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Detection and Identification

Conventional detection methods

Detection is tracing of plant pathogenic bacteria in or on plant material, especially when they occur latently, without causing symptoms. It should be clearly distinguished from identification, what is characterization and naming of bacteria. Traditional detection involves plant tissue that is washed or macerated, filtrated and often centrifuged. Thereafter a small aliquot of the suspension is streaked onto a suitable non selective or (semi-)selective medium. Often also some of the suspension is inoculated into a test plant, which may act as a selective medium. These methods however are labour-intensive and take a lot of time.

Conventional identification methods

In identification of a pure culture using conventional methods the following phenotypic characteristics can be determined:

- **Morphology of the bacterial colony-** Form, colour and smell of a colony may be an indication of a bacterial pathogen, but many other non-pathogenic bacteria may form similar colonies. Diffusible pigment production is also of importance in the first screening of a bacterium
- **Morphology of the bacterial cells** -Plant pathogens are Gram-positive or -negative rods or they are filamentous (*Streptomyces* spp.). Again many other non-pathogenic bacteria may show a similar morphology and further tests are necessary.
- **Physiological characteristics-** Determination of growth at different temperatures e.g. 4ºC or 37oC; thermal death point (for plant pathogenic bacteria usually 50-55ºC, when kept for 10 minutes at this temperature in liquid medium), growth at different levels of NaCl e.g. 2, 5 and 7%etc. Furthermore screening for toxin production and ice-nucleation activity will yield additional characteristics useful in the identification of plant pathogenic bacteria, especially those belonging to the *Pseudomonas syringae* group and *Clavibacter* spp. Detection of these substances nowadays often takes place via molecular detection of the responsible genes.
- **Biochemical characteristics-** In biochemical tests the expression of diverse enzyme systems of the bacterium is determined. By offering the nutrients to the bacteria in a culture tube or agar plate, the action of these enzyme systems can be checked.

Some typical tests used for biochemical characterization of plant pathogenic bacteria are mentioned below:

Arginine dihydrolase

Test for enzymes that enable certain otherwise aerobic bacteria (e.g. pseudomonads) to grow anaerobically. The enzymes generate ATP by decomposition of arginine to CO2 and NH3. The change in pH due to NH3 formation is determined by a colour reaction in a semi solid agar medium from light orange to cherry red.

Catalase

The catalase enzyme decomposes hydrogen peroxide (H_2O_2) to water and O2. Hydrogen peroxide is very toxic for bacteria. Bacteria are smeared in a drop of hydrogen peroxide and checked for formation of oxygen gas bubbles. Most bacteria are catalase positive.

Pectin hydrolysis

Pectic enzymes are important for degradation of middle lamellae and cell walls of plants by bacteria. Media with sodium polypectate have been developed (single or double layer) that allow detection of pectinolysis in the form of pits in the medium.

Oxidase test

This test demonstrates the presence of cytochrome c respiratory enzyme by oxidation of tetramethyl-ρ-phenylenediamine. Some bacterial growth is rubbed into a drop of the chemical on a filter paper. In a positive reaction a deep purple substance is formed. The test is especially useful for identification of pseudomonads and erwinias.

Nitrate reduction and denitrification

Obligate aerobic bacteria can use nitrate instead of oxygen in anaerobic conditions as an electron acceptor. They reduce nitrate to nitrite and then to N2. Bacteria are grown in medium with KNO3, where after nitrite is demonstrated with sulfanilic acid and α- naphtylamine.

Non conventional/ Modern/ Molecular Identification Techniques-

Serological Techniques

The basic principle of serological techniques lies on the fact that the antigen (target pathogens) is detected using antibodies (specific to the target pathogens concerned). Mono- and poly-clonal antibodies are required to develop for specific immunological diagnostic assays. The following serological methods and techniques are mostly used:

a) **Agglutination test** on a microscopic slide. If antibodies (in antiserum) and a suspension of bacteria are mixed in a certain concentration they will clump together and an agglutination reaction takes place
b) **Precipitation test-** In this test only certain soluble antigenic proteins or polysaccharides of the bacteria react with the antibodies. Bacteria, often killed and disrupted by phenol, are placed in wells in the agar plate. Soluble antigens and antibodies diffuse in the agar. Where they meet in a certain concentration they bind form flakes and precipitate, visible as a white line.
c) **Immunofluorescence (IF)-** This is a very **sensitive and robust** serological test because the primary reaction of antigen and antibody is made visible. Binding reactions can be observed at very high titres of antiserum. In the IF test antibodies are marked with a chemical dye fluorescein isothiocyanate (FITC) that fluoresces in blue light.

d) **Enzyme linked immunosorbent assay (ELISA)** Through this technique, the bacterial pathogens can be detected directly in the infected plant materials without culturing the bacteria. Owing to its simplicity, sensitivity and adaptability, this technique has gained popularity in different diagnostic

laboratories. Both poly and monoclonal antibodies are used for the detection of phytopathogenic bacteria using ELISA assay. In this technique reaction is visualized through an enzyme-substrate hybridization. The enzyme is usually alkaline phosphatase and substrate is p-nitrophenylphosphate.

- First antibodies are coated in the wells of a plastic ELISA plate.
- Bacterial antigens in buffered sample solution are subsequently added in the wells and trapped by the coated antibodies.
- After incubation and washing an enzyme-labelled antiserum (conjugate) is added. Only wells where antigens reacted with the enzyme will change in colour after incubation with a suitable enzyme substrate.

Monoclonal antibodies (MA)

When a plant pathogenic bacterium is injected into a rabbit, different cell types of the rabbit produce normally many types of antibodies. The antiserum taken from such a rabbit is called a **polyclonal serum**. In the MA technique one antibody-producing cell is selected (B-lymphocyte from a mouse) which produces only one type of antibodies against one antigen (or epitope) of the bacterium. The B-lymphocyte is then fused with a cancer cell (plasma cell) of the mouse to produces a hybrid cell called hybridoma. With these hybridomas many copies of monoclonal antibodies can be produced with very constant quality and of a high specificity. Reactivity of monoclonal sera is usually lower than that of polyclonal sera, because only one epitope of the bacterium will show the binding reaction.

Nucleic Acid based Techniques:

DNA/RNA (dot - blot) hybridization

This is a technique that has become less common for detection after the introduction of the PCR technique. In blot hybridization antibodies are not used to bind with the target bacterium, but genetic material (DNA, RNA) of the bacterium itself. Due to its high specificity the DNA or RNA hybridization can also potentially be used simultaneously for detection and identification. A sample of plant tissue suspected of bacterial contamination is treated in such a way that as much as possible non-DNA material is removed. Thereafter DNA cleavage takes place under low pH conditions. The now single-stranded (target) DNA is fixed on a nitrocellulose filter and then a DNA probe is added. This probe contains a large number of specific nucleic acid molecules of the target bacterium. The nucleic acid probe binds to the complementary parts on the single strands present on the filter, this process is called hybridization.

Polymerase chain reaction (PCR)

With this method a specific part of the nucleic acid of the target bacterium is artificially multiplied before detection takes place. This multiplication is reached by repeated cycles of:

1) **Denaturation** (melting) of nucleic acid, usually at 95ºC.

2) **Annealing** of short (specific) strands of nucleic acids, so-called **primers** (these primers have been artificially assembled and consist of a sequence known to be specific for the target organism), at 68ºC (depending on the primers used).

3) **Extension** of nucleic acid strands at 72ºC in the presence of free nucleotides and a thermostable nucleic acid polymerase (usually ***Taq* polymerase**, originally isolated

from the hot-spring thermophylic bacterium *Thermus aquaticus*, but also available as an artificially synthesized product). After multiplication the PCR products can be visualized on an ethidium bromide-stained agarose gel via electrophoresis.

Restriction fragment length polymorphism (RFLP)

RFLP analysis has been extensively used in detection and identification of plant pathogens. A small DNA segment from a known bacterium, pathogenic to the host plant in question, is used as a probe. The DNA from both the known (as positive control) and suspected bacterial pathogen (isolated from infected plant samples) are digested with the same restriction enzyme(s) that cut DNA in to fragments of varying size. The samples of both control as well as unknown digested bacterial DNA are placed side by side in an agarose gel, and are then separated by size using electrophoresis. Then the DNA fragments in gel are transferred to a nylon membrane for hybridization with radioactive DNA probe. The probe will hybridize with its complementary sequence (if present). Then hybridization is visualized through X- ray film, a dark band will appear at each location where the probe hybridized to complementary DNA. These banding patterns will determine whether the unknown specimen is same to the bacterium that was used as probe.

Random amplified polymorphic DNA (RAPD) analysis

With the RAPD method patterns are obtained by PCR-amplification of genomic DNA with arbitrary, short (10 bp) primers (this means that in this case the primers are nonspecific and will bind at arbitrary places on the DNA strand) at a permissive annealing temperature of 36-45ºC. The pattern of amplification products is usually discriminative at low taxonomic level, often strain level. No prior sequence information about the target, no probe, no blotting and hybridization and no restriction are necessary, making it a very fast method.

Repetitive extragenic palindromic (REP)-PCR

REP-PCR is a fingerprinting technique that uses repetitive sequences (mostly of unknown function, that are interspersed throughout the DNA) present in the genomic DNA of bacteria. Whole cells can be used or DNA extracted from a pure culture of the bacteria. Primers are used that specifically anneal to these sequences and multiply the DNA between two repetitive sequences. The primers are rather long and, therefore, higher annealing temperatures that enable greater stringency and less variation can be used. Approximately 10-40 PCR fragments are generated ranging in size from 200 bp to > 6 kbp. These fragments are separated by size in electrophoresis on a gel and visualized in ethidium bromide-stained gels. The band patterns can be scanned, digitized and used for identification.

Polyacrylamide gel electrophoresis (PAGE) of whole cell proteins

With PAGE usually total protein profiles of bacteria are compared. Approximately 20-40 different protein classes can be made visible. The proteins have first to be extracted and denatured with a detergent (sodium dodecyl sulfate, SDS) and mercaptoethanol. The proteins lose their 3-dimensional structure and are negatively charged. Subsequently the mixture of the denatured, negatively charged proteins is loaded on a porous polyacrylamide gel in an electric field. The proteins will migrate to the positive pole, where the smaller ones will migrate faster than the bigger ones (less resistance in

the gel). After separation the proteins are stained and the protein bands can be compared.

Gas chromatographic Fatty Acid Analysis (FAA)

In this technique the total fatty acid profiles of bacteria are compared. Bacteria contain lipids in concentrations of 0.2 to 50% of the dry weight, usually 5%, mainly in their membranes. The lipids important for the technique are those containing esterified fatty acids, such as a) phospholipids, present in the cell membrane, b) lipid A in lipopolysaccharide of Gram-negative bacteria and c) ß polyhydroxybutyrate, present in storage material. Generally a bacterium contains a certain number (10-30) of different fatty acids, which may differ quantitatively and qualitatively from those of other bacteria.

Analysis of the total fatty acid profile is performed by gas chromatography. The bacteria are lysed by boiling, the fats saponified to release the fatty acids, the fatty acids methylated to make them more volatile, and fatty acid methyl ester (FAME)extracted. After gas chromatography the profile obtained can be compared (matched) with a large number of reference profiles present in the database (library) of a connected computer. The computer identifies the fatty acids, determines deviations from a reference fatty acid mixture and presents the identification of the bacterium and the percentage of similarity.

Bacteriophage typing- Specific bacterial viruses (bacteriophages) have sometimes been used in identification. Phages, however, are usually too specific or not specific enough. An example where bacteriophage typing for strain identification was successful is described by Du *et al.* (1982). Three bacteriophages of *Xanthomonas arboricola* pv. *pruni* isolated from soil under diseased plum trees were used to type six South African and four other *X. a.* pv. *pruni* isolates which were previously shown to be serologically closely related. According to patterns of lysis and efficiency of plaque formation by the phages, the bacterial isolates are categorised in to distinct groups.