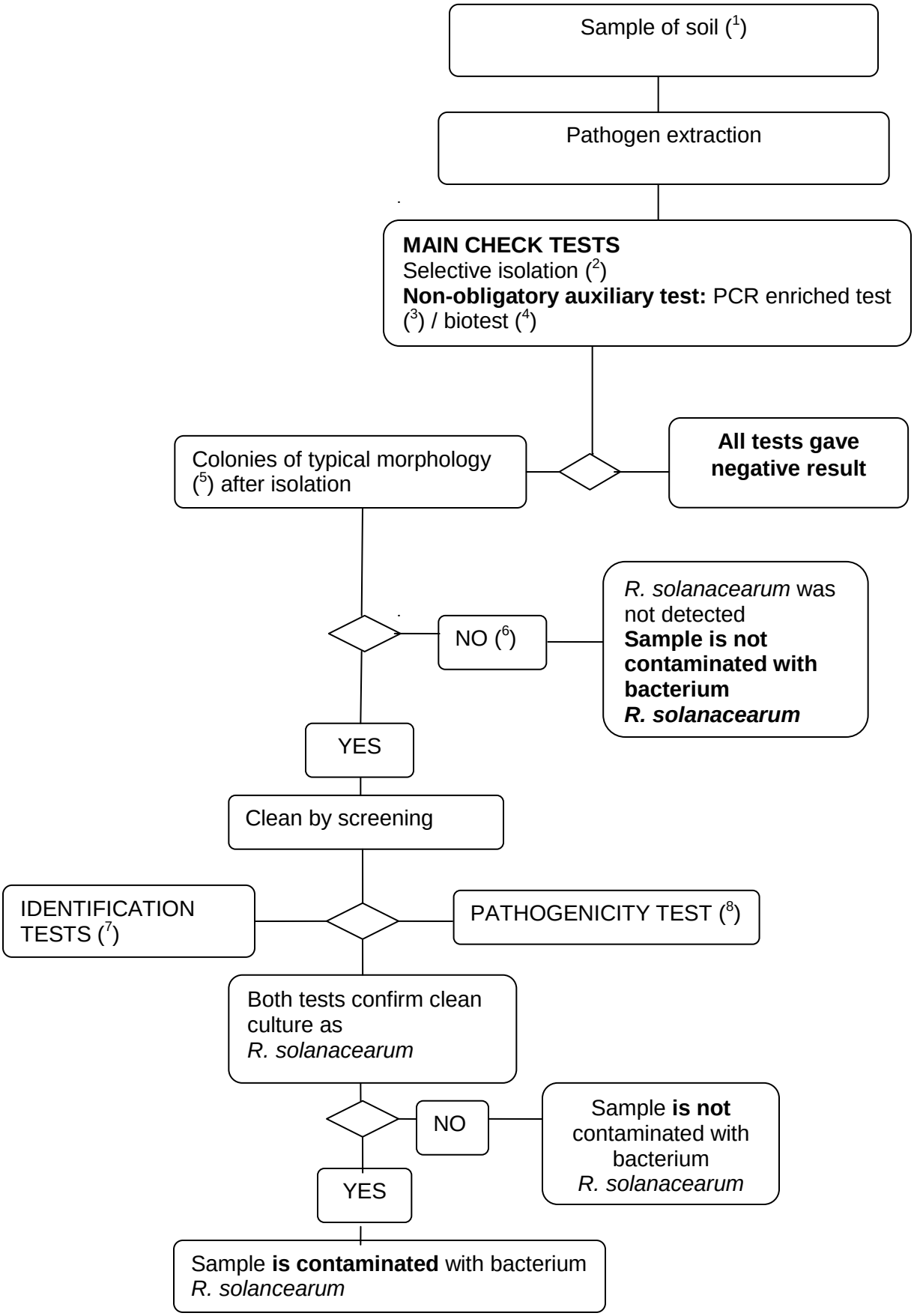
Concentrating of dilution is usually not recommended for samples of liquid waste (industrial) arising from processing of potato or for samples of waste waters, because larger populations of competitive saprophytic bacteria will hamper detection of bacterium *R. solanacearum.*

2.2. Testing

See diagram and description of tests and optimal protocols in corresponding annexes.

**SECTION V**

**1. Scheme for detection and identification of bacterium *R. solanacearum* in ground**


Sample of soil (1)
Pathogen extraction
MAIN CHECK TESTS
Selective isolation (2)
Non-obligatory auxiliary test: PCR enriched test (3) / biotest (4)
Colonies of typical morphology (5) after isolation
All tests gave negative result
NO (6)
R. solanacearum was not detected
Sample is not contaminated with bacterium R. solanacearum
YES
Clean by screening
IDENTIFICATION TESTS (7)
PATHOGENICITY TEST (8)
Both tests confirm clean culture as R. solanacearum
NO
Sample is not contaminated with bacterium R. solanacearum
YES
Sample is contaminated with bacterium R. solancearum

([1]) For recommended procedures of sampling Section IV.2.1.
([2]) Selective isolation is described in Section VI.A.4.
([3]) Enriching methods for PCR test are described in Section VI.A.4.2. and Section VI.A.6.
([4]) Biotest is described in Section VI.A.9.
([5]) Typical morphology of a colony is described in section II.3.d.
([6]) Growing of a bacterial culture can be unsuccessful due to competition or inhibition of saprophytic bacteria. If it is suspected that large populations of saprophytic bacteria will influence reliability of isolation, repeat isolation right after you additionally dilute the sample.
([7]) Reliable identification of clean cultures of probable isolates of bacterium *R. solanacearum* is achieved by application of tests described in Section VI.B.
([8]) Pathogenicity test is described in Section VI.C.

## 2. **Methods for detection and identification of bacterium *R. solanacearum* in ground**

Principles:

Valid procedure described in this section can be applied to detection of pathogens in samples of soil as well as for examination of solid waste samples arising from processing of potatoes or sewerage sludge samples. However, it is important to mention that these methods are not sensitive enough to guarantee detection of smaller and/or unequally distributed populations of bacterium *R. solanacearum* which can be naturally present in samples of these substrates.

We should take into account limitations in sensitivity of this testing procedure while evaluating reliability of obtained negative results as well as when we apply this procedure in researches aimed at establishing presence or absence of pathogens in soil or in sludge. The most reliable method of establishment of presence of pathogens in soil on a plot is to plant the host plant which is sensitive to that pathogen and monitor whether infection will occur, but neither with this method will we discover low levels of contamination.

### 2.1. Preparation of sample

2.1.1. Sampling of soil from a lot should be performed in accordance with basic principles which are applied at sampling for examination of nematodes. From 60 locations at every 0.3 ha take 0.5 to 1 kg of soil per sample, from depth from 10 to 20 cm (or on a 7 x 7 meter web). If presence of pathogen is suspected, increase the number of sampling locations t o`120, at every 0.3 ha.

Prior to testing, keep the samples at temperature from 12 to 15 °C. Sampling of solid waste arising from processing of potatoes or sampling of sewerage sludge, perform by taking totally 1 kg at places which represent total quantity of sludge which should be tested. Mix every sample well before testing.

2.1.2. Shake sub-samples of 10 to 25 g of soil or sludge on a rotational mixer (250 turns/min) in 60 to 150 ml of extraction buffer (Annex 4) for two hours at the longest. Adding of 0.02% sterile Tween -20 and 10 to 20 g of sterile sand can, if needed, contribute to loosening.

2.1.3. Maintain the suspension at the temperature of 4 °C during testing.

### 2.2. Testing

See diagram and description of tests in corresponding annexes.

## SECTION VI

## OPTIMAL PROTOCOLS FOR DETECTION AND IDENTIFICATION OF *R. SOLANACEARUM*

A. DIAGNOSTIC TESTS AND DETECTION TESTS

### 1. Testing of bacterial exudate from stem

Probable presence of *R. solanacearum* in stems of withered potato, tomato and other host plants stems can be established by the following simples test: cut stem just above the ground level. Put the cut part into a tube with clean water. Observe whether, after several minutes, characteristic fibres of bacterial exudate will begin to come out of cut conductive sheaves spontaneously.

### 2. Detection of poly-β –hydroxybutyrate (PHB) grains

1. On a microscopic glass, prepare smear of bacterial exudate from infected tissue from a 48-hour culture from nutritive medium YPGA or SPA (Annex 2).
2. For positive control, prepare smears of biovar 2, bacterium *R. solanacearum* and if you find it useful, for negative control, a smear of bacterial type which is known to be negative to PHB.
3. Leave the smears to dry in air and pull rapidly the bottom part of every glass above fire, so that smears are fixed.
4. Colour the preparation with Nile blue or Sudan black and watch under microscope as described in text below.

Test with Nile blue colour:

a) Cover every glass with 1% water solution of Nile blue colour A and incubate for 10 minutes at 55 °C.
b) Drain colour solution. Wash out shortly under a light tap water trickle. Remove excessive water with absorbing paper.
c) Cover smear with 8 % water solution of vinegar acid and incubate one minute at room temperature.
d) Wash out shortly under a light tap water trickle. Remove excessive water with absorbing paper.
e) Moist again with a drop of water and cover with cover glass.
f) Check the coloured smear under an epifluorescence microscope at 450 nm, with immersion objective magnification from 600 to 1000 x (oil or water immersion).
g) Check whether there is light orange fluorescence of PHB grains. Also, check the glass under normal light to confirm presence of PHB granules in cells and whether cell morphology is typical of bacterium *R. solanacearum*.

Test with Sudan black colour:

(a) Cover every glass with 0.3% water solution of Sudan black B in 70 % ethanol and incubate for 10 minutes at room temperature.
(b) Drain water solution, shortly wash under a tap water trickle and remove excessive water with absorbing paper.

(c) Dip glasses shortly into xylol and dry them on the absorbing paper. Caution! Xylol is dangerous for health. Take necessary safety measures and work in a digestor.

(d) Cover glasses with 0.5 % (w/v) water solution of safranin and leave it at the room temperature for 10 seconds. Caution! Safranin is dangerous for health. Take necessary safety measures and work in a digestor.

(e) Wash under a light tap water trickle, dry on absorbing paper and cover with cover glass.

(f) Check coloured smears under microscope using transient light, oil immersion objective with magnitude 1000 x.

(g) Check whether there are blue-red grains of PHB in cells of *R solanacearum* with cell walls coloured pink.

**3. Serological agglutination test**

Agglutination of cells of bacterium *R. solanacearum* in bacterial exudate or extracts of tissues with symptoms is best perceived by use of confirmed and approved antibodies ( Annex 3) marked with corresponding coloured labels such as red cells of *Staphylococcus aureus* or coloured latex particles. If set available in the market is used ( Annex 3), follow the producer's instructions. Otherwise, apply the following procedure:

(a) mix drops of suspension of the marked antibody and bacterial exudate (around 5 µl of each) on microscope glasses with several openings

(b) prepare positive and negative controls by using suspensions of *R. solanacearum* biovar 2 and heterologous strain

(c) observe whether agglutination occurs in positive samples after 15 seconds of light mixing.

**4. Selective isolation**

4.1. Isolation on selective medium

Note:

Prior to application of this method, perform the necessary tests to ensure repeatable detection of $10^3$ to$10^4$ colony forming units (CFU) of bacterium *R. solanacearum* per ml, which are added to extracts of samples which are negative, according to previous testing. Use the confirmed and approved selective nutritive medium such as SMSA (adjusted by Elphinstone et al., 1996; Annex 2). On the occasion of check of sown nutritive plant, pay attention to distinguishing *R. solanacearum* from other bacteria whose colonies can grow on that nutritive medium. Namely, colonies of *R. solanacearum* can be of atypical morphology if there is an intense increase in bacteria on the medium or if there are antagonistic bacteria present. If it is suspected that negative impact of competition or antagonism occurred, sample should be tested again with another test.

Greatest sensitivity of detection by this method can be expected if fresh prepared extracts of samples are used. However, this method can be applied on extracts which were kept with glycerol at the temperature from– 68 to – 86 °C. for positive control, prepare decimal dilutions of suspension of $10^6$ CFU/ml of virulent strain biovar 2*, R. solanacearum* (e.g. NCPPB 4156 = PD 2762 = CFBP 3857). In order to prevent every possibility of contamination, prepare positive controls completely separately from samples which are examined. For every newly prepared series of selective nutritive medium, before it is used for testing of routine samples, check

whether it is suitable for growth of pathogens. Test the control material in the same manner as samples.

4.1.1. Apply the corresponding technique of application of dilution in order to make sure that all the populations of saprophytic bacteria are diluted enough. Apply 50 – 100 µl of sample extract by medium and by every dilution.

4.1.2. Incubate mediums on 28 °C. Read mediums after 48 hours, and after that, every day in the period up to six days. Typical colonies of *R. solanacearum* on nutritive medium SMSA are milky white, flat, of irregular shape and watery and after three days of incubation their centre becomes pink to blood-red with internal lines and spirals.

Note:

Sometimes, atypical colonies of *R. solanacearum* grow on this nutritive medium. They can be small, round, completely red and non-watery or only partly watery, and that is why it is difficult to distinguish them from colonies of saprophytic bacteria.

4.1.3. Colonies which are considered to be *R. solanacearum* colonies should be transferred to usual nutritive medium and cleaned in order to obtain individual separate colonies ( Annex 2).

4.1.4. Cultures can be briefly kept in sterile water (pH 6 to 8, without chlorine) in room temperature in the dark , or for a longer period in the temperature from– 68 to – 86 °C or lyophilized.

4.1.5. Identify probable cultures (Section VI.B) and carry out the pathogenicity test (Section VI.C).

Interpretation of selective isolation results

Selective isolation is negative if six days after there are no bacterial colonies perceived or if no suspicious colonies typical of *R. solanacearum* are found, on condition that there is no suspicion of inhibition due to competition or antagonism of other bacteria and that typical colonies of *R. solanacearum* are found in positive controls. Selective isolation is positive if suspicious colonies of *R. solanacearum* are isolated.

4.2. Enrichment procedure

Use valid (confirmed and approved) medium for enrichment such as modified Wibrink liquid medium (Annex 2). This procedure can be applied for selective increase in population of *R. solanacearum* in extracts of samples and to increase sensitivity of detection. by this procedure, also, inhibitors of PCR reaction are efficiently diluted (1:100). However, it should be mentioned that enrichment of *R. solanacearum* can be unsuccessful due to competition or antagonism of saprophytic organisms which are often enriched at the same time. Because of that, it can be hard to isolate *R. solanacearum* from cultures enriched in liquid medium. In addition, since populations of serologically related saprophytes can be increased, it is recommended to use specific monclonal antibodies instead of polyclone antibodies if ELISA test is going to be carried out.

4.2.1. For enrichment for PCR test, transfer 100 µl of sample extract into 10 ml of liquid medium for enrichment (Annex 2) which has previously been divided in aliquots in tubes or bottles without DNA. For enrichment for ELISA test, bigger parts of sample extract in liquid medium can be used (e.g. 100 µl in 1,0 ml of liquid medium for enrichment).

4.2.2. Incubate for 72 hours at temperature from 27 to 30 °C with or without shaking, with loose cap (so that it is not stiffly sealed), to enable breathing.

4.2.3. Mix well prior to use in ELISA or PCR tests.

4.2.4. Handle enriched liquid medium equally as samples in previously described tests.

Note:

If it is expected that enrichment of *R. solanacearum* will be unable due to high populations of certain competitive saprophytic bacteria, better results can be achieved if sample extracts are enriched prior to centrifuging or other concentration procedures.

**5. IF Test principle**

Use of IF test as basic check test is recommended due to its proven consistency in achievement of required detection thresholds. When IF test is used as the main check test and if IF reading is positive, isolation must be carried out, PCR test or FISH test 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:

Use valid (confirmed and approved) antibodies for *R. Solanacearum.*

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 bacterium *R. solanacearum* with use of Fluorescein isothiocyanate (FITC) conjugated antibodies according to producer's instructions. All valid (confirmed and approved) polyclone serums had an IF titre of at least 1: 2000. During the testing, antibodies should be used in working dilutions which are close or equal to title. 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 buffer for dilution of sediment (Annex 4), repeated 15 minute centrifuging at 7000 g and resuspending in equal volume of buffer for sediment (pellet buffer). That is rarely necessary, especially if samples are fixed to glasses by flame. For positive control, prepare separate glasses with homologous strain or some other reference strain of bacterium *R. solanacearum* suspended in potato extract, as given in Annex 3B 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, aliquots (parts) of sample extract can be used which proved negative result in previous testing to *R. solanacearum.* In Annex 3, Standardized positive and negative control materials which can be used in this test are given. 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.

5.1. Prepare 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.

5.2. 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 storage frozen in a desiccators.

5.3 IF procedure

(i) In accordance with the procedure for preparation of glasses for testing, which is described under 5.1.(i): Prepare a series of double dilutions of antibodies. First opening has to have 1/2 titre (T/2), and others 1/4 titre (T/4), 1/2 titre (T/2), titrer (T) and double titre (2T).

(ii) In accordance with the procedure for preparation of glasses for testing which is described under 5.1.(ii): Prepare working dilution of antibodies in IF buffer. Working dilution affects specificity.

Figure1.
Preparation of glasses in accordance with paragraphs 5.1.(i) and 5.3.(i)

Dilution of resuspended sediment

| | **1/100** | **1/1** | **1/1** | **1/1** | **1/1** □ dilutions of resuspended sediment |
|---|---|---|---|---|---|
| (T=titar) | T/2 | T/4 | T/2 | T | 2T □ double dillutions of serum/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. (ii) and 5.3.(ii)

**Working dilutions of serums/antibodies**

| 1/1 | 1/10 | 1/100 | empty | empty | □decimal dilutions of dissolved sediment |
|---|---|---|---|---|---|

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

5.3.1. 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:

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

5.3.3. Dry glasses carefully with absorbing paper. Shake drops off every glass and carefully wash them out with IF buffer. Submerge them for 5 minutes in IF buffer-Tween (Annex 4) and after that in IF buffer. Take care that aerosol is not created and that there is no transfer of drops because that could result in cross-contamination.

5.3.4. Line up glasses on moist absorbing paper. Cover openings with diluted FITC conjugate which is used for titre determination. Volume of conjugate applied to openings must be equal to volume of applied antibody.

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

5.3.6. Shake off the conjugate drops from glasses. Wash out and wash as previously described (5.3.3). Carefully dry glasses.

5.3.7. Apply to every opening with a pipette 5-10 µl 0.1 M glycerol with phosphate buffer (Annex 4) or means against discoloration which is available in the market and put the cover glass.

5.4. Reading of IF test

5.4.1. Check the prepared glasses under epifluorescence 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 which 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 coloured on the established titre of antibodies or working dilution. In case that there is no aberration with colouration, IF test must be repeated (Section VI.A.5).

5.4.2. Check whether there are clearly visible fluorescent cells with morphology characteristic of bacterium *R. solanacearum* in the openings

Intensity of fluorescence has to be equal 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.

5.4.3. There are several problems regarding specificity of the immunofluorescence test. In concentrated extract of isolated tuber sprouts cones or parts of stem, 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 *R. solanacearum.*

5.4.4. Take into account only the fluorescent cells of typical size and morphology in titre or working dilution of antibodies as described in paragraph 5.3

5.4.5. 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 5). Reading of IF test is positive for samples which have at least $5 \times 10^3$ 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 $5 \times 10^3$ cells per ml of resuspended sediment and sample is considered negative. No further testing is necessary.

## 6. PCR Tests

Principles

When PCR test is used as main check test and result is positive, IF test must be carried out or 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 of cells of bacterium *R. solanacearum* 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 optimization (standardization) of the method. Use valid (confirmed and approved) reagents and protocols for PCR (Annex 6). 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 *R. solanacearum*
- buffer used for extraction of bacteria and DNA from the sample
- Reaction mix for PCR.

The following positive controls should be included:

- aliquots of resuspended sediments to which bacterium *R. solanacearum* is added (for preparation, Annex 3B).
- suspension in water of $10^6$ cells/ml of bacteria *R. solanacearum* virulent isolate (e.g.
- NCPPB 4156 = PD 2762 = CFBP 3857; Annex 3 B).

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. Sample extract must contain the less possible soil. If there is intention to apply the PCR protocol, in some case it would be advised to wash tubers before extract preparation. In Annex 3, Standardized positive and negative control materials which can be used in these tests are given.

### 6.1. Methods of DNA cleansing

Use samples for positive and negative control as previously described (Annex 3). Test the control material in the same manner as samples. There are various methods for cleansing of targeted DNA from complex samples, by which inhibitors of PCR reaction and other enzyme reactions and methods of concentration of targeted DNA in sample extract are removed.

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

**a) Method by Pastric (2000)**

1) Measure with a pipette 220 µl for buffer lisis [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 for 30 minutes at 37 °C5) Add 220 µl Easy DNA® solution A (Invitrogen), mix well on vortex i.e. centrifugal mixer and incubate for 30 minutes at 65 °C.
5) Add 100 µl Easy DNA® solution B (Invitrogen), mix roughly on centrifugal mixer (vortex) until achievement of uniform viscosity of the sample.
6) Add 500 µl of chloroform and mix on centrifugal mixer (vortex) until viscosity is reduced and mixture becomes homogenous.
7) Centrifuge 15000 g for 20 minutes at 4 °C do divide phases and create interphase.
8) Transfer the upper phase to the new Eppendorf tube.
9) Add 1 ml 100 % ethanol (- 20 °C), shortly stir on centrifugal mixer (vortex) and incubate on ice for 10 minutes.
10) Centrifuge at 15000 g for 20 minutes at 4 °C and remove ethanol from sediment.
11) Add 500 µl 80 % ethanol (- 20 °C) and stir by turning of the tube.
12) Centrifuge at 15000 g for 10 minutes at 4 °C, retain sediment, remove ethanol.
13) Leave the sediment to dry on air or in vacuum centrifuge (DNA speed vac).
14) Resuspend sediment in 100 µl of sterile ultra clean water and leave at room temperature for at least 20 minutes.
15) Keep at - 20 °C until it is needed for PCR.
16) Separate possible white precipitate (sediment) by centrifuging and use for PCR 5 µl of supernatant containing DNA.

**(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 valid (approved) protocol (Section VI.A.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). It is recommended to use, if possible, the protocol for PCR multiplex which includes also the internal PSR contorl.

6.2.3. According to the protocol for PCR (Annex 6) add 2-5 µl of DNA extract on 25 µl of reaction mixture in sterile tubes for PCR.

6.2.4. Include the sample for negative contorl which controls 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 60 minutes taking adequate precaution measures for work with this mutagen.

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

6.3.4. For every new finding/case, check the authenticity of multiplied PCR of the product, by restrictive 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 agarose gel, as previously mentioned, and after colouring with ethidium bromide on an UV transilulminator, observe the characteristic UV pattern 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 *R. solanacearum* 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 *R. solanacearum* of expected length and expected pattern and rescstricition 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 these wit another pair of PCR primers (Annex 6)

Note:

It can be suspected that inhibition of PCR reaction occurred if expected product is obtained from the positive control sample containing *R. solanacearum* in water, and negative results are obtained from positive controls with *R. solanacearum* in potato extract. In multiplex PCR protocols with internal controls, inhibition of reaction is indicated if none of the two products is obtained. If expected product is obtained from one or several negative controls, one can suspect that contamination has occurred.

**7. 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 or isolation as the other 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 (confirmed and approved) olygo-trials specific for bacterium *R. solanacearum* (Annex 7). Preliminary testing with this method should enable a repeatable detection of at least $10^3$ to $10^4$ cells of bacterium *R. Solanacearum* per ml which are added to sample extracts which proved negative in previous testings. The following procedure is best 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 aliquotas (parts) of sample extract which were negative in the previous testing to *R. solanacearum.*

For positive control, prepare suspensions containing at least $10^5$ to $10^6$ cells/ml 0.01 M phosphate buffer (PB) of bacterium *R. solanacearum* biovar 2 (e.g. strain NCPPB 4156 = PD 2762 = CFBP 3857; Annex 3) from a culture which is 3 – 5 days old. Prepare separated glasses for positive control with a homologues strain or some other reference strain of bacterium *R. solanacearum* solved in potato extract, as given in Annex 3B. Use of ecubacteria olygo-check marked with FITC enables contorl of hybridization process, because all the ecubacteria present in the sample will be coloured. In Annex 3A, the Standardized positive and negative control materials which can be used in this test are given. Test the contorl material in the same manner as samples.

7.1. Fixation of potato extract

The following protocol is based on Wullings et al. (1998):

7.1.1. Prepare solution for fixation ( Annex 7).

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

7.1.3. Remove the supernatant and solve sediment in 200 µl of fixative prepared 24 hours earlier, at most. Stir on centrifugal mixer and incubate one hour in a refrigerator.

7.1.4. Centrifuge for 7 minutes at 7000g, remove the supernatant and resuspend the sediment in 75 µl 0,01 M PB (Annex 7).

7.1.5. Apply 16 µl of fixed suspensions in openings of a clean glass as demonstrated in figure 7.1. 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 7.1. Glasses for FISH test

<table>
<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 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>

7.1.6.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.

7.2. Hybridization

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

7.2.2. 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 hybridization device for at least 10 minutes at 45 °C.

7.2.3. Apply 10 µl of hybridization solution (Annex 7) in each of eight openings (openings 1, 2, 4, 5, 6, 7, 9 and 10; image 7.1.) of every glass, and leave the two central openings (3 and 8) empty.

7.2.4. Cover first and last four openings with a cover glass (24 x 24 mm), taking care not to leave any air in openings. Leave the glasses in heated wet chamber and hybridize for five hours in hybridization device at 45 °C in the dark.

7.2.5. Prepare three receptacles with q L of ultra clean water (for use in molecular biology), 1 L 1x hybmix (334 ml 3x hybmix and 666 ml of ultra clean water) and 1 L 1/8x hybmix (42 ml 3X hybmix and 958 ml of ultra clean water) and 1 L 1/8x hybmix (42 ml 3X hybmix and 958 ml of ultra clean water). Primarily, incubate every receptacle in a water bath at 45 °C.

7.2.6. Remove cover glasses, and place the glasses concerned on a holder.

7.2.7. Washout the surplus trial by incubation for 15 minutes at 45 °C in a receptacle with 1x hybmix.

7.2.8. Transfer glass holder in washing solution (1/8x hybmix) and incubate for 15 minutes more.

7.2.9. Shortly immerse glasses in ultra clean water and place them on filter paper. Remove excessive moist by covering the surface lightly with 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 microscope glass with a big cover glass (24 x 60 mm).

7.3. FISH test reading

7.3.1. 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 gram negative cells) in the sample, show as fluorescent green. By use of filter for tetrametilrodamin-5-izotiocianat , cells of bacterium *R. solanacearum* colored with Cy3, show as fluorescent red. Compare cell morphology with positive controls morphology. Cells must be clearly fluorescent and entirely coloured. If there is divergence in coloration, FISH test (Section VI.A.7) must be repeated. Check every opening vertically and horizontally under normal angle and along the outer edge. Fore samples in which a small count of cells is visible or there are not any, check at lest 40 microscopic fields of view.

7.3.2. Check whether there are clearly visible fluorescent cells of morphology characteristic of bacterium *R. Solanacearum.*

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

7.3.3. 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.

7.3.4. There are several problems connected to specificity of the FISH test. In concentrated extract of separated tuber sprouts cones of tubers or stem parts, populations of fluorescent cells of atypical morphology and saprophytic\ bacteria which are similar to bacterium *R. solanacearum* by size and morphology, although more rarely than with an IF test.

7.3.5. Take into account only the fluorescent cells of typical size and morphology.

7.3.6. Interpretation of the FISH test results.

(i) FISH test results are considered valid if, by application f a FITC filter green fluorescent cells are perceived, size and morphology of which is typical of bacterium *R. solanacearum* and if by application of rhodamine filter, red fluorescent cells are perceived in all positive controls and in none of the negative controls.

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 $5 \times 10^3$ of typical cells per ml of resuspended sediment are considered potentially contaminated. Further tests are necessary.

Samples with less than $5 \times 10^3$ 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 *R. solanacearum* appear, on condition that typical prominently fluorescent red cells in positive control preparations are perceived.

## 8. ELISA Tests

Principle

Due to relatively low sensitivity, ELISA can be applied only as an non-obligatory additional test, in addition to IF, PCR or FISH tests. If DAS ELISA is applied, it is obligatory to primarily enrich the extract and use monoclonal antibodies.
Sample enriching prior to application of ELISA test can be useful for the purpose of increase in test sensitivity, but it can be unsuccessful due to competition of other organisms in the sample.
Note: Use valid (confirmed and approved) source of antibodies for *R.solanacearum*

It is recommended that titre be determined for every new series of antibodies. Titre is defined as the greatest dilution with which optimal reaction is achieved by testing a suspension containing $10^5$ to 10 cells/ml of homologous strain of bacterium R. solanacearum with use of corresponding conjugates of secondary antibodies, by producer's recommendations. In the course of testing, antibodies should be used in working solutions which are near or equal to titre with formulation which is available in the market. Establish titre of antibodies in a suspension of $10^5$ to $10^6$ of homologous strain of bacterium R. solanacearum.

For negative control, use the sample extract which was negative in previous testing to *R. solanacearum*, and bacterium suspension, with which there is no cross-contamination, in phosphate buffer with salt addition (PBS). For positive control, use the aliquots of sample extract which was negative in earlier testing, mixed with $10^3$ to $10^4$ cells/ml of bacterium *R. solanacearum* biovar 2 (e.g. strain NCPPB 4156 = PD 2762 = CFBP 3857; Annex 2A and B). For comparison of results, use on every microtitre panel, the standard suspension from $10^5$ to$10^6$ cells/ml of bacterium *R. solanacearum* in PBS. Take care that positive controls on microtitre panel are well separated from samples which are being tested. In Annex 3A, the Standardized positive and negative control materials which can be used in this test are given. Test the control material in the same manner as samples. Two protocols for ELISA test are valid.

**(a) INDIRECT ELISA (Robinson Smith et al., 1995)**

1) Use aliquots form 100 to 200 µl of sample extract. (by heating for four minutes at 100 °C in water bath or heating block in some cases, non-specific results can be reduced).
2) Add equal buffer volume for double strength wrapping (Annex 4) and stir on a centrifugal mixer (vortex).
3) Apply aliquots of 100 µl into every of at least two openings on a microtitre panel (e.g. Nunc-Polysorp or equivalent) and incubate for one hour at 37 °C or overnight at 4 °C.
4) Wash out the openings three times with a solution PBS-Tween (Annex 4), and leave the last solution for wash out i openings for at least five minutes.
5) Prepare appropriate antibodies dilution for *R. solanacearum* in blocking buffer (Annex 4). For valid antibodies which are available in the market, use recommended solutions (usually double titre concentration).
6) Add µl to every opening and incubate for one hour at 37 °C.
7) Remove the antibodies solution from the opening and wash the openings three times with PBS-Tween solution, and leave the last solution for wash out in openings for at least five minutes.
8) Prepare the appropriate solution of secondary antibodies-conjugate of alcal phosphatase in blocking buffer. Add 100 µl in every opening and incubate for one hour at 37 °C.
9) Remove the conjugate of antibodies from the openings and wash the openings as previously explained.
10) Add 100 µl of solution of substrate of alcal phosphatase (Annex 4)in every opening, incubate in the dark at room temperature and read absorption on 405 nm in regular intervals in the course of 90 minutes.

**(b) DASI ELISA**

1) Prepare appropriate solution of polyclonal immunoglobulins for bacterium *R. solanacearum* in the buffer for wrapping pH 9,6 (Annex 4). Apply 200 µl in every opening. Incubate for four to five hours at 37 °C or 16 hours at 4 °C.
2) Wash out the openings three times with solution PBS-Tween (Annex 4). Apply 190 µl of sample extract in at least two openings. On every panel, add a positive and a negative contorl, each in every two openings. Incubate for 16 hours at 4 °C.
3) Wash out the openings with solution PBS-Tween (Annex 4).

4) Prepare the corresponding solution of monoclonal bodies specific for bacterium *R. solanacearum* in PBS -u (Annex 4) containing 0.5 % of bovine serum albumin (BSA) and apply 190 µl to every opening. Incubate for two hours at 37 °C.
5) Wash out openings three times with solution PBS-Tween (Annex 4).

6) Prepare the appropriate solution of anti-mouse immunoglobulins conjugated with alcal phosphatase in PBS. Apply 190 µl in every opening. Incubate for two hours at 37 °C.
7) Wash out the openings three times with solution PBS-Tween (Annex 4).
8) Prepare solution of alcal phosphatase substrate containing 1 mg p-nytrophenyl phosphate per ml of substrate buffer (Annex 4). Apply 200 µl in every opening. Incubate in the dark at room temperature and working absorption at 405 nm in regular intervals in the course of 90 minutes.

Interpretation of ELISA tests results

ELISA test is negative if average optical density (OD) in openings with sample duplicate < 2x OD in the opening with negative sample extract negative control, on condition that all values of OD positive controls are above 1.0 (after 90 minutes of incubation with the substrate) and that bigger double values of OD obtained for negative sample extracts. ELISA test is positive is average optical density (OD) in openings with sample duplicate > 2x OD in an opening with sample extract negative contorl, on condition that OD values for all openings with negative control < 2x OD in openings with positive control. If negative results are read in ELISA test in openings with positive contorl, it indicates that the test has not been correctly carried out or that inhibition occurred. If positive results are read in the ELISA test in openings with negative control, it indicates that negative contamination has occurred or non-specific antibodies binding.

**9. BIOTEST**

Note:
Preliminary testing by this method should enable repeatable detection of $10^3$ to $10^4$ colony forming units (CFU) of bacterium *R. solanacearum* per ml, which are added to sample extracts which were negative in previous testing (for preparation- Annex 3). 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. The next step is based on Janse (1988):

9.1. Use 10 plants of sensitive tomato variety for every sample (e.g. Moneymaker or other variety which has been confirmed in the laboratory to be equally sensitive) in the third real leaf phase. For details on growing, see Annex 8. It is possible to use aubergines (e.g. Black Beauty variety or equivalent sensitivity variety), but only plants at the phase 2-3 leaves to complete development of the third real leaf. It turned out that symptoms are weaker with aubergines and that they develop more slowly. That is why it is recommended to use young tomato plants.

9.2. Distribute 100 µl of sample extract on test plants.

9.2.1. Injection inoculation.
Inoculate plants right above the cotyledon with an injection with a thin needle (at least 23G). Distribute the sample to test plants, so that every plant is attributed the corresponding quantity.

9.2.2. Inoculation by section.

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. With a sterile scalpel, make a diagonal cut, around 1 cm long and around 2/3 of plant width deep, starting from the resuspended sediment drop. Firmly close the cut with sterile vaseline from the syringe.

9.3. Using the same method, inoculate five plants with suspension in water of $10^5$ to $10^6$ cells/ml prepared form an 48-hour culture of virulent strain biovar 2, of bacterium *R. solanacearum* for positive contorl, and with a buffer for sediment for negative contorl (Annex 4). In order that the cross-contamination would not appear, separate the plants for positive and negative control from other plants.

9.4. Grow test plants in quarantine conditions up to four weeks at the temperature from 25 to 30 °C and with high relative humidity and adequately water them, taking care not to cause water saturation or wrinkling due to lack of water. In order to avoid contamination, grow plants for positive and negative contorl on clearly separated shelves in a greenhouse or growing chamber, or, if the space is limited, make sure that certain procedures are clearly separated. in a greenhouse or growing chamber, or, if the space ifs limited, make sure that It is crucial that there are no insects in greenhouses and growing chambers, because they can transfer bacterium from one sample to another. Monitor whether there are symptoms of withering, epinasty, chlorosis and/or growth lag.

9.5. Isolate from infected plants (Section II.3) and identify the treated cultures of probable bacterium *R. solanacearum* (Section VI.B).

9.6. If, after three weeks, there are no symptoms perceived, carry out the IF/PCR test/isolation on a prepared sample of stem parts 1 cm long, taken above the place of inoculation, from every test plant. If the test is positive, apply the method of screening of the dilution on selective medium (Section 4.1.).

9.7. Identify all cleansed cultures of probable bacterium *R. solanacearum* (Section VI.B).

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 *R. solanacearum,* on condition that bacterium *R. solanacearum* is detected in positive controls. biotest is positive if test plants are infected with bacterium *R. solanacearum.*

**B. IDENTIFICATION TESTS**

To identify clean cultures of probable isolates of bacterium *R. solanacearum* by at least two from the following tests based on different biological principles. If needed, include in every test the known referent strains – isolates (Annex 3).

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

Establish phenotype properties, universally present or absent, at bacterium *R. solanacearum*, according to methods Lelliot and Stead (1987), Klement et al. (1990), Schaad (2001).

| Test | Expected results |
|---|---|
| Creation of fluorescent pigment | – |
| Inclusions of poly-β-hiydroxybutyrate | + |
| Oxidation/fermentation (O/F) test | O+/F– |
| Catalse activity | + |
| Oxidises test by Kovac | + |
| Nitrate reduction | + |
| Use of citrates | + |
| Growth at 40 °C | – |
| Growth in 1 % NaCl | + |
| Growth in 2 % NaCl | – |
| Activity of arginine-dyhydrolasis | – |
| Lyquefaction (decomposition) of gelatine | – |
| Hydrolysis of starch | – |
| Hydrolysis of esculin | – |
| Creation of levan | – |

**2. IF test**

2.1. Prepare suspension of around $10^6$ cells/ml in buffer for IF (Annex 4).
2.2. Prepare a set of double dilutions of corresponding serum
2.3. Conduct IF test (Section VI.A.5).
2.4. IF test is positive if IF titre of the culture is equal to that of positive control.

**3. ELISA test**

Note:
If only 2 identification tests are carried out, the other serological test is not carried out with this one.

3.1. Prepare suspension of around $10^8$ cells/ml in 1x PBC (Annex 4).
3.2. Conduct the corresponding procedure of ELISA with monoclonal bodies specific for bacterium *R. solanacearum.*
3.3. ELISA test is positive if ELISA value of reading of a culture is equal to at least half value of positive control.

**4. PCR tests**

4.1. Prepare suspension of around $10^6$ cells/ml in sterile water aimed at use in molecular biology (ultra clean water).
4.2. Heat 100 µl of cell suspension in closed tubes in thermo block or hot water bath for four minutes at 100 °C. Samples can be kept, after use, at - 16 to - 24 °C.
4.3. Apply corresponding PCR procedures for multiplication of specific fragments of *R. solanacearum* [e.g. Seal et al. (1993), Pastrik & Maiss (2000), Pastrik et al. (2002), Boudazin et al. (1999), Opina et al. (1997), Weller et al. (1999)].
4.4. *R. solanacearum* is considered identified if multiplied PCR products are of same length and have the same polymorphism of restriction fragments length as well as the positive control layer (isolate).

**5. FISH test**

5.1. Prepare suspension of around $10^6$ cells/ml in ultra clean water.
5.2. Apply the FISH procedure (Section VI.A.7.) with at least two olygo-checks specific for *R. solanacearum* (Annex 7).
5.3. FISH test is positive if both cultures and positive controls result in same reactions.

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

6.1. Grow cultures for 48 hours at 28 °C on trypticase-soy-agar (Oxoid).
6.2. Apply the corresponding FAP procedure (Janse, 1991; Stead, 1992).
6.3. FAP test is positive if profile of probable culture is identical to profile of positive contorl. Presence of characteristic fat acids 14:0 3OH, 16:0 2OH, 16:1 2OH and 18:1 2OH and absence of 16:0 3OH indicates *Ralstonia* sp. to a large extent.

## 7. Strain categorization methods

With every new case of isolation of *R. solanacearum* , it is recommended to perform characterization of strain by one of the following methods. If needed, include the known reference strains in every used test ( Annex 3).

7.1. Biovar establishment

*R. solanacearum* is divided to biovars on the basis of the ability of use and/or oxidation of three disaccharides and three hexose alcohols (Hayward, 1964 and Hayward et al., 1990). Nutritive mediums for biovar establishment are described in Annex 2. Test can e successfully performed by inoculation of medium with clean cultures of isolate of *R. solanacearum* and incubation at 28 °C. If medium is distributed in 96 sterile openings on panel for cell growing (200 µl per opening), in the course of 72 hours, change of colour from khaki-green to yellow can be perceived, which means that test result is positive.

| Biovar | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Use: | | | | | |
| Maltose | – | + | + | – | + |
| Lactose | – | + | + | – | + |
| D (+) celobiosis | – | + | + | – | + |
| Manitole | – | – | + | + | + |
| Sorbitol e | – | – | + | + | – |
| Dulcitole | – | – | + | + | – |

Biovar 2 is divided to sub-phenotypes by additional tests.

| | Biovar 2A(spread worldwide) | Biovar 2A(found in Chile and Columbia) | Biovar 2T(found in tropical areas) |
|---|---|---|---|
| Use of trechalose | – | + | + |
| Use of meso-inositol | + | – | + |
| Use of D-ribose | – | – | + |
| Pectolite activity ([1]) | low | low | high |

([1]) See Lelliot and Stead (1987)

7.2. Genome fingerprint

Molecular differentiation of strains in a complex of *R. solanacearum* can be achieved by application of several methods, including the following:

7.2.1. Analysis of restriction fragments length polymorphism (RFLP) (Cook et al., 1989).

7.2.2. PCR or repetitive series, by use of primers REP, BOX and ERIC (Louws et sur., 1995, Smith et al., 1995).

7.2.3. Analysis of fragment length polymorphism (AFLP) (Van der Wolf et al., 1998).

7.3. PCR methods

Specific PCR primers (Pastrik et al., 2002; Annex 6) can be sued for differentiation of strains which belong to group 1 (biovars 3, 4 and 5) and group 2 (biovars 1, 2A and 2T) of bacterium *R. solanacearum,* as it has been primarily established by the RFLP method (Cook et al., 1989) and sequencing of 16S rDNK (Taghavi et al., 1996).

## C. CONFIRMATION TEST (PATHOGENICITY TEST)

Pathogenicity test must be carried out as final confirmation of diagnosis of *R. solanacearum* and for assessment of virulence of cultures identified as *R. solanacearum.*

1) Prepare the inoculum of around $10^6$ cells/ml from 24 to 48 hours old culture of isolates which are intended for testing and appropriate positive control strain ob bacterium *R. solanacearum* (e.g. NCPPB 4156 = PD 2762 = CFBP 3857; see Annex 3).
2) Inoculate 5 do 10 young seedlings of sensitive varieties of tomato or aubergine in the phase of third real leaf (See Section VI.A.9).
3) Incubate up to two weeks at 25 to 28 °C and with high relative humidity, and adequately water them, taking care not to cause saturation with water or withering due to lack of water. Clear cultures should show typical withering in the course of 14 days. If symptoms are not present in the course of this period, it can not be confirmed that the culture is a pathogenous form of bacterium *R. solanacearum*.
4) Monitor whether there are symptoms of withering/epinasty, chlorosi or growth lag.
5) From plants with symptoms, separate a part of stem around 2 cm above the place of inoculation. Powder and dissolve in a smaller volume of sterile distilled water or 50 mM of phosphate buffer (Annex 4). Perform isolation from suspension with spread of dilution to the appropriate nutritive medium, selective if possible (Annex 2), incubate for 48 to 72 hours at 28 °C and monitor growth of colonies typical for *R. solanacearum*.

## Annex 1

## Laboratories included in optimization and validation of protocols

| Laboratory (1) | Place | State |
|---|---|---|
| Agentur für Gesundheit und Ernährungssicherheit | Wienna 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 a protection des végétaux, Station de quarantaine de la pomme de terre | Le Rheu | France |
| Biologische Bundesanstalt | Kleinmachnow | Germany |
| Pflanzenschutzamt Hannover | Hannover | Germanu |
| State Laboratory | Dublin | Ireland |
| Dipartimento di Scienze e Tecnologie Agroambientali | Bologna | Italy |

| | | |
|---|---|---|
| Regione Veneto Unita Periferica per i Servizi Fitosanitari | Verona | Italy |
| Nederlandse Algemene Keuringsdienst | Emmeloord | Netherlands |
| Plantenziektenkundige Dienst | Wageningen | Nietherlands |
| Direcção-Geral de Protecção das Culturas | Lisabon | Portugal |
| Centro Diagnostico de Aldearrubia | Salamanca | Spain |
| Instituto Valenciano de Investigaciones Agrarias | Valencia | Spain |
| Swedish University of Agricultural Sciences | Uppsala | Sweden |

## Annex 2.

## Mediums for isolation and growing of bacterium *R. Solanacearum*

(a) Usual 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.

Yeast, peptone and glucose agar (YPGA)

Yeast extract (Difco) 5.0 g
D (+) glucose (monohydrate) 10.0 g
Bacto agar (Difco) 15.0 g
Distilled water 1.0 L
Dissolve ingredients sterilize in an autoclave at 121 °C for 15 minutes.

Saharosis and peptone agar (SPA)

Saharosis 20.0 g
Bacto peptone (Difco) 5.0 g
$K_2HPO_4$ 0.5 g
$MgSO_47H_2O$ 0.25 g
Bacto agar (Difco) 15.0 g

Distilled water 1.0 L
pH 7.2 – 7.4
Dissolve ingredients sterilize in an autoclave at 121 °C for 15 minutes.

Kelman's tetrazolium medium

Casamino acids (Difco) 1.0 g
Bacto peptone (Difco) 10.0 g
Dextrose 5.0 g
Bacto agar (Difco) 15.0 g
Distilled water 1.0
Dissolve ingredients sterilize in an autoclave at 121 °C for 15 minutes.
Cool at 50 °C and add to the solution 2,3,5-tryphenyl-tetrazolium-chloride (Sigma),sterilised by filtration, in order to gain final concentration of 50 mg/l.

(b) Valid (confirmed and approved) selective nutritive mediums

SMSA medium (Englebrecht, 1994, amendments by Elphinstone et al., 1996)
Basic medium
Casamino acids (Difco) 1.0 g
Bacto peptone (Difci) 10.0 g
Glycerole 5.0 ml
Bacto agar (Difco),(check note 2) 15.0 g
Distilled water 1.0 L

Dissolve ingredients and sterilize in an autoclave at 121 °C for 15 minutes. Cool at 50 °C and add basic water solutions of the following ingredients, sterilized by filtration, in order to gain the envisaged final concentrations:

Crystal violet (Sigma) 5 mg on L
Polymixin-B-sulphate(Sigma P-1004) 600 000 U (around 100 mg) on L
Bacytracil (Sigma B-0125) 1 250 U (around 25 mg) on L
Chloramphenicol (Sigma C-3175) 5 mg on L
Penicillin-G (Sigma P-3032) 825 U( around 0.5 mg) on L
2,3,5-triyphenile-tetrazolium-chloride (Sigma) 50 mg on L

Note:

1. Reagents different from the above mentioned can affect growth of bacterium *R. solanacearum.*

2. Instead of Bacto agar (Difco) Oxoid agar no. 1 can be used. In that case, *R. solanacearum* will grow slower, but growth of competitive saprophytes can be reduced. For forming of typical colonies of bacterium *R. solanacearum* maybe it will take 1 to 2 days more, and red coloration can be lighter and more diffuse than on Bacto agar.

3. By increase in concentration of bacitracin to 2500 U/L populations of competitive bacteria can be reduced, without affecting growth of bacterium *R. solanacearum.*

Keep nutritive medium and basic antibiotic solution at 4 °C in a dark place and use in the course of one month. Prior to use, surface condensation must be removed from mediums. Mediums must not be over-dried. After preparation of each new series of nutritive mediums, only quality control should be performed, by applying suspension of the reference culture of *R. solanacearum* (see Annex 3) and monitor whether typical creation of colonies will occur in the course of two to five days of incubation at 28 °C.

(c) Valid (confirmed and approved) nutritive mediums enrichment

SMSA liquid medium (Elphinstone et al., 1996)
Prepare as for selective medium SMSA with agar, but leave out Bacto agar and 2,3,5-tryphenyl-tetrazolium-chloride.

Modified Wilbrink liquid medium (Caruso et al., 2002)

| | |
|---|---|
| Sacharose | 10 g |
| Proteosis pepthon | 5 g |
| $K_2HPO_4$ | 0.5 g |
| $MgSO_4$ | 0.25 g |
| $NaNO_3$ | 0.25 g |
| Distilled water | 1 L |

Sterilize in an autoclave at 121 °C for 15 minutes and cool at 50 °C. Add basic solutions of antibiotics such as SMSA for liquid medium.

## Annex 3.

A. **Standardized control materials available in the market**

(a) Bacterial isolates.

It is recommended to use the following bacterial isolates as standard reference material either for positive controls (Table 1) or during optimization of tests for purpose of avoiding cross-reactions (Table 2). All strains are available in the market and can be procured from:

1. National Collection of Plant Pathogenic Bacteria (NCPPB), Central Science Laboratory, York, UK

2. Culture Collection of the Plant Protection Service (PD), Wageningen, Netherlands

3. Collection française de bactéries phytopathogčnes (CFBP), INRA – Station de phytobactériologie, Angers, France

Table 1. SMT inventory of reference isolates of bacterium *R. solanacearum*

| Label NCPPB | No. SMT | Other labels | State of origin | Biovar |
|---|---|---|---|---|
| NCPPB 4153 | 6 | CFBP 4582, Pr 3020, EURS11 | Egypt | 2 |
| NCPPB 4154 | 10 | CFBP 4585, 550, EURS21 | Turkey | 2 |
| NCPPB 3857 | 12 | CFBP 4587, Pr 1140, EURS26 | England | 2 |
| NCPPB 1584 | 23 | CFBP 4598, EURS49 | Cyprus | 2 |
| NCPPB 2505 | 24 | CFBP 4599, EURS50 | Sweden | 2 |
| NCPPB 4155 | 26 | CFBP 4601, 502, EURS55 | Bedium | 2 |
| NCPPB 4156* | 71 * | PD 2762, CFBP 3857 | Netherlands | 2 |
| NCPPB 4157 | 66 | LNPV 15.59 | Fraance | 2 |
| NCPPB 4158 | 39 | CFBP 4608, Port 448, EURS55 | Portugal | 2 |
| NCPPB 4160 | 69 | IVIA -1632-2 | Spain | 2 |
| NCPPB 4161 | 76 | B3B | Netherlands | 2 |
| NCPPB 325 | 41 | CFBP 2047, KEL60-1, R842 | USA | 1 |
| NCPPB 3967 | 42 | CFBP 4610, R285, GONg7 | Costarica | 1 |
| NCPPB 4028 | 43 | CFBP 4611, R303/571, CIP310, SEQ205 | Columbia | 2 |
| NCPPB 3985 | 44 | CFBP 4612, R578, CIP312 | Peru | 2T |
| NCPPB 3989 | 45 | CFBP 4613, R568, CIP226 | Brazil | 2T |
| NCPPB 3996 | 46 | CFBP 3928, R276/355, CIP72, SEQ225 | Peru | 3 |
| NCPPB 3997 | 47 | CFBP 4614, R280/363, CIP49, HAY0131a | Australia | 3 |
| NCPPB 4029 | 48 | CFBP 4615, R297/349, CIP121, CMIb2861 | Sri Lanka | 4 |
| NCPPB 4005 | 49 | CFBP 4616, R470 | Philippines | 4 |
| NCPPB 4011 | 50 | CFBP 4617, R288, HEmps2 | China | 5 |

* Use as standard reference strain of bacterium *R. solanacearum* biovar 2 (strain 3)

Note: Authenticity of the above mentioned strains can be guaranteed only if they are obtained from an authentic collection of cultures.

Table 2. SMT inventory of reference serologically or genetically related bacteria for use in optimization of detection tests.

| Label NCPPB | No. SMT | Other labels | Identification |
|---|---|---|---|
| NCPPB 4162 | 51 | CFBP 1954 | *Bacillus polymyxa* ([1]) |
| NCPPB 4163 | 52 | CFBP 1538 | *Pseudomonas marginalis pv. marginalis* ([1]) |
| NCPPB 4164 | – | CFBP 2227 | *Burkholderia cepacia* ([2]) |
| NCPPB 4165 | – | CFBP 2459 | *Ralstonia pickettii* ([2]) |
| NCPPB 4166 | 58 | CFBP 3567<br>CSL<br>Pr1150 | *Ralstonia pickettii* ([1]) |
| NCPPB 4167 | 60 | CFBP<br>4618 PD<br>2778 | *Ralstonia* sp. ([1]) |
| NCPPB 1127 | 53 | CFBP 3575 | *Burkholderia andropogonis* ([1]) |
| NCPPB 353 | 54 | CFBP 3572 | *Burkholderia caryophylli* ([1]) |
| NCPPB 945 | 55 | CFBP 3569 | *Burkholderia cepacia* ([1]) |
| NCPPB 3708 | 56 | CFBP 3574 | *Burkholderia glumae* ([1]) |
| NCPPB 3590 | 57 | CFBP 3573 | *Burkholderia plantarii* ([1]) |

| | | | |
|---|---|---|---|
| NCPPB 3726 | 59 | CFBP 3568 | *Banana Blood Disease Bacterium* ([1]) ([2]) ([3]) |
| NCPPB 4168 | 61 | CFBP 4619 IPO S339 | *Enterobacter* sp. ([1]) |
| NCPPB 4169 | 62 | IPO 1695 | *Enterobacter* sp. ([1]) |
| NCPPB 4170 | 63 | CFBP 4621 IPO S306 | *Ochrobactrum anthropi* ([1]) ([2]) |
| NCPPB 4171 | 64 | CFBP 4622 IPO 1693 | *Curtobacterium* sp. ([1]) ([2]) |
| NCPPB 4172 | 65 | IPO 1696a | *Pseudomonas* sp. ([1]) |
| NCPPB 4173 | – | PD 2318 | *Aureobacterium* sp. ([2]) |
| NCPPB 4174 | 81 | IVIA 1844.06 | *Flavobacterium* sp. ([1]) ([2]) |
| ([1]) Strain which can cross-react in serological tests (IF and/or ELISA) with polyclone serums. | | | |
| ([2]) Strain from which in some laboratories PCR products of length similar to length expected when using specific primer OLI-1 i Y-2 in some laboratories (see Annex 6). | | | |
| ([3]) In most tests it could cross-react, but it is known to appear only on bananas in Indonesia. | | | |

(b) Standardized contorl materials available in the market.

Standard control material given in the text below can be obtained for NCPPB collections of cultures. Lyophilized granules of potato extract from 200 healthy potato tubers as a negative control for all tests.

Lyophilized granules of potato extract from 200 healthy potato tubers with $10^3$ to $10^4$ and $10^4$ to $10^6$ cells of *R. solanacearum* biovar 2 (strain NCPPB 4156 = PD 2762 = CFBP 3857) as positive control for serological and PCR tests. Since lyophiliztion affects vitality of cells, they are not appropriate as a standard control for isolation or biotests. Formalin-fixed suspensions of bacterium *R. solanacearum* biovar 2 (strain NCPPB 4156 = PD 2762 = CFBP 3857) with $10^6$ cells /ml as a positive contorl for serological tests.

B. Preparation of positive and negative controls for basic check tests (PCR/IF and FISH)

Make suspension of a culture of a virulent strain of *R. solanacearum* strain 3/biovar 2 (e.g. strain NCPPB 4156 = PD 2762 = CFBP 3857) grown for 48 hours at SMS A basic medium in 10 mM phosphate buffer in order to gain cell density of around 2 x $10^8$ cfu/ml. This is usually a lightly intransparent suspension with optical density of 0.15 per 600 nm.

Take sprout cones from 200 tubers of white variety potato which are known not to be infected with bacterium *R Solanacearum.* Process cones with usual method and resuspend the sediment in 10 ml. Fill 10 sterile tubes of 1.5 ml with 900 µl of resuspended sediment. Add 100 µl of suspension into the first micro-tube of *R. Solanacearum.*

Stir on a centrifugal mixer, i.e. vortex. Prepare decimal solutions 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 *R. Solanacearum* 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 contorl samples for every series of samples for testing. In IF, FISH and PCR tests, *R. Solanacearum* must be detected in at least $10^6$ and $10^4$ cells/ml of positive controls and in none of the negative controls.

## Annex 4

## Buffers for testing procedures

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

1. **Buffers for extraction procedure**

1.1. Extraction procedure (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 | 1.00 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 litre)* |
|---|---|---|
| Lubrol flakes | deflocculant (*) | 0.5 g |
| DC silicone against foaming | anti-foaming agent (*) | 1.0 ml |
| Tetrasodium pyrophosphate | antioxidant | 1.0 g |
| Polyvynylpyrolydon–40000 (PVP-40) | binding of PCR inhibitors | 50 g |

(*) 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.

| | |
|---|---|
| $Na_2HPO_4.12H_2O$ | 2.7 g |
| $NaH_2PO_4.2H_2O$ | 0.4 g |
| Distilled water | 1.0 L |

Dissolve ingredients, check pH and sterilize in an autoclave for 15 minutes at 12 °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

| | |
|---|---|
| $Na_2HPO_4.12H_2O$ | 2.7 g |
| $NaH_2PO_4.2H_2O$ | 0.4 g |
| NaCl | 8.0 g |
| distilled water | 1.0 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. Glycerol with phosphate buffer pH 7.6

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

| | |
|---|---|
| $Na_2HPO_4 \cdot 12H_2O$ | 3.2 g |
| $NaH_2PO_4 \cdot 2H_2O$ | 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).

## 3. **Buffers for indirect ELISA test**

3.1. Buffer for wrapping of double strength, pH 9.6

| | |
|---|---|
| $Na_2CO_3$ | 6.36 g |
| $NaHCO_3$ | 11.72 g |
| Distilled water | 1.00 L |

Dissolve ingredients, check pH and sterilize in an autoclave for 15 minutes at 121°C. Sodium sulphite can be added (0.2 %) as an antioxidant if it is necessary to prevent creation of oxydated aromatic compounds.

.

3.2. 10X phosphate buffer with salt addition (PBS), pH 7.4

| | |
|---|---|
| NaCl | 80.0 g |
| $KH_2PO_4$ | 2.0 g |
| $Na_2HPO_4 \cdot 12H_2O$ | 29.0 g |
| KCl | 2.0 g |
| Distilled water | 1.0 L |

3.3. PBS-Tween

| | |
|---|---|
| 10X PBS | 100 ml |
| 10 % Tween 20 | 5 ml |
| Distilled water | 895 ml |

3.4. Blocking (for antibodies) buffer (must be freshly prepared)

| | |
|---|---|
| 10X PBS | 10.0 ml |
| Polyvynypyrolydon-44000 (PVP-44) | 2.0 g |
| 10 % Tween 20 | 0.5 ml |
| Powder milk | 0.5 g |
| Distilled water | add to 100 ml |

3.5. Solution of substrate of alcal phosphatase, pH 9.8

| | |
|---|---|
| Diethanolamine | 97 ml |
| Distilled water | 800 ml |

Mix and set pH to 9.8 concentrated HCl
Add water until you reach one liter.
Add 0.2 g $MgCl_2$.
Solve two tablets of 5 milligram phosphatase substrate (Sigma) on 15 ml of solution

**4. Buffers for DASI ELISA test**

4.1. Buffer for wrapping, pH 9.6

| | |
|---|---|
| $Na_2CO_3$ | 1.59 g |
| $NaHCO_3$ | 2.93 g |
| Distilled water | 1 000 ml |

Dissolve ingredients and set pH to 9.6.

4.2. 10X phosphate buffer with salt added (PBS), pH 7.2 to 7.4

| | |
|---|---|
| NaCl | 80.0 g |
| $NaH_2PO_4 \cdot 2H_2O$ | 4.0 g |
| $Na_2HPO_4 \cdot 12H_2O$ | 27.0 g |
| Distilled water | 1 000 ml |

4.3. PBS-Tween

| | |
|---|---|
| 10X PBS | 50 ml |
| 10 % Tween 20 | 5 ml |
| Distilled water | 950 ml |

5) Substrate buffer, pH 9.8
Diethanolamine
100 ml
Distilled water
900 ml
Mix and set pH on 9.8 concentrated HCl.

## Annex 5

### Establishment of contamination level of IF and FISH tests

1. Establish average count of typical fluorescent cells per field of vision (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 = quantity of resuspended sediment in every opening

and F = factor of dilution of resuspended sediment.

## 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 *R. solanacearum* per ml of sample extract. Preliminary testing must not give falsely positive results at certain selected bacterial strains and isolates (see Annex 3).

### 1. Protocol for PCR by Seal et al. (1993)

1.1. Oligonucleotide primers

Upstream OLI-1 5'-GGG GGT AGC TTG CTA CCT GCC-3'

Downstream primer Y-2 5'-CCC ACT GCT GCC TCC CGT AGG AGT-3'

Expected length of multiplied product from DNA mould of bacterium *R solanacearum* = 288 bp (a couple of PSA primers).

1.2. Reaction mixture for PCR

| Reagent | Quantity per reaction | Final concentration |
|---|---|---|
| Sterile ultra clean water | 17.65 µl | |
| 10X PCR buffer *(1)* (15 mM $MgCl_2$) | 2.5 µl | 1X (1,5 mM $MgCl_2$) |
| dNTP mixture (20 mM) | 0.25 µl | 0.2 mM |
| Primer OLI-1 (20 µM) | 1.25 µl | 1µM |
| Primer Y-2 (20 µM) | 1.25 µl | 1µM |
| Taq polymerase (5U/µl) | 0.1 µl | 0.5 U |
| Sample volume | 2.0 µl | |
| Total volume | 25 µl | |

(1) Method was valid Perkin Elmer (AmpliTaq) and Gibco BRL

1.3. Conditions for PCR reactions

Carry out the following programme:

1 cycle of: (i) 2 minutes at 96 °C (denaturation of DNA chain)

35 cycles of: (ii) 20 seconds at 94 °C (denaturation of DNA chain)

(iii) 20 seconds at 68 °C (primer binding)

(iv) 30 seconds at 72 °C (copy elongation - hybridization)

1 cycle of: (v) 10 minutes at 72 °C (final elongation)

(vi) keep at 4 °C

Note:

Optimal conditions for use of this programme are defined for use on PCR (thermal cycler) Perkin Elmer 9600. Modification of duration of cycle steps (ii), (iii) and (iv) is especially needed in case of use of other models of thermal cycler.

1.4. Analysis of multiplication products by restriction enzymes

PCR produces multiplied from DNA of bacterium *R. solanacearum* give characteristic polymorphisms in length of restriction fragments with enzyme Ava II after incubation on 37 °C.

**2. Protocol for PCR by Pastrik and Maiss (2000)**

2.1. Oligonucleotide primers

Up-water primer Ps-1 5'- agt cga acg gca gcg ggg g -3'

Down-water primer Ps-2 5'- ggg gat ttc aca tcg gtc ttg ca -3'

Expected length of multiplied product form DNA chain of bacterium *R solanacearum* = 553bp.

2.2. Reaction mixture for PCR (reaction mix)

| Reagent | Quantity per reaction | Final concentration |
|---|---|---|
| Sterile ultra clean water | 16.025 µl | |
| 10X PCR buffer (1) | 2.5 µl | 1X (1,5 mM $MgCl_2$) |
| BSA (fraction V) (10 %) | 0.25 µl | 0.1 % |
| d-NTP mix (20 mM) | 0.125 µl | 0.1 mM |
| Primer Ps-1 (10 µM) | 0.5 µl | 0.2 µM |
| Primer Ps-2 (10 µM) | 0.5 µl | 0.2 µM |

| | | |
|---|---|---|
| Taq polymerase (5U/µl) | 0.1 µl | 0.5 U |
| Sample volume | 5.0 µl | |

| | | |
|---|---|---|
| Total volume | 25,0 µl | |
| ([1]) Method validation was carried out with use of Taq polymerase Perkin Elmer (AmpliTaq or Gold) and Gibco BRL | | |

Note: Optimal conditions of use of this programme are defined for use on PCR MJ Research PTC 200 thermal cycler with Gibco Taq polymerase. On same concentrations, Perkin Elmer AmpliTaq and buffer can be used.

2.3. conditions for PCR reactions

Cary out the following programme:

1 cycle of: (i) 5 minutes at 95°C (denaturation of DNA chain)
35 cycles of: (ii) 30 seconds at 95°C (denaturation of DNA chain)
(iii) 30 seconds aa 68°C (primer binding)
(iv) 45 seconds at 72°C (copy elongation)
1 cycle of: (v) 5 minutes at 72°C (final elongation)
(vi) keep at 4 °C

Note:

Optimal conditions for use of this programme are defined for use at PCR MJ Research PTC 200 thermal cycler. Modification of duration of cycle steps (ii), (iii) and (iv) may be necessary in case of use of other thermal cycler models.

2.4. Analysis of products of multiplication by restrction enzyme

PCR produces multiplied DNA of bacterium *R. solanacearum* show characteristic polymorphisms in length of restriction fragments with enzyme Taq I after 30 minutes incubation at 65 °C. Restrction fragments obtained from fragment specific for *R. solanacearum* have size 457 bp and 96 bp.

**3. Protocol for multiplex PCR with internal control (Pastrik et al., 2002)**

3.1. Oligonucleotide primers

Upstream primer RS-1-F 5'-ACT AAC GAA GCA GAG ATG CAT TA-3'
Downstream primer RS-1-R 5'-CCC AGT CAC GGC AGA GAC T-3'
Upstream primer NS-5-F 5'-AAC TTA AAG GAA TTG ACG GAA G-3'
Downstream primer NS-6-R 5'-GCA TCA CAG ACC TGT TAT TGC CTC-3'

Expected length of multiplied product of DNA of bacterium *R. solanacearum* = 718 bp (a couple of RS primers).

Expected length of multiplied product from internal PCR control 18S rRNA = 310 bp (a couple of NS primers).

3.2. Reaction mixture for PCR(reaction mix)

| Reagent | Quantity per reaction | Final concentration |
|---|---|---|
| Sterile ultra clean water | 12.625 µl | |
| 10X PCR buffer ([1])(15mM $MgCl_2$) | 2.5 µl | 1X (1.5 mM $MgCl_2$) |

| | | |
|---|---|---|
| BSA (fraction V) (10 %) | 0.25 µl | 0.1% |
| d-NTP mix (20 mM) | 0.125 µl | 0.1 mM |
| Primer RS-1-F (10 µM) | 2.0 µl | 0.8 µM |
| Primer RS-1-R (10 µM) | 2.0 µl | 0.8 µM |
| Primer NS-5-F (10 µM) ([2]) | 0.15 µl | 0.06 µM |
| Primer NS-6-R (10 µM) ([2]) | 0.15 µl | 0.06 µM |
| Taq polymerase (5U/ µl) ([1]) | 0.2 µl | 1.0 U |
| Sample volume | 5.0 µl | |
| Total volume | 25.0 µl | |

([1]) Method is confirmed by Taq polymerase Perkin Elmer (AmpliTaq or Gold) and Gibco BRL

([2]) Concentrations of primer NS-5-F and NS-6-R are optimized for extraction of potato tuber sprout cones by method of and treatment of DNA by Pastriku (2000) ( Section VI.A.6.1.a). If extraction is carried out by shaking or other methods it is necessary to optimize reagents concentration anew.

3.3. PCR reaction conditions

Carry out the following programme:

1 cycle of: (i) 5 minutes at 95 °C (denaturation of DNA chain)

35 cycles of: (ii) 30 seconds at 95 °C (denaturation of DNA chain)

(iii) 30 seconds at 58 °C (primer binding)

(iv) 45 seconds at 72 °C (copy elongation - hybridization)

1 cycle of: (v) 5 minutes at 72 °C (final elongation)

(vi) keeping at 4 °C

Note:

Optimal conditions for use of this programme are defined for use on PCR MJ Research PTC 200 thermal cycler. Modification of duration of cycle steps (ii), (iii) and (iv) may be useful in case of use of other thermal cycle models.

3.4. Analysis of product of multiplication by restriction enzyme

PCR products multiplied from DNA of bacterium *R. solanacearum* show characteristic polymorphisms of length in length of restriction fragments with enzyme Bsm I or isoschizomer (e.g. Mva 1269 I) after 30 minutes incubation at 65 °C.

## 4. Protocol for specific PCR of biovar (Pastrik et al. 2001)

4.1. Oligonucleotide primers

Up-water primer Rs-1-F 5'-ACT AAC GAA GCA GAG ATG CAT TA-3'
Down-water primer Rs-1-R 5'-CCC AGT CAC GGC AGA GAC T-3'
Down-water primer Rs-3-R 5'-TTC ACG GCA AGA TCG CTC-3'
Expected length of multiplied product of DNA of bacterium *R. solanacearum:*
with Rs-1-F/Rs-1-R = 718 bp
with Rs-1-F/Rs-3-R = 716 bp

4.2. Reaction mixture for PCR (reaction mix)

(a) Specific PCR for biovar 1/2

| Reagent | Quantity per reaction | Final concentration |
|---|---|---|
| Sterile ultra clean water | 12.925 µl | |
| 10X PCR buffer ([1]) | 2.5 µl | 1X (1,5 mM $MgCl_2$) |
| BSA (fraction V) (10 %) | 0.25 µl | 0.1 % |
| d-NTP mix (20 mM) | 0.125 µl | 0.1 mM |
| Primer Rs-1-F (10 µM) | 2 µl | 0.8 µM |
| Primer Rs-1-R (10 µM) | 2 µl | 0.8 µM |
| Taq polymerase (5U/µl)([1]) | 0.2 µl | |
| Sample volume | 5.0 µl | |
| Total volume | 25.0 µl | |
| ([1])Methods are confirmed by Taq polymerase Perkin Elmer (AmpliTaq) and Gibco BRL | | |

(b) Specific PCR for biovar 3/4/5

| Reagens | Quantity per reaction | Final concentration |
|---|---|---|
| Sterile ultra clean water | 14.925µl | |
| 10X PCR buffer (1) | 2.5 µl | 1X (1,5 mM $MgCl_2$) |
| BSA (fraction V) (10 %) | 0.25 µl | 0.1 % |
| d-nTP mix (20 mM) | 0.125 µl | 0.1 mM |
| Primer Rs-1-F (10 µM) | 1 µl | 0.4 µM |
| Primer Rs-3-R (10 µM) | 1 µl | 0.4 µM |
| Taq polymerase (5U/µl) | 0.2 µl | 1.0 U |
| Sample volume | 5.0 µl | |
| Total volume | 25.0 µl | |
| ([1])Methods are confirmed by Taq polymerase Perkin Elmer (AmpliTaq) and Gibco BRL | | |

4.3. Conditions for PCR reactions.
Carry out the following programme for specific reactions for biovar 1/2 andi biovar 3/4/5:

| | |
|---|---|
| 1 cycle of: | (i) 5 minutes at 95 °C (denaturation of DNA chain) |
| 35 cycles of: | (ii) 30 seconds at 95 °C (denaturation of DNA chain) |
| | (iii) 30 seconds at 58 °C (primer binding) |
| | (iv) 45 seconds at 72 °C (copy elongation - hybridization) |
| 1 cycle of: | (v) 5 minutes at 72 °C (final elongation) |
| | (vi) keep at 4 °C |

Note:
Optimal conditions for use of this programme are defined for use on PCR MJ Research PTC 200 thermal cycler. Modification of duration of cycle steps (ii), (iii) and (iv) may be needed in case of use of other thermal cycle models.

4.4. Analysis of multiplication product by restriction enzyme

PCR products multiplied from DNA of bacterium *R. solanacearum* with primers Rs-1-F and Rs-1-R show characteristic polymorphisms in length of restriction fragments with enzyme Bsm I or isoschizomer (e.g. Mva 1269 I) after 30 minute incubation at 65 °C. PCR multiplied from DNA of bacterium *R. solanacearum* with primers Rs-1-F and RS-3-R do not have restriction spots.

**5. Preparation of buffer for coloration for electrophoresis**

5.1. Bromphenol blue (10% concentrated solution)
Bromphenol blue 5 g
Distilled water (bidistilled) 50 ml

5.2. Buffer for coloration
Glycerol (86 %) 3.5 ml
Bromphenol blue (5.1) 300 µl
Distilled water (bidistilled) 6.2 ml

6. 10x Tris-Acetate and EDTA buffer (TAE), pH 8.0
Tris buffer 48.40 g
Glacial vinegar acid 11.42 ml
EDTA (disodium salt) 3.72 g
Distilled water 1.00 L
.
Dilute 1x before use.
Available in the market (e.g. Invitrogen or equivalent).

## Annex 7

## Valid (confirmed and approved) reagents for FISH test

1. **Olygo-checks**

Specific check for *R. solanacearum* OLI-1-CY3 *:* 5'-ggc agg tag caa gct acc ccc-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.

3. **3X Hybmix**

| | |
|---|---|
| NaCl | 2.7 M |
| Tris-HCl | 60 mM (pH7.4) |
| EDTA (sterilized by filtration in an autoclave) | 15 mM |

Dilute by 1x, as needed.

4. **Hybridization solution**

1X Hybmix
Sodium dodecyl sulphate (SDS) 0.01 %
Formamide 30 %
Check EUB 338 5 ng/µl
Check OLI-1 or OLI-2 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.

IMPORTANT: FORMAMIDE IS VERY TOXIC SO USE GLOVES AND TAKE ALL NECESSARY PRECAUTION MEASURES

Table 1. Proposed quantities for preparation of hybridization mixture

| Number of glasses | 1 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|
| Sterile ultra clean water | 23.1 | 92.4 | 138.6 | 184.8 | 231.0 |
| 3 x hybmix | 30.0 | 120.0 | 180.0 | 240.0 | 300.0 |
| 1 % SDS | 0.9 | 3.6 | 5.4 | 7.2 | 9.0 |
| Formamide | 27.0 | 108.0 | 162.0 | 216.0 | 270.0 |
| Check EUB 338 (100 ng/µl) | 4.5 | 18.0 | 27.0 | 36.0 | 45.0 |
| Check OLI-1 or OLI-2 (100 ng/µl) | 4.5 | 18.0 | 27.0 | 36.0 | 45.0 |
| Total volume (µl) | 90.0 | 360.0 | 540.0 | 720.0 | 900.0 |
| *Note:*<br>Keep in the dark all dilutions containing olygo-checks sensitive to light at - 20 °C.<br>Protect them from direct sunlight or electric light in the course of use. | | | | | |

5. 0.1M 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

## Conditions of growing of aubergine and tomato

Plant seed of tomato (Lycopersicon esculentum) and aubergine (Solanum melongena) in pasteurized compost for seeds. Plant seedlings in pasteurized compost in pots after cotyledons are fully developed (10 to 14 days). Before inoculation, tomato and aubergine should be grown in a greenhouse, in following conditions:
Day length: 14 hours or natural day length, if it is linger than 14 hours;
Temperature: day 21 to 24 °C , night 14 to 18 °C.

Sensitive tomato variety » Moneymaker «.
Sensitive aubergine variety » Black Beauty «.

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**ANNEX II**

## MEASURES TAKEN IN SAFETY ZONE

**1. Measures at places of production marked as contaminated:**

1.1 On a plot or in a greenhouse aimed at plant production 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 tomato as well as other wild-growing host plants to harmful organism, including weeds from family *Solanaceae* and
- it is prohibited to:
  - plant potato tubers or plants, as well as to sow seeds in botanical sense;
  - plant plants and sow tomato seeds;
  - taking into account biology of the harmful organism;
  - plant other host plants;
  - plant these plant varieties from genus *Brassica* for which it is established that they enable survival of harmful organism;
  - plant plants for which it is established that they enable spreading of harmful organism;

a) in the first season of potato or tomato 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 and tomato were found, nor other host plants , including weeds form family *Solanaceae*:
   - in case of production of potato, exclusively production of mercantile potato is allowed;
   - produced potato tubers or tomato plants are tested, when needed in accordance with prescribed procedures;

b) in the season of production of potato or tomato 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, systematic research is carried out in accordance with Article 3 of this rulebook or

1.1.2 in the course of at least five growing years after the year when contamination was established:

- other measures of removal o wild-growing potato and tomato plants are carried out, as well as of all other host plants to harmful organism, including weeds from family *Solanaceae* and
- plot is maintained as idle land or permanent pasture with intensive pasture or frequent low mowing, or cereals are sawn , depending on harmful organism risk assessment, or it is used for production of seeds grass; in following two consecutive years, production of plants which are not harmful organism hosts and for which it is known that they do not enable survival or spreading of harmful organism is allowed.

U In the first season of production of potato or tomato, 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 and tomato

found on the plot, nor other wild-growing host plants, including weeds from family *Solanaceae*:

- in case of production of potato, production of seeds and mercantile potato is allowed;
- produced potato tubers or tomato plants are tested, when needed, in accordance with prescribed procedures.

At all other plots within the contaminated place of production, on condition that phytosanitary inspector established that danger from wild-growing plants of potato and tomato as well as other harmful organism host plants exists, including weeds from family *Solanaceae*:

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
- in case of production of potato, it is allowed to plant certified seeds potato aimed exclusively at production of mercantile potato; or
- in case of production of tomato, it is allowed to plant tomato plants grown from seeds meeting requirements from the Rulebook on phytosanitary measures for introduction and spreading and measures of harmful organisms control with lists of harmful plant organisms to plants, plant products and facilities under supervision, exclusively for fruit production;

1.2.2 in the second growing year which ensues after the year in which contamination was established:

- in case of production of potato, 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,
- in case of tomato production, it is allowed to plant only these potato plants which are grown from a seed which meets requirements of the Rulebook on phytosanitary measures for introduction and spreading and measures of harmful organisms control with lists of harmful plant organisms to plants, plant products and facilities under supervision, or from plants obtained by vegetative reproduction of tomato plants produced from such seed and grown under technical supervision at places of production which are not marked as contaminated, for production of nursery plants or fruit.

1.2.3 at least, still in the third year ensuing after established contamination:

- in case of potato production, 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;
- in case of tomato production, it is allowed to plant only the potato plants grown which are grown from seed which meets requirements from the Rulebook on phytosanitary measures for introduction and spreading and measures of harmful organisms control with lists of harmful plant organisms to plants, plant products and facilities under supervision, or from tomato plants grown under technical supervision from such plants, for production of nursery plants or fruit ;

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 crop is carried out in corresponding time periods, of all plots where potato is grown; produced potato from every plot must be tested in accordance with prescribed procedures.

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 or tomato must be cleaned, and when necessary, disinfected in accordance with Article 18 of this rulebook;
- phytosanitary inspector supervises carrying out of irrigation and sprinkling programme, which can be prohibited, when necessary, for the purpose of prevention of spreading of harmful organism .

1.4 In a greenhouse aimed at plant production, marked as contaminated, 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, as well s to plant other host plants to harmful organisms, including tomato seed and plants, until in that greenhouse, under supervision of the phytosanitary inspector, measures are taken to destroy harmful organism and remove all host plants and their parts, while, as a minimum measure, complete replacement of substrate for growing is performed, as well as cleaning and disinfection of that greenhouse an entire equipment, and until phytosanitary inspector approves, after carrying out of these measures, production of potato and tomato;
- it is allowed to produce potato from certified seeds potato produced under technical supervision or of mini-tubers or mini-plants obtained from a plant tissue culture, originating form tested sources;
- it is allowed to produce tomato from seeds which meets requirements from the Rulebook on phytosanitary measures for introduction and spreading and measures of harmful organisms control with lists of harmful plant organisms to plants, plant products and facilities under supervision or from plants obtained by vegetative reproduction of tomato plants grown from such seeds under technical supervision;
- phytosanitary inspector supervises conducting of the irrigation and sprinkling programme, which can be prohibited, when necessary, for the purpose of prevention of spreading of harmful organism.

**2. Measures in entire safety zone (including measures at places of production marked as contaminated):**

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 18 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:

2.2.1 in safety zone established in accordance with Article 12 of this rulebook:

- supervise estates where potato tubers or tomato are grown, storage, located or treated, including the estates where the same devices are used to perform activities related to listed 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 11 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;
- order planting of only the tomato plants grown from seed which meets requirements from the Rulebook on phytosanitary measures for introduction and spreading and measures of harmful organisms control with lists of harmful plant organisms to plants, plant products and facilities under supervision, or plants obtained by vegetative reproduction of tomato plants produced from such seeds and grown under technical supervision, in all tomato crops within the safety zone;
- conduct systematic research referred to in Article 3 of this rulebook;

2.2.2 for surface water which was marked as contaminated pursuant to Article 14 paragraph 1 indent 2 of this rulebook or represents on of factors, referred to in Article 12 paragraph 2 indent 3 and Article 14 paragraph 2 of this rulebook ,which influence possible spreading of harmful organism:

- perform systematic research referred to in Article 3 of this rulebook in accordance with annual programme in appropriate time intervals, take samples of surface water, and when necessary, harmful organism host plants from family *Solanaceae*, from corresponding water sources, for testing purposes. Testing of main host plants and all other samples taken must be carried out in compliance with prescribed procedures;
- supervise conducting of irrigation and sprinkling programme and may prohibit use of water marked as contaminated for irrigation and sprinkling of main host plants, and where necessary, of the host plants for the purpose of prevention of spreading of harmful organism. Phytosanitary inspector may revoke that prohibition, if on the basis of results of conducted systematic research referred to in Article 3 of this rulebook in accordance with the annual plant, the inspector established that surface water is no longer contaminated. Use of water for irrigation and sprinkling of host plants, use of which is prohibited, can be allowed under official supervision , by application of officially approved procedures by which harmful organism is removed and its spreading is prevented;
- in case when liquid waste is contaminated, disposal of solid or liquid waste arising from industrial processing or packaging of main host plants is carried out under supervision of phytosanitary inspector.

2.3 Phytosanitary Inspectorate can, in case of need, order replacement of all stocks of seeds potato in certain time period.