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Detection and identification of Erwinia amylovora

梨火疫病菌检疫鉴定方法

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1 Scope

The document describes methods for the detection of the fire blight bacterium by isolation and culture, by immunological means and by molecular biology.

It applies to the quarantine detection and identification of the fire blight bacterium carried by plants, rootstocks, scions and young fruit of pear, apple and other rosaceous species, and to field survey and monitoring of the disease.

2 Normative references

The document has no normative references.

3 Terms and definitions

No terms or definitions need to be defined for this document.

4 Basic information on the fire blight bacterium

Scientific name: Erwinia amylovora (Burrill, 1883) Winslow et al., 1920.

Routes of spread: diseased plant propagating material, including seedlings, rootstocks and scions, insects, wind, rain, and contaminated packaging materials and means of transport, together with other natural and human factors, can all cause the spread of fire blight. Among these, diseased plant propagating material is the main route of long-distance spread.

Further information on the fire blight bacterium is given in Annex A.

5 Principle of the methods

Immunological detection is carried out on the basis of the specific reaction between the fire blight bacterium and antibodies; molecular biological detection is carried out on the basis of specific DNA sequences of the bacterium; and isolation, culture, identification and pathogenicity determination are carried out on the basis of the cultural characteristics, biological properties, host range and damage symptoms of the pathogen.

6 Instruments, equipment and main reagents

Instruments, equipment and utensils: clean bench, constant-temperature incubator, shaker, ultra-low-temperature freezer, ordinary refrigerator, autoclave, constant-temperature water bath, small centrifuge, high-speed centrifuge, PCR instrument, real-time fluorescence PCR instrument, electrophoresis apparatus, horizontal electrophoresis tank, gel imaging system, Biolog microplates, automatic microbial identification system (Biolog), ice maker, electronic balance, vortex mixer and micro-injectors.

Main reagents: two times concentrated PCR reaction premix and two times concentrated fluorescence PCR reaction premix; buffers and culture media are prepared according to Annex B; unless otherwise specified, all reagents are of analytical grade.

7 Quarantine detection and identification methods

Symptom inspection. Plants are inspected for the typical symptoms of fire blight described in Annex A, early spring being the key monitoring period. The shoots, flowers, fruit and trunk are the main parts to inspect: whether shoots show wilting in the shape of a shepherd's crook and blacken and die; whether flowers and young fruit blacken and die;

whether the leaf veins blacken upward from the base of the petiole; and whether the trunk bears canker patches and whether bacterial ooze is present on the diseased tissue. Plants showing typical or suspect symptoms are photographed and recorded, and symptomatic shoots, flower clusters, branches and fruit are collected and sent to the laboratory for detection and identification. On young shoots that do not show typical symptoms, sampling focuses on the tip and the base.

Preparation of samples from symptomatic plants. Samples are tested as soon as possible after collection; if immediate testing is not possible they are kept at 4 °C to 8 °C, if possible for no more than a week, and cross-contamination is avoided during collection, transport and handling. Sample processing uses a general procedure valid for isolation and culture, immunological detection and molecular biological detection: the sample is treated with sterile distilled water or with phosphate buffered saline at pH 7.2 and used directly. Plant parts showing the most typical symptoms and bearing bacterial ooze, such as flowers, shoots, young branches, leaves or fruit, are chosen; tissue is picked at the boundary between diseased and healthy parts, cut into pieces of 0.1 g to 1.0 g, placed in a centrifuge tube or Petri dish containing an appropriate amount of phosphate buffered saline or sterile distilled water, ground or squeezed, and left to stand for at least 5 min to give a sample suspension for the subsequent tests.

Preparation of samples from symptomless plants. Symptomless samples are processed singly or in groups of at most ten, and cross-contamination is avoided during processing and extraction. From the beginning of flowering to the mid-stage of fruit swelling, flower parts, young fruit and young shoot segments are collected into sterile bags or containers; shoots of about 20 cm in length or flower parts at flowering are cut from the plants to be tested. For winter sampling, five to ten flower buds are collected from each plant. In the laboratory, flower parts at flowering, flower stalks and the petiole bases or stem segments of several leaves at the lower part of the shoots are cut from the selected plants; 0.1 g to 1.0 g of plant material is weighed and soaked in the sterile distilled water or phosphate buffer described above to give a sample suspension. Where a sample consists of several plant materials, it can be placed in a sterile conical flask with an appropriate amount of phosphate buffered saline or sterile distilled water and shaken vigorously on a shaker at room temperature for 1 h to give a sample suspension for direct analysis; alternatively the suspension is transferred to a 50 mL centrifuge tube and centrifuged at 12 000 r/min for 20 min, the supernatant is discarded, and 2 mL of sterile distilled water or phosphate buffer is added to resuspend the pellet, giving an enriched sample for the subsequent tests.

Immunological detection. For samples with suspect symptoms, such as fresh leaves and young fruit, a commercial immuno-colloidal gold test strip can be used for on-site preliminary screening. A commercial enzyme-linked immunosorbent assay (ELISA) is used for detection, carried out and interpreted according to the instructions supplied with it. If the result is negative, the sample is judged as not carrying the fire blight bacterium. If the result is positive, isolation, culture, identification and pathogenicity determination are required.

Immunological detection may also be omitted and molecular biological detection carried out directly.

Template preparation. For plant samples, after a sample suspension or enriched sample has been prepared as in 7.2, total DNA is extracted directly with a commercial plant genomic DNA extraction kit according to its instructions and kept at minus 20 °C for later use. Suspect strains obtained by isolation and culture are used directly or after lysis, an appropriate amount of bacterial suspension being placed in a 1.5 mL centrifuge tube, held in a boiling water bath for 10 min and centrifuged at 12 000 r/min for 10 min, the supernatant being kept at minus 20 °C as the template for molecular biological detection.

PCR and gel electrophoresis. A standard strain of E. amylovora serves as the positive control, a plant bacterium other than E. amylovora as the negative control, and double distilled water in place of template as the blank control. Two pairs of specific amplification primers targeting the plasmid pEA29 of the fire blight bacterium and its chromosomal genes are used for PCR amplification of the samples under test, followed by gel electrophoresis; the detailed steps follow Annex C.

Real-time fluorescence PCR. The detailed steps follow Annex D, with the same control arrangements as for PCR and gel electrophoresis.

Isolation, culture and identification. After a sample suspension or enriched sample has been prepared as in 7.2, a sterile inoculating loop is used to streak the suspension on LB agar plates in a clean bench, or the suspension is diluted tenfold in series with sterile water and 100 µL of each dilution is spread on plates, each in three replicates. The plates are placed in a constant-temperature incubator at 25 °C for 24 h to 48 h, suspect single colonies are picked and purified, and are subcultured two to three times before pathogenicity determination, Biolog identification, and immunological or molecular biological identification. On LB medium the fire blight bacterium forms milky white colonies that are relatively large and raised, smooth and lens-shaped, whereas the common contaminant Erwinia herbicola forms yellow colonies on LB medium.

Biolog identification. After culture, the isolate is transferred to BUG medium and grown at 30 °C for 24 h; following the Biolog instructions, the turbidity of the isolate suspension is adjusted and 100 µL is added to each well of a Biolog GN microplate or GEN III microplate. The microplates are incubated at 30 °C for 18 h and then read with the Biolog instrument to obtain the test result.

Pathogenicity determination, young fruit inoculation test. Young pear fruit of about 3 cm to 5 cm in diameter are washed and air-dried, cut transversely with a sterile blade and placed on a Petri dish lined with sterile filter paper with a small amount of sterile water. Colonies to be identified, grown on LB medium for 24 h to 36 h, are picked up with an inoculating needle and inoculated into the flesh of the young fruit by pricking, three to four points on one cut face. After inoculation the fruit are kept moist at 26 °C to induce disease and the