TWINNING PROJECT BA/12/IB/AG01

“ Act. 3.3 „Train laboratory staff on laboratory methods for diagnosing harmful organisms”

Diseases symptoms cased by the quarantine bacteria.

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(All pictures from EPPO web side – only for training purposes)

Quarantine bacteria according the 2000/29/EC Directive Of 8 May 2000

On protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community

ANNEX I

PART A

HARMFUL ORGANISMS WHOSE INTRODUCTION INTO, AND SPREAD WITHIN, ALL MEMBER STATES

SHALL BE BANNED

S e c t i o n I

HARMFUL ORGANISMS NOT KNOWN TO OCCUR IN ANY PART OF THE COMMUNITY AND RELEVANT FOR THE

ENTIRE COMMUNITY

Bacteria

1. *Xylella fastidiosa* (Well and Raju)

S e c t i o n I I

HARMFUL ORGANISMS KNOWN TO OCCUR IN THE COMMUNITY AND RELEVANT FOR THE ENTIRE

COMMUNITY

Bacteria

1. *Clavibacter michiganensis* (Smith) Davis et al. ssp. *sepedonicus* (Spieckermann and Kotthoff) Davis et al.

2. *Pseudomonas solanacearum* (Smith) Smith

ANNEX II

PART A

HARMFUL ORGANISMS WHOSE INTRODUCTION INTO, AND SPREAD WITHIN, ALL MEMBER STATES

SHALL BE BANNED IF THEY ARE PRESENT ON CERTAIN PLANTS OR PLANT PRODUCTS

S e c t i o n I

HARMFUL ORGANISMS NOT KNOWN TO OCCUR IN THE COMMUNITY AND RELEVANT FOR THE ENTIRE

COMMUNITY

Species and Subject of contamination

1. **Citrus greening bacterium /**Plants of Citrus L., Fortunella Swingle, Poncirus Raf., and their hybrids, other than fruit and seeds

2. **Citrus variegated chlorosis /**Plants of Citrus L., Fortunella Swingle, Poncirus Raf., and their hybrids, other than fruit and seeds

3. ***Erwinia stewartii*** (Smith) Dye / Seeds of Zea mais L.

4. ***Xanthomonas campestris*** (all strains pathogenic to Citrus) / Plants of Citrus L., Fortunella Swingle, Poncirus Raf., and their hybrids, other than seeds

5**.** ***Xanthomonas campestris*** pv. ***oryzae*** (Ishiyama) Dye and pv. oryzicola (Fang. et al.) Dye / Seeds of Oryza spp.

S e c t i o n I I

HARMFUL ORGANISMS KNOWN TO OCCUR IN THE COMMUNITY AND RELEVANT FOR THE ENTIRE COMMUNITY

Species and Subject of contamination

1. ***Clavibacter michiganensis*** spp. ***insidiosus*** (McCulloch) Davis et al. / Seeds of Medicago sativa L.

2. ***Clavibacter michiganensis*** spp. ***michiganensis*** (Smith) Davis et al. / Plants of Lycopersicon lycopersicum (L.) Karsten ex Farw., intended for planting

3. ***Erwinia amylovora*** (Burr.) Winsl. et al. / Plants of Chaenomeles Lindl., Cotoneaster Ehrh., Crataegus L., Cydonia Mill., Eriobotrya Lindl., Malus Mill., Mespilus L., Pyracantha Roem.,Pyrus L., Sorbus L. other than Sorbus intermedia (Ehrh.) Pers. and Stranvaesia Lindl., intended for planting, **other** than seeds

4. ***Erwinia chrysanthemi*** pv. ***dianthicola*** (Hellmers) Dickey / Plants of Dianthus L., intended for planting, other than seeds

5. ***Pseudomonas caryophylli*** (Burkholder) Starr and Burkholder / Plants of Dianthus L., intended for planting, other than seeds

6. ***Pseudomonas syringae pv. persicae*** (Prunier et al.) Young et al. / Plants of Prunus persica (L.) Batsch and Prunus persica var. nectarina (Ait.) Maxim, intended for planting, other than seeds

7. ***Xanthomonas campestris pv. phaseoli*** (Smith) Dye / Seeds of Phaseolus L.

8. ***Xanthomonas campestris*** pv. ***pruni*** (Smith) Dye / Plants of Prunus L., intended for planting, other than seeds

9. ***Xanthomonas campestris*** pv. ***vesicatoria*** (Doidge) Dye / Plants of Lycopersicon lycopersicum (L.) Karsten ex Farw. and Capsicum spp., intended for planting

10. ***Xanthomonas fragariae*** Kennedy and King / Plants of Fragaria L., intended for planting, other than seeds

11. ***Xylophilus ampelinus*** (Panagopoulos) Willems et al. / Plants of Vitis L., other than fruit and seeds

ANNEX II

PART B

HARMFUL ORGANISMS WHOSE INTRODUCTION INTO, AND WHOSE SPREAD WITHIN, CERTAIN

PROTECTED ZONES SHALL BE BANNED IF THEY ARE PRESENT ON CERTAIN PLANTS OR PLANT

PRODUCTS

Species, Subject of contamination and Protected zone(s)

1. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Hedges) Collins and Jones

-Seeds of Phaseolus vulgaris L. and Dolichos Jacq.

Greece, Spain, Portugal

2. ***Erwinia amylovora*** (Burr.) Winsl. et al. / Parts of plants, other than fruit, seeds and plants intended for planting, but including live pollen for pollination of *Chaenomeles*, *Cotoneaster, Crataegus*, *Cydonia*, *Eriobotrya*, *Malus*, *Mespilus*, *Pyracantha*, *Pyrus*, *Sorbus* (other than *Sorbus intermedia*) (Ehrh.) Pers. and *Stranvaesia* Lindl.

E, F,IRL, I, P, UK,A, FI

Tomato

Clavibacter michiganensis* ssp. *michiganensis

bacterial canker of tomato

- causes systemic infection of tomato plants. The pathogen can also cause spots on leaves, petioles, peduncles and fruits as a result of a local infection, usually under overhead irrigation. There are a wide range of symptoms, which vary with place of production (glasshouse or field), age of the plant at time of infection, cultural practices, cultivar, etc.

Tomato

Clavibacter michiganensis* ssp. *michiganensis

- Symptoms can be divided into **two types**, depending on whether infection is systemic within the vascular tissue or superficial.

Tomato

Clavibacter michiganensis ssp. *michiganensis*

- In systemic infections, the disease can often be recognized at an early stage by dull green, oily areas between the leaf veins that rapidly desiccate, appearing subsequently as pale brown necrotic areas, frequently marginal, giving the plant a scorched appearance. Small affected areas may coalesce and produce larger necrotic zones

Tomato

Clavibacter michiganensis ssp. *michiganensis*

- Downward turning of one or a few of the lower leaves occurs as the systemic infection progresses, and often the leaflets along one side of a leaf become flaccid, at least during periods of enhanced evapotranspiration.

Tomato

Clavibacter michiganensis* ssp. *michiganensis

- Under favourable conditions for symptom development (25–30°C and evapotranspiration stress), entire leaves wilt and shrivel within a few days. Finally, the whole plant wilts and desiccates.

Tomato

Clavibacter michiganensis ssp. *michiganensis*

Under less favourable conditions, irreversible wilting will be delayed and the plant may not show any wilting when defoliation is carried out for crop management. Fruits of systemically infected plants may fail to develop, ripen unevenly or fall. They can appear marbled with longitudinal chlorotic streaks and internal bleaching of vascular and surrounding tissues.

Tomato

Clavibacter michiganensis ssp. *michiganensis*

Typical leaf or fruit spot symptoms, arising from external infection, are found less frequently in glasshouse crops than in open-field crops.

Tomato

Clavibacter michiganensis ssp. *michiganensis*

Following systemic infections, the vascular tissues of transversely cut stems of wilted plants usually appear dark yellow to brown, in particular at the nodes. The vascular parenchyma in particular has a mealy appearance resulting from bacterial degradation and ooze production.

Tomato

Clavibacter michiganensis* ssp. *michiganensis

The pith may collapse completely. Systemic presence of *C. michiganensis* subsp. *michiganensis* can be detected by suspending a stem section with the cut surface in a glass tube with tap water. Bacterial exudate will spontaneously make a milky suspension after a few minutes.

Tomato

Clavibacter michiganensis* ssp. *michiganensis

Superficial infections occur when bacteria multiply on the plant surface or within surface wounds and stomata. Leaves, stems and calyces may show a mealy appearance, as if dusted with coarse flour. Close examination reveals raised or sunken blisters, which are usually white to pale orange. Mealy spots on stems are usually more discrete than those on leaves

Tomato

Clavibacter michiganensis* ssp. *michiganensis

A common leaf symptom is a dark brown spot surrounded by a yellow–orange area, usually at the edge of the leaf, which results from infection of a water-excreting gland (hydathode). Leaflets of the oldest infected leaves then curl, and their margins yellow and become necrotic. Plants grow poorly and gradually entire plants desiccate.

Tomato

Clavibacter michiganensis ssp. *michiganensis*

Yellow streaks may develop along the stem and occasionally these split open at the nodes, forming cankers. Blister lesions on stems are sometimes observed.

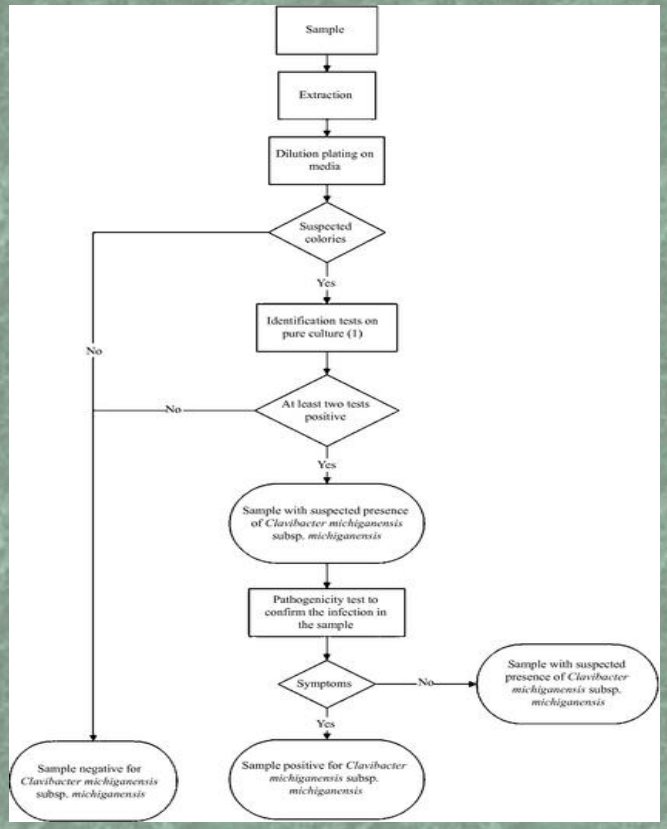
Tomato

Clavibacter michiganensis ssp. *michiganensis*

Symptoms on affected fruits may begin as small, slightly raised lesions with a white margin or halo. These lesions may expand to a few millimeters with brown, roughened centers known as ‘bird's-eye’ spots. Several lesions may be formed near the calyx where fruits touch in a cluster. The vascular tissues under the calyx scar and those leading to the seeds may be dark yellow to brown.

Tomato

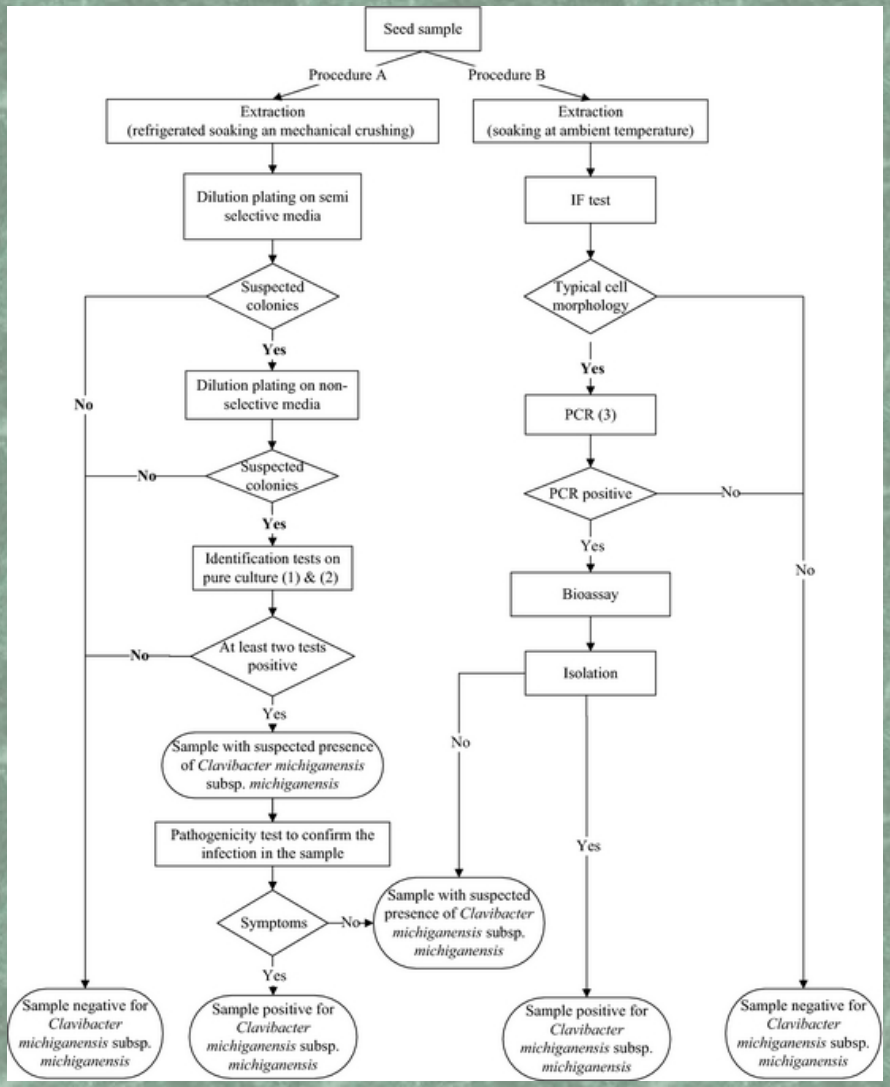
Clavibacter michiganensis ssp. *michiganensis*



Flow diagram for detection and identification of *Clavibacter michiganensis* subsp. *michiganensis* in symptomatic samples

Tomato

Clavibacter michiganensis ssp. *michiganensis*



Flow diagram for detection and identification of *Clavibacter michiganensis* subsp. *michiganensis* in seed samples.

Tomato

Xanthomonas campestris pv. *vesicatoria*

Xanthomonas spp. causing bacterial spot of tomato and sweet pepper (*Xanthomonas euvesicatoria*, *Xanthomonas gardneri*, *Xanthomonas perforans*, *Xanthomonas vesicatoria*).

Tomato

Xanthomonas campestris pv. *vesicatoria*

Symptoms

Lycopersicon esculentum

On tomato leaves, lesions appear as irregular, water-soaked areas that are green at first, becoming brown and necrotic later. Lesions are frequently surrounded by large chlorotic haloes. Foliar blight can occur when the lesions coalesce. Necrosis of the petioles and canker-like splits can be observed along the stem. On tomato leaves, bacterial speck lesions (*P. syringae* pv. *tomato*) look similar initially but are surrounded by a more distinct yellow halo.

Tomato

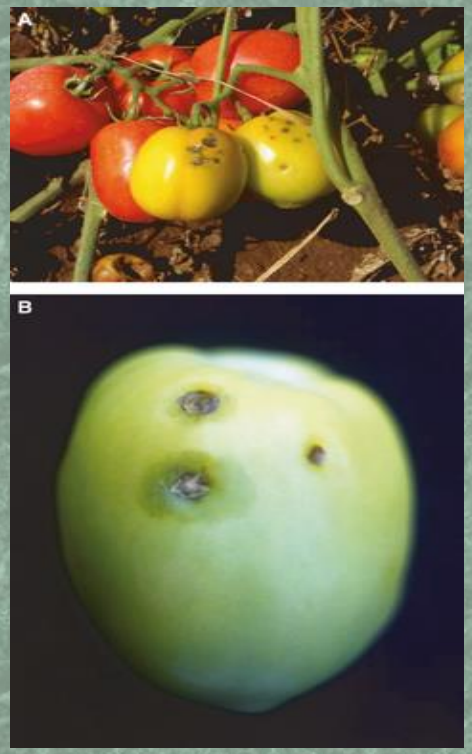
Xanthomonas campestris pv. *vesicatoria*

Lesions on fruits begin as tiny, slightly raised blisters. Subsequently, the spots increase in size and become brownish, scab-like, raised and surrounded by a water-soaked halo. Several lesions can coalesce. On tomato fruits, *P. syringae* pv. *tomato* causes smaller, blackish lesions without a scab-like appearance

Tomato

Xanthomonas campestris pv. *vesicatoria*

Typical bacterial spot lesions on the surface of tomato fruits.



Tomato

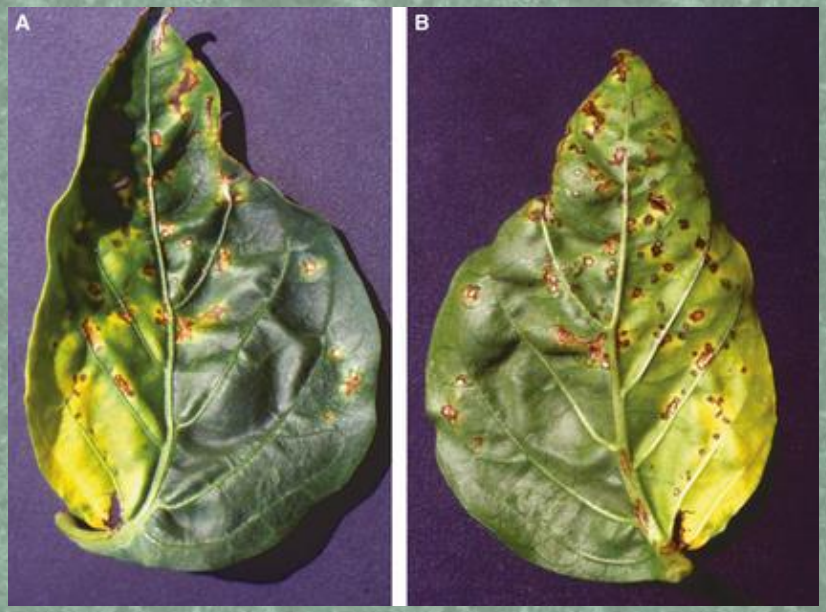
Xanthomonas campestris pv. *vesicatoria*

Capsicum annuum

Lesions on *Capsicum annuum* leaves are of irregular shape and necrotic, in some cases surrounded by a chlorotic halo. When the infection is severe, foliar blight can occur and leaves may fall. On fruits, scab-like, raised, whitish lesions appear.

Tomato

Xanthomonas campestris pv. *vesicatoria*



Bacterial spot lesions on pepper leaf (upper and lower surface).

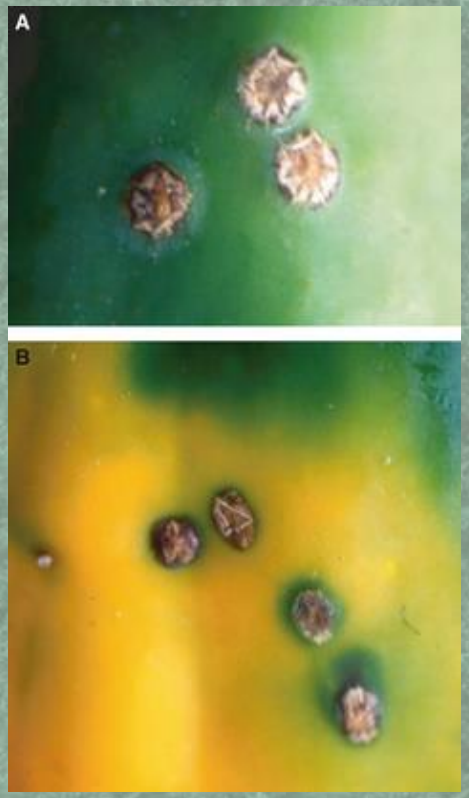
Tomato

Xanthomonas campestris pv. *vesicatoria*

Xanthomonas perforans leaf spot symptoms on pepper. Figure A shows small necrotic leaf spots while figures B-D show large necrotic leaf sports with perforated centers.

Tomato

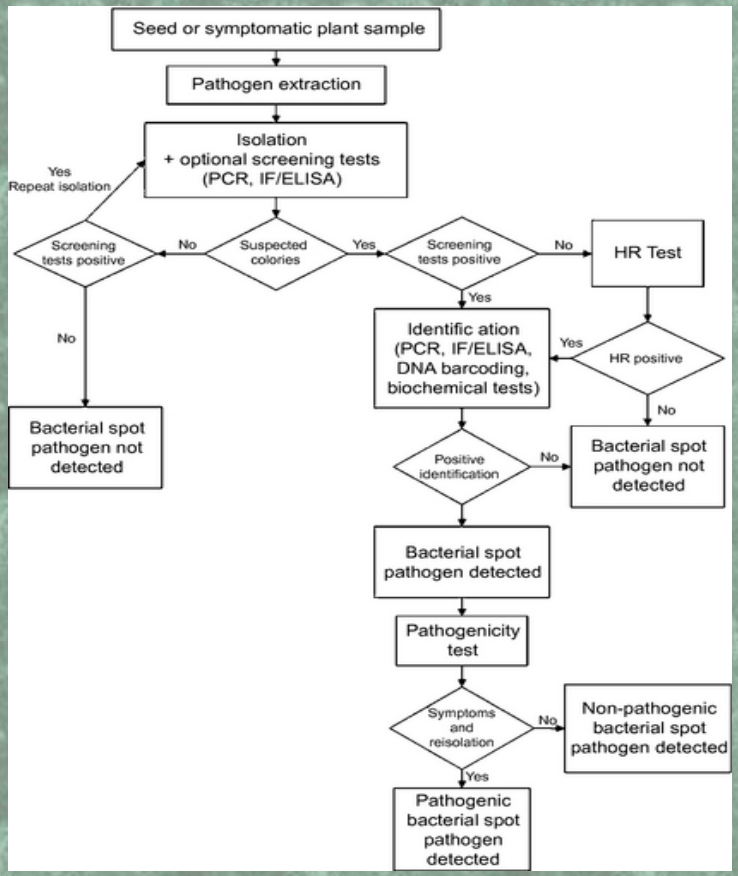
Xanthomonas campestris pv. *vesicatoria*



Typical bacterial spot lesions on the surface of pepper fruit.

Tomato

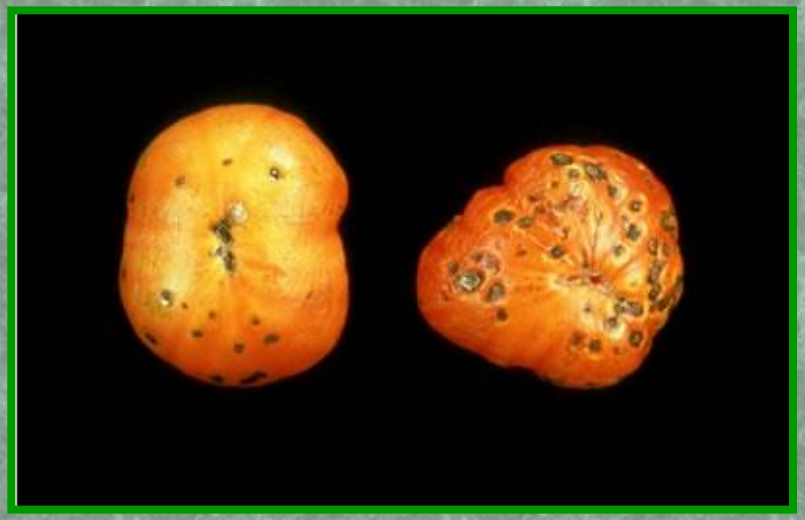
Xanthomonas campestris pv. *vesicatoria*



Flow diagram for testing tomato and pepper seed and plant samples to detect *Xanthomonas* spp. causing bacterial spot.

Tomato

Xanthomonas campestris pv. *vesicatoria*



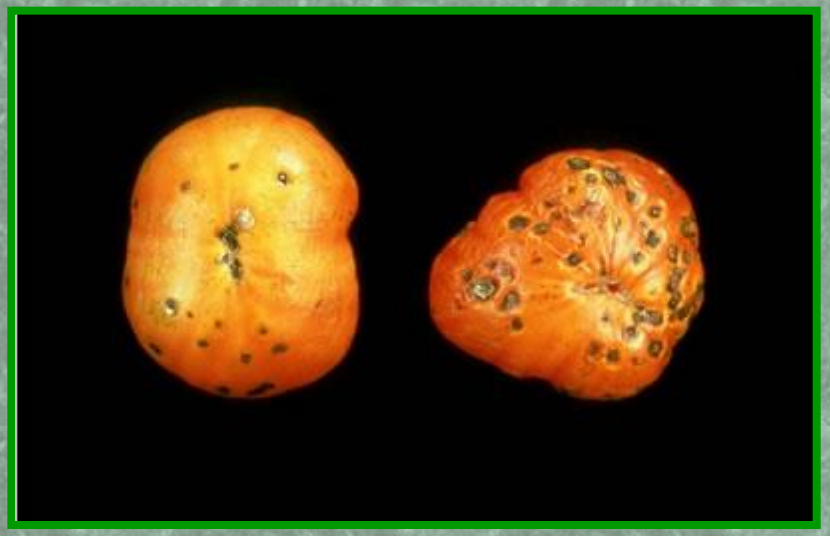
Tomato

Xanthomonas campestris pv. *vesicatoria*

Tomato

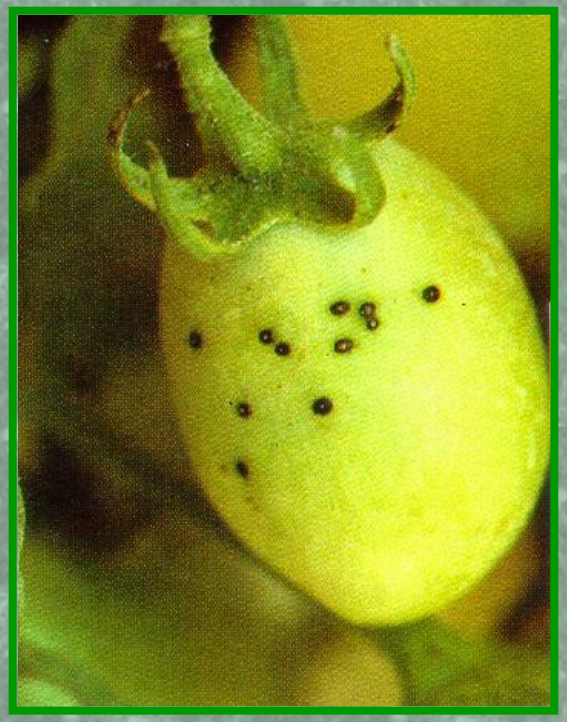
Xanthomonas campestris pv. *vesicatoria*

necrotic spots caused by *Pseudomonas syringae pv. tomato* of diameter <1 mm.



Xanthomonas campestris pv. *vesicatoria*

Clavibacter michiganensis ssp. *michiganensis*



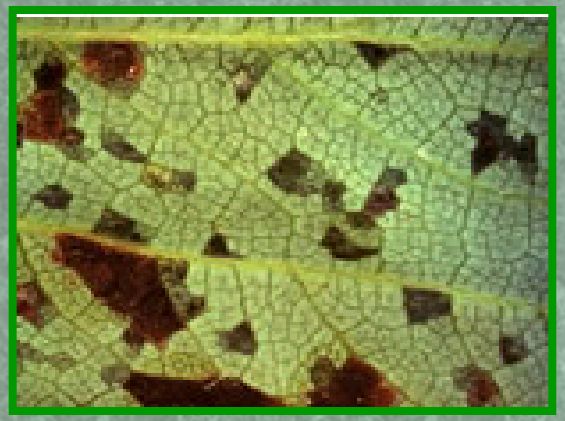
Pseudomonas syringae pv. *tomato*

Strawberry

Xanthomonas fragariae

Xanthomonas fragariae is the causal agent of bacterial angular leaf spot of strawberry.

The natural hosts of *X. fragariae* are *Fragaria x ananassa*, its parents *Fragaria chiloensis* and *Fragaria virginiana*, and various wild strawberries such as *Fragaria vesca.*



Strawberry

Xanthomonas fragariae

Small (1–4 mm) angular water-soaked spots appear initially only on the lower leaf surface surrounded by the veins. In the early stage, the spots are only visible on the lower surface and appear translucent when viewed with transmitted light. The bacteria are disseminated from the spots by irrigation, rain or dew to initiate new infections, frequently along the main veins of the leaf

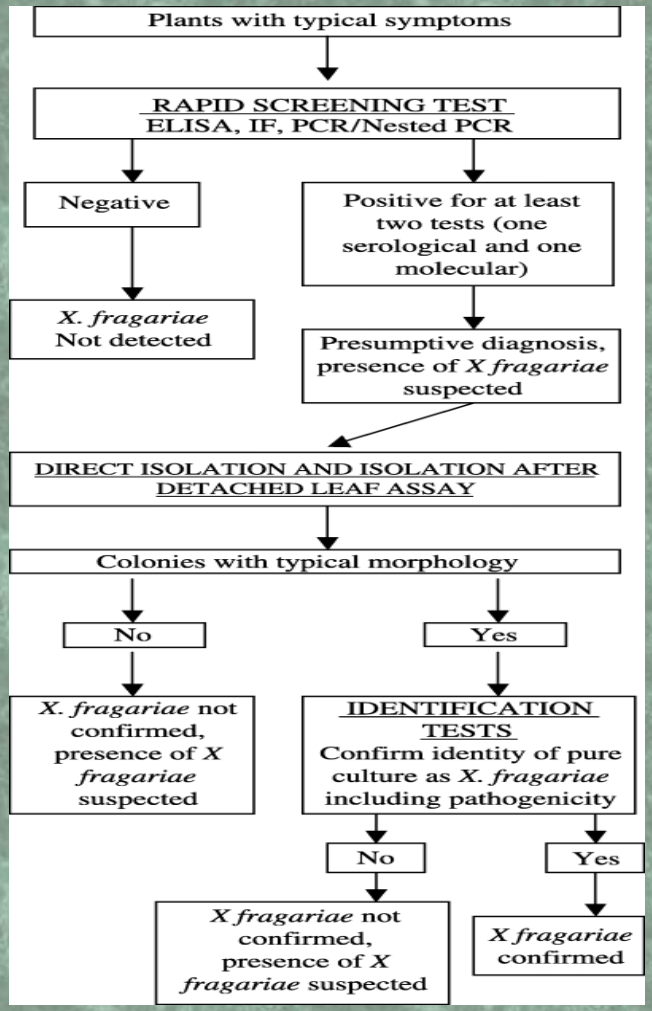
Strawberry

Xanthomonas fragariae

The leaves are most sensitive when they are two weeks to two months old. Older and younger leaves are resistant to infection. The size of the lesions increases progressively and, subsequently, the spots may coalesce and become apparent in the upper surface of the leaf. Dead tissues appear as reddish-brown irregular spots, and tear and break off. Symptoms of angular leaf spot caused by *X. fragariae* may be confused with those caused by fungi like *Mycosphaerella fragariae* and with the symptoms caused by a new pathovar of *X. arboricola*, pv. *fragariae*

Strawberry

Xanthomonas fragariae



Flow diagram for the diagnosis of bacterial angular leaf spot (*Xanthomonas fragariae*) on host plants with symptoms.

Carnation

Burkholderia caryophylli

Causes a wilt of carnation. It also may cause stem cracking and a progressive rot of stems and roots. It used to be a major problem in carnation production in the USA

Carnation

Burkholderia caryophylli

B. caryophylli is now considered to be an uncommon pathogen in commercial carnation production. *Dianthus caryophyllus* and a few other *Dianthus* spp. are the only known natural hosts. The pathogen is a typical member of the genus *Burkholderia*, taxonomically distinct from all other known species.

Carnation

Burkholderia caryophylli

Infection usually occurs during taking of cuttings, and symptoms may then take several years to develop. Leaves and stems become greyish-green with subsequent yellowing, usually followed by wilting and death. Stem bases may show internodal stem cracking, developing into cankers. The early phase of this cracking may be confused with physiological cracking. Cutting diseased stems often reveals a brownish-yellow ooze.

Carnation

Burkholderia caryophylli

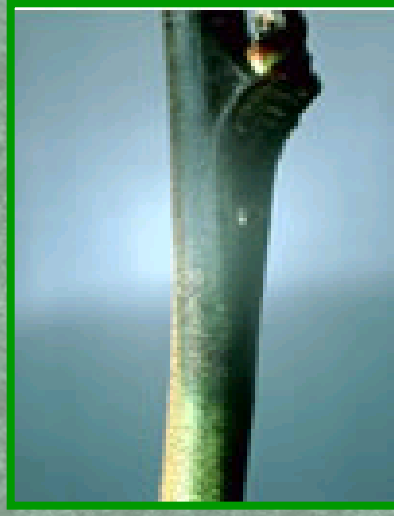
Although there may be an extended period of latency once plants begin showing symptoms, death usually occurs within 1–2 months, often assisted by secondary fungal invasion. Symptoms can be confused with those of *Pectobacterium* (*Erwinia*) *chrysanthemi* pv. *dianthicola (Dickeya dianthicola)* and *Phialophora cinerescens*. Visual symptoms are most readily seen in mature plants by inspection of aerial parts having a greyish-green colour. Wilting symptoms are found more reliably in crops grown at high temperatures (> 30°C) whereas stem-cracking symptoms are more common at lower temperatures (< 20°C).

Carnation

Erwinia chrysanthemi pv. dianthicola

Carnation

Erwinia chrysanthemi pv. dianthicola



Prunus

Pseudomonas syringae pv. *persicae*

Bacterial dieback of peach

In nectarine and peach, symptoms include shoot dieback, limb and root injury, tree death, leaf spots and fruit lesions.
Japanese plum symptoms are mainly confined to dieback, occasional limb death, and leaf spots (Young, 1995). Dieback of terminal shoots can occur already in autumn and in spring following the development of girdling lesions from nodal infections.

Prunus

Pseudomonas syringae pv. *persicae*

Small elliptical lesions may develop at internodes.
The rootstock can also be infected showing symptoms similar to those on woody shoots. Leaf infection results in small, angular, water-soaked spots, the tissue of which becomes brown. The necrotic tissue subsequently falls out, causing a ‘shot hole’ effect.

Prunus

Pseudomonas syringae pv. *persicae*

On fruits, small, round, dark, oily spots occur. These can be spread within the fruit tissue, causing sunken, deforming lesions that ooze gum. Some symptoms of bacterial dieback due to *P. s. persicae* can be confused with those of bacterial canker of stone fruits (*Pseudomonas syringae*pv.*syringae*,*Pseudomonas syringae* pv. *mors-prunorum*) and symptoms of leucostoma canker (*Leucostoma*spp.) or frost injury.

Prunus

Pseudomonas syringae pv. *persicae*

Distinctive characteristics of dieback are discoloration of wood in branches above the necrosis and the absence of an obvious boundary between the morbid and healthy bark in the lower parts of the tree. Bacterial dieback can be disseminate with infected plants for planting or contaminated pruning tools.

Prunus

Xanthomonas campestris arboricola pv. *pruni*

X. a. pruni attacks only *Prunus* species and particularly the fruit crops.

Symptoms on leaves:

On peach leaves, infection is first apparent on the lower surface as small, pale-green to yellow, circular or irregular areas with a light-tan center. These spots soon become evident on the upper surface as they enlarge, becoming angular and darkening to deep-purple, brown or black. The immediately surrounding tissue may become yellow. The diseased areas drop out, usually after darkening in color, but they may drop out prior to the color change, giving a shot-hole appearance to the leaf.

Prunus

Xanthomonas campestris pv. *pruni*

Often, a dark ring of diseased tissue is left with the formation of the shot hole. Spots are usually concentrated towards the leaf tip, because the bacteria accumulate in this region in droplets of rain or dew. Bacterial ooze may be associated with the spots. Severely infected leaves turn yellow and drop off. Atypical symptoms reported for peach include a grey leaf spot on the upper surface, and a case in which bacteria infiltrated a large area, giving the leaf a greenish-yellow, translucent appearance. A severe defoliation can occur, leaving a carpet of yellow chlorotic leaves under the trees of susceptible cultivars.

Prunus

Xanthomonas campestris pv. *pruni*

On plum leaves, initial symptoms are angular water-soaked spots, rapidly turning reddish-brown, then dark brown and necrotic, whereas chlorosis is minimal and less apparent than on peach leaves. The necrotic spots frequently perforate, so that a shot-hole effect can be pronounced. On almonds, apricots and cherries, leaf symptoms are similar to those on peach, but rarely of importance.
Symptoms of bacterial spot on leaves can sometimes be confused with injuries caused by fungi or copper preparations. However, copper lesions are larger (2–6 mm in diameter) and often round in shape

Prunus

Xanthomonas campestris pv. *pruni*

Symptoms on fruits:

On peach fruits, small circular brown spots appear on the surface. They become sunken, the margins are frequently water-soaked, and there are often light-green haloes which impart a mottled appearance to the fruit. As a result of natural enlargement of the fruit, pitting and cracking occur in the vicinity of the spots. These cracks are often very small and difficult to see, but where heavy infection has occurred on young fruits they can be extensive, severely damaging the fruit surface. Gum flow, particularly after rain, may occur from bacterial wounds; this may easily be confused with insect damage. Similar symptoms may appear on apricots and almonds.

Prunus

Xanthomonas campestris pv. *pruni*

On plum fruits, symptoms may be quite different; large, sunken black lesions are common on some cultivars, while, on others, only small pit-like lesions occur. On cherries, early fruit infection results in distorted fruit, and bacteria may be found from the epidermis to the stone.

As a general rule, symptoms on fruits appear 3–5 weeks after petal fall and develop until the skin colour changes, when ripening process begins and some physiochemical parameters change. Symptoms often occur after hail damage

Prunus

Xanthomonas campestris pv. *pruni*

Symptoms on twigs:

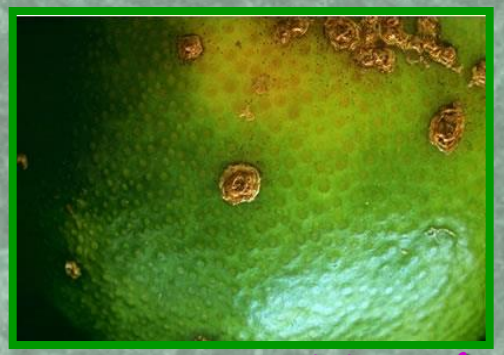
On peach twigs, spring cankers occur on the top portion of overwintering twigs and on water sprouts before green shoots are produced; initially small, water-soaked, slightly darkened, superficial blisters, they extend 1–10 cm parallel to the long axis of the twig and may even girdle it. In this case the tip of the twig may die, while the tissue immediately below the dead area, in which the bacteria are present, is characteristically dark; this is the so-called ‘black tip’ injury.

Prunus

Xanthomonas campestris pv. *pruni*

Twig infections later in the season result in summer cankers, which appear as water-soaked, dark-purplish spots surrounding lenticels. These later dry out and become limited, dark, sunken, circular to elliptical lesions with a water-soaked margin.

On plum and apricot twigs and branches, cankers are perennial, in contrast to peach, and continue developing in twigs 2 and 3 years old. The inner bark is penetrated, resulting in deep-seated cankers which deform and kill twigs.



Citrus

Xanthomonas axonopodis pv. *citri*

citrus bacterial canker

The pathogen causes
necrotic lesions on leaves, stems and fruits. Severe infections can cause defoliation, badly blemished fruits, premature fruit drop, twig dieback and general tree decline. Known hosts are in the family *Rutaceae*, *Citrus* spp. being the hosts of major economic importance. Natural infections are known to occur on *Citrus* hybrids, and on *Poncirus trifoliata* , *Fortunella japonica* , *Fortunella margarita* , *Severinia buxifolia* and *Swinglea glutinosa*. The disease is present in Asia, Africa, North America, South America and Oceania.

Rosacae

Erwinia amylovora

causal agent of fire blight in most species of the subfamily *Maloideae* of the family *Rosaceae*.

Rosacae

Erwinia amylovora

The most economically important hosts :

Amelanchier
Chaenomeles
Cotoneaster
Crataegus
Cydonia
Eriobotrya
Malus
Mespilus
Photinia davidiana
Pyracantha
Pyrus
Sorbus

Rosacae

Erwinia amylovora

Fire blight is probably the most serious disease affecting *Pyrus* spp. (pear) and *Malus* spp. (apple) cultivars in many countries. Although the life cycle of the bacterium is still not fully understood, it is known that it can survive as endophyte or epiphyte for variable periods depending on environmental factors

Rosacae

Erwinia amylovora

The development of fire blight symptoms follows the seasonal growth development of the host plant. It begins in the spring with production of primary inoculum and infection of flowers, continues in summer with infection of shoots and fruits, and ends in autumn with the development of cankers. The pathogen is apparently quiescent through the dormant period of the host

Rosacae

Erwinia amylovora

Symptoms of fire blight on the principal hosts are relatively similar and easily recognized. The name of the disease is descriptive of its major characteristic: the brownish appearance of twigs, flowers and leaves as though burned by fire. Typical symptoms on pome fruit trees are the brown to black color of leaves on affected branches, the production of exudates under humid conditions, and the typical ‘shepherd's crook’ in the shoots. Depending on the affected plant part, the disease causes blossom blight, shoot or twig blight, leaf blight, fruit blight, limb and trunk blight, collar or rootstock blight

Rosacae

Erwinia amylovora

In apple and pear, the first symptoms usually appear in early spring during warm and humid weather, and can progress very quickly under favourable conditions. Flowers appear to be water-soaked, then wilt, shrivel and turn pale brown to black. Peduncles may also appear water-soaked, become dark green, and finally brown or black, sometimes oozing droplets of sticky bacterial exudates. Leaves wilt and shrivel, and entire spurs turn brown in most hosts, or dark brown to black in pear, but remain attached to the tree for some time.

Rosacae

Erwinia amylovora

Immature fruits (or less frequently mature fruits) have infected parts that appear oily or water-soaked, becoming brown to black and often exuding droplets of bacterial ooze.

They also remain attached to the tree. Characteristic reddish-brown streaks are often found in the subcortical tissues when bark is peeled from infected twigs, branches or trunks (van der Zwet & Keil). Brown to black, slightly depressed cankers can develop in the bark of twigs or branches, or even the trunk, in autumn and winter. These cankers may later become defined by cracks near the margin of diseased and healthy tissue

Rosacae

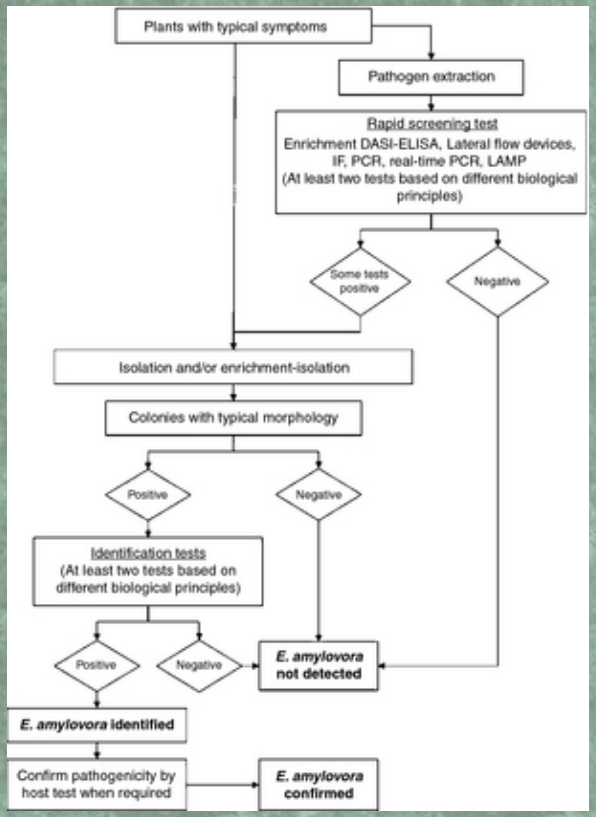
Erwinia amylovora

Confusion between fire blight and blight- or blast-like symptoms, especially in blossoms and shoots, may occur with diseases/disorders caused by other bacteria, fungi, insect damage and physiological disorders, and consequently laboratory analysis is always necessary. Other bacteria can cause blight-like symptoms, including *Erwinia pyrifoliae*, causal agent of bacterial shoot blight of *Pyrus pyrifolia* (Asian pear); *Erwinia piriflorinigrans*, isolated from necrotic pear blossoms in Spain; *Erwinia* sp. and *Erwinia uzenensis*, causing different types of pear symptoms in Japan; Matsuura; and *Pseudomonas syringae* pv. *syringae*, the causal agent of blossom blast.

Rosacae

Erwinia amylovora

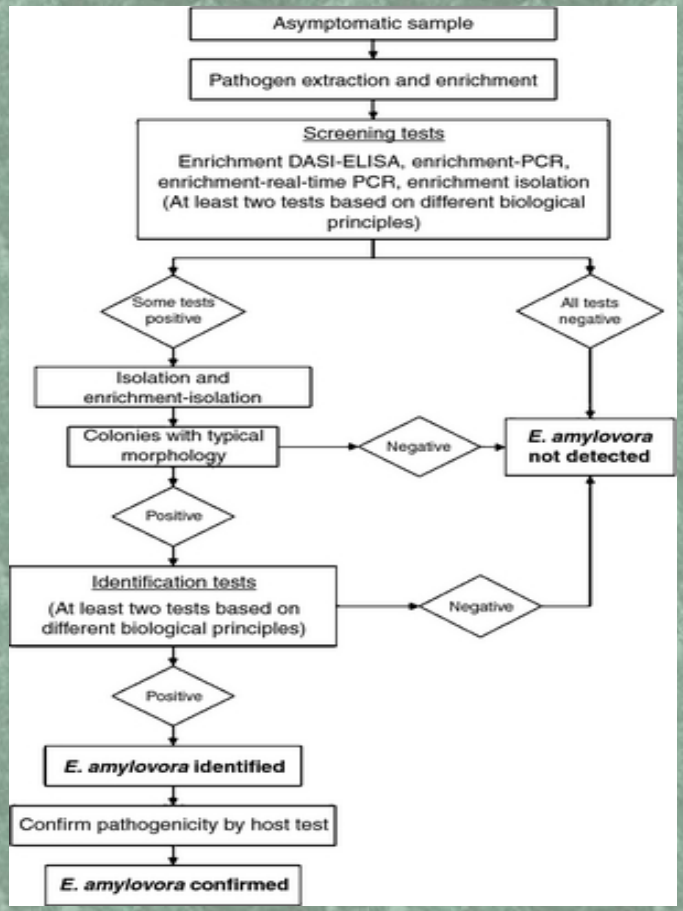
Flow diagram for diagnosis of fire blight in plants with symptoms



Rosacae

Erwinia amylovora

Flow diagram for analysis of *Erwinia amylovora* in asymptomatic samples.



Grapevine

Xylophilus ampelinus

bacterium causing ‘bacterial blight’ of grapevine

In the field, symptoms can appear on all aerial parts of the plant. Buds in infected shoots either fail to sprout or give stunted growth in the spring. Cracks appear along infected shoots, because of the force exerted by the hyperplasia of the cambial tissues, resulting in canker formation. These cracks mainly appear in the lowest parts of the shoots. Infection spreads along the branches which show a brown discoloration of tissues and may eventually die. Young shoots on infected spurs, develop pale yellowish-green areas on the lower inter-nodes. Spurs easily crack at the canker sites.

Grapevine

Xylophilus ampelinus

These expand upwards to become darker,

crack and develop into cankers. When these cankers split, the xylem tissues are revealed. Later in summer, cankers are often seen on one side of petioles causing a characteristic one-sided necrosis of the leaf. They may also appear on main and secondary flower and fruit stalks. Depending on the age of the infected shoots, the bacterium may survive and develop into the cane. Such canes could either show cankers (usually on the lower inter-nodes), or have no visible symptoms, being latently infected. Almost all plant parts with disease symptoms exhibit a brown discoloration of the xylem tissues in longitudinal sections.

Grapevine

Xylophilus ampelinus

Late and irregular lignification is observed in the canes of the affected plants of some cultivars. The general aspect of affected grapevines may change, affected plants being less erect than healthy ones.

On leaves, necrotic spots surrounded by a discolored halo can be observed when contamination occurs via drops of contaminated sap falling down onto the young leaf or from other external contamination. Eventually the central dried part of the spot drops out and a ‘shot hole’ symptom appears. However, when contamination reaches the leaf via the petiole, necrotic sectors surrounded by a halo occur.

Grapevine

Xylophilus ampelinus

Bacterial blight can affect both the cultivar and rootstock.

Grapevine

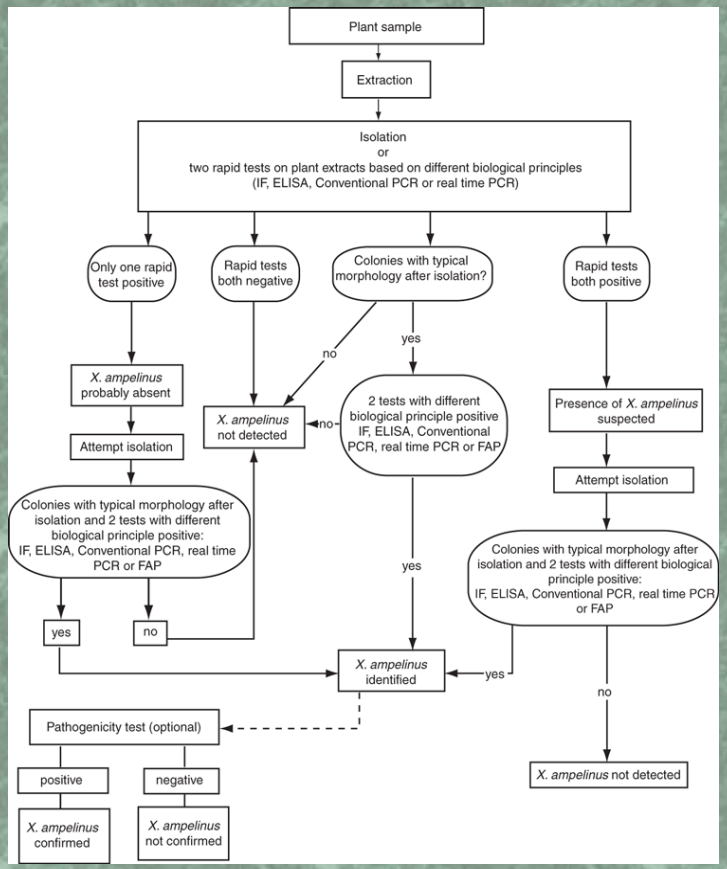
Xylophilus ampelinus

Confusion may occur with other diseases or disorders. Cankers on shoots and leaf spots similar to those caused by *X. ampelinus* could be induced in cases of heavy infections by the fungi *Sphaceloma ampelinum* (without brown discoloration of the xylem vessels) and *Phomopsis viticola*. Failure of spurs to sprout and dead branches could also be caused by the wood fungi *Togninia minima* (anamorph: *Phaeoacremonium aleophilum*), *Phaeomoniella clamydospora, Fomitiporia mediterranea, Eutypa lata, Botryosphaeria* spp. or *Verticillium* spp. In these cases no cankers are evident or they are different but the brown discoloration of the xylem tissues is present. Canker-like symptoms could also be caused by hailstorms.

Grapevine

Xylophilus ampelinus

Flow diagram for the detection and identification of *X. ampelinus*



Grapevine

Xylella fastidiosa

Symptoms caused by*Xylella fastidiosa* were first observed in 1892 in the grape-growing region of southern California (US) and the syndrome was called ‘Pierce’s disease’. Subsequently, similar diseases were reported on many fruit-tree and ornamental species, such as phony peach, alfalfa dwarf, periwinkle wilt, citrus variegated chlorosis, leaf scald of plum and maple, leaf scorch of pear, almond, elm, mulberry, sycamore, oak, oleander and coffee, especially in north and south America.

Grapevine

Xylella fastidiosa

X. fastidiosa is a xylem-limited fastidious bacterium. From a taxonomic point of view, it is a complex species and several research studies have suggested that the different strains which are found on different host plants might be grouped into subspecies (e.g. *X. fastidiosa* subsp. *fastidiosa, X. fastidiosa* subsp. *multiplex, X. fastidiosa* subsp. *pauca, X. fastidiosa* subsp. *sandyi*), however this concept is apparently still being debated among specialists.

Xyphon fulgida.

Grapevine

Xylella fastidiosa

Draeculacephala minerva.

X. fastidiosa causes several diseases of economic importance: grapevine Pierce's disease; citrus variegated chlorosis; peach phony disease; plum leaf scald; as well as leaf scorch diseases on oleander, almond, coffee, pecan, and amenity tree species

Insect vectors: Numerous species of Cicadellidae and Cercopidae (Insecta: Hemiptera) are known to be vectors of *X. fastidiosa*.

Grapevine

Xylella fastidiosa

In Californian vineyards, *Homalodisca vitripennis (=H. coagulata), Carneocephala fulgida, Draeculacephala minerva,* and *Graphocephala atropunctata* are considered to be the most important vectors of Pierce's disease. In Brazilian citrus orchards, *Acrogonia terminalis, Dilobopterus costalimai, Oncometopia fascialis* are considered to be the most important vectors of citrus variegated chlorosis. It is thought that virtually all sucking insects that feed predominantly on xylem fluid are potential vectors of the bacterium. In Italy, the putative vectors of the disease which is currently observed on olive trees are not known.

Grapevine

Xylella fastidiosa

Main symptoms:

Symptoms vary according to the host plants but in general, as the bacterium invades xylem vessels and blocks the transport of water and soluble mineral nutrients, affected plants show drying, scorching, wilting of the foliage, eventually followed by plant death.

Bean

Xanthomonas campestris pv. *phaseoli*

Common bacterial blight and fuscous blight of bean

Small wather-coakt spots on leaves, petioles, pods and stems are the first symptoms. On leaves thes spots become reddish-brown and necrotic, surrounded by a bright yellow halo. On pods spots become sunken and reddish-brown. On stems the yellowing is absent. The bacterium can invade vascular tissues, which may result in wilting.

Bean

Xanthomonas campestris pv. *phaseoli*

Confusion with halo blight is possible. Severely infected seed may be shrivelled and show poor germination or produce weakened plants, but many infected seeds are symptomless (latently infected). On varieties with white seeds, yellow or brown spots can be observed, especially near the hilum. On varieties with dark seed, this discoloration is not visible. *Xanthomonas campestris* pv. *phaseoli* is seed transmitted. Dispersal of bacterium is also by splash or wind-driven rain.

Bean

Xanthomonas campestris pv. *phaseoli*

This bacterium is accidentally dispersed by insects. Seed infection can occur internally and externally.

The pathogen can survive in seed, plant debris and epiphytically on hosts and non-hosts. Infection is through stomata and wounds at higher temperatures (28-32 °C) and high humidity.

Bean

Curtobacterium flaccumfaciens pv. *flaccumfaciens*

bacterial wilt disease of *Phaseolus* spp.

Young *Phaseolus* plants, 5–8 cm tall, may be attacked and are usually killed. If plants survive an early attack, or are infected at a later stage of growth, they may live throughout the season and bear mature seed. The disease is characterized by the wilting of leaves, or parts of them, initially during the warmest hours of the day, followed by a recovery as the temperature drops in the evening.

Bean

Curtobacterium flaccumfaciens pv. *flaccumfaciens*

Wilting becomes permanent during the following days as a result of bacterial plugging of the vessels when the water supply is cut off; the leaves turn brown and then drop.

Occasionally, these typical wilting symptoms may be absent and replaced by golden-yellow necrotic leaf lesions closely resembling those of common blight, *Xanthomonas axonopodis* pv. *phaseoli* however, the lesion margin is more irregular in *C. flaccumfaciens* pv. *flaccumfaciens* infections.

Bean

Curtobacterium flaccumfaciens pv. *flaccumfaciens*

In general, there is no water-soaking of stems and leaves, as found in common blight and halo blight (*Pseudomonas syringae* pv. *phaseolicola*) infections.

On pods, the disease is much more conspicuous than in common blight. In fact, all the seeds in a pod may be infected, while the pod remains apparently healthy. This is due to the pathogen infecting the seed via the vascular system, following the sutures of the pods.

Bean

Curtobacterium flaccumfaciens pv. *flaccumfaciens*

The sutures may be discolored with darkening, sometimes extending laterally. On young pods, water-soaked spots occasionally appear, the area turning either a yellowish-green or darker than the rest of the pod.

On ripe pods, lesions are more evident, being an olive-green colour, in contrast to the yellow colour of the normal pod. It should be noted that seemingly vigorous plants may bear one or more shriveled shoots, or infected pods which are hidden by healthy foliage.

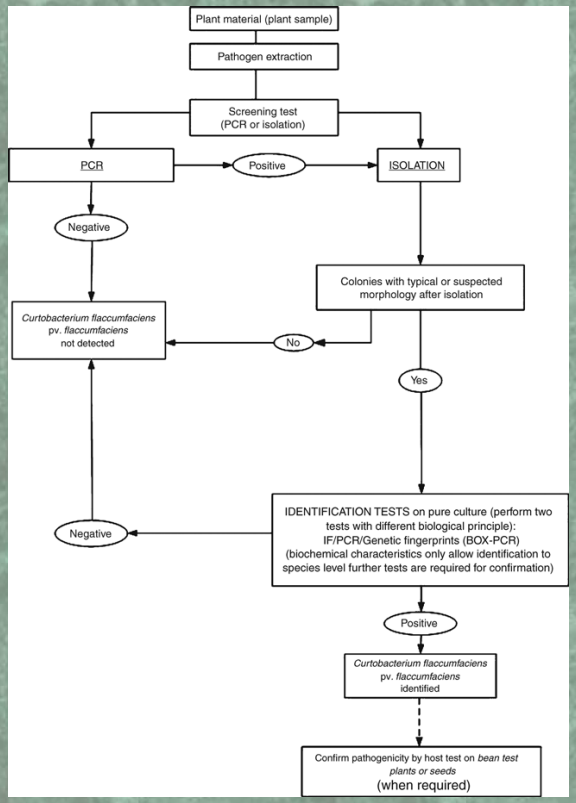
Bean

Curtobacterium flaccumfaciens pv. *flaccumfaciens*

Seeds of white-seeded cultivars, when infected systemically, are bright yellow, while the coloration is less intense in cultivars with coloured seed coats. There may be a small amount of yellow slime at the hilum, and seeds may be shrivelled. The colour mutants formerly described as *Corynebacterium flaccumfaciens* subsp. *aurantiacum* and *violaceum* produce an orange and purple discoloration, respectively, in the seed coat.

Bean

Curtobacterium flaccumfaciens pv. *flaccumfaciens*



Lucerne

Clavibacter michiganensis ssp. insidiosus

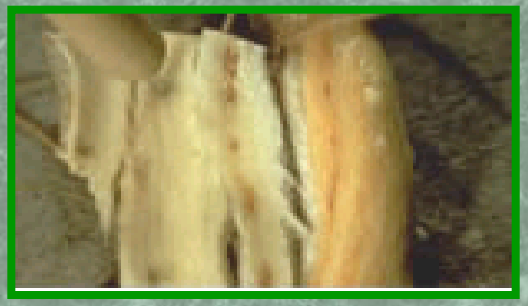
Bacterial wilting of lucerne

Generally, *C. michiganensis* subsp. *insidiosus* causes systemic infection of alfalfa plants. The disease may induce wilting under dry and hot conditions, but most often symptoms consist only of stunting and proliferation of stems. Chlorosis, reduction of size and cupping of leaflets are quite common as well as marginal, papery, white grey necrosis of leaflets

Lucerne

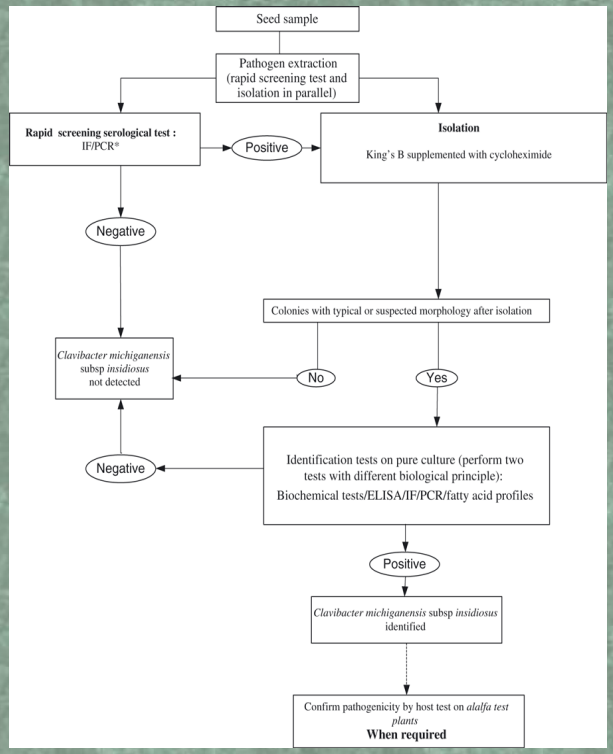
Clavibacter michiganensis ssp. insidiosus

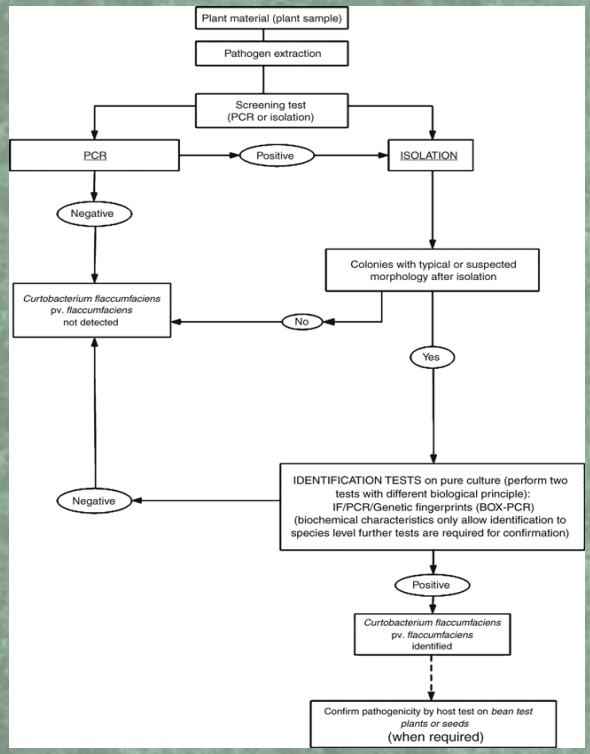
In some varieties, the vascular root system may be discoloured yellowish-brown. Moist or full-ring discolourations may appear in the outer cortex of cut roots. Wilt symptoms caused by *C. michiganensis* subsp. *insidiosus* may be confused with other systemic diseases caused, for instance, by *Verticillium albo-atrum* lucerne strains



Lucerne

Clavibacter michiganensis ssp. insidiosus





Maize

Pantoea stewartii subsp. *stewartii*

The principal host is maize, especially sweetcorn, but also dent, flint, flour and popcorn cultivars.

The bacterium may be found on seeds of maize. No characteristic symptoms are visible on seeds.

The first phase - wilt phase, of this bacterial disease may affect plants at the seedling stage. The disease spreads systematically through the vascular system. If infected late, plants may reach a reasonable size.

Maize

Pantoea stewartii subsp. *stewartii*

Leaves develop pale-green to yellow, longitudinal streaks, with irregular or wavy margins, which are parallel to the veins and may extend the length of the leaf. These streaks dry out and turn brown. Small water-soaked spots may develop on the husk of the cobs. Bacteria may exude in fine droplets on the inner face of the husk. Plants that are not killed may produce bleached, dead tassels.

Maize

Pantoea stewartii subsp. *stewartii*

Cavities may appear close to the soil in the stalk pith of severely infected plants. *P. s. stewartii* penetrates the seed deeply, but not the embryo. Sweetcorn is particularly susceptible to this phase of the disease.

Field and dent corn are more susceptible to the second phase, a leaf blight, usually most apparent after tasseling. Short to long, irregular, pale-green to yellow streaks, which originate from feeding marks of the corn flea beetle (*Chaetocnema pulicaria*), appear along the veins of leaves.

Maize

Pantoea stewartii subsp. *stewartii*

Whole leaves sometimes become straw-coloured and die. The weakened plants are more susceptible to fungal stalk rots.

Sampling

5–10 leaves, cobs, tassels, stems with typical symptoms are collected from the inspected plot (field) for laboratory examination. For seed lots, 400 seeds per lot are needed for testing.

Maize

Pantoea stewartii subsp. *stewartii*

Extraction of bacteria from plant samples

Plant parts (leaves, husk, tassel) showing symptoms are excised at the advancing edge of the lesions and macerated in a plastic bag with a hand homogenizer (or in a mortar with a pestle) with a few mL of sterile water or comminuted in a sterile Petri dish with sterile water or sterile phosphate-buffered saline. An appropriate aliquot of the macerating liquid is transferred into a centrifuge tube for immunofluorescence staining.

Maize

Pantoea stewartii subsp. *stewartii*

The remaining macerating liquid is collected in sterile Wassermann or disposable plastic tubes for further isolation and stored at +4°C or on ice. Macerates should be processed as soon as possible (immediately if stored at room temperature and within the day if stored at 4°C). Although it is not recommended, macerates may be stored longer at a temperature below −18°C.

Maize

Pantoea stewartii subsp. *stewartii*

Extraction of the bacteria from seeds

The sample is divided into subsamples of up to 100 seeds each and put into plastic bags. Seeds treated with any plant protection product should be washed under running tap water until water runs clear to remove product from the seed surface (in such cases, the report should mention that the seeds were treated). Sterile distilled water (IF method) or extraction buffer (ELISA method), equal to 2 × seed weight is added to the subsamples, and the bag is then closed.

Maize

Pantoea stewartii subsp. *stewartii*

The subsamples are incubated in refrigerator at +4°C overnight. After incubation, the soaked seeds are shaken on rotary shaker at room temperature for 10–15 min at 200 rev min^{-1} or processed in a Stomacher laboratory blender for 2 min at maximum speed. Appropriate aliquots of the soaking liquid are transferred to centrifuge tubes for immunofluorescence staining. The remaining soaking liquid is collected in sterile Wassermann or disposable plastic tubes for testing by ELISA and/or for further isolation, and stored at +4°C or on ice.

Maize

Pantoea stewartii subsp. *stewartii*

Macerates should be processed as soon as possible (immediately if stored at room temperature and within the day if stored at 4°C). Although it is not recommended, macerates may be stored longer at a temperature below −18°C.

For samples showing symptoms, direct isolation is the best method and should be performed immediately after maceration.

Maize

Pantoea stewartii subsp. *stewartii*

Immunofluorescence cell staining

The soaking liquid intended for immunofluorescence staining is centrifuged at 10 000 *g* for 10 min. The pellet is resuspended in 1/10 of the original liquid volume sterile distilled water. The concentrated suspension, and its 10 and 100 times dilutions, are used for indirect IF staining (using specific polyclonal antibody).

Maize

Pantoea stewartii subsp. *stewartii*

ELISA

The soaking liquid is tested by ELISA using the Agdia kit (following the manufacturer's instructions).

Maize

Pantoea stewartii subsp. *stewartii*

The soaking liquid is tested by ELISA using the Agdia kit (following the manufacturer's instructions).

Isolation of bacteria by dilution plating

As the amount of viable cells decreases quickly in macerates, isolation should be done immediately. From the IF-positive subsamples, suspensions (kept on ice) are serially diluted in 10-fold steps up to 1 : 10 000 in sterile physiological solution. 100 µL of each dilution is spread over the surface of a King's B agar plate supplemented with 200 mg/L of nystatin (or 200 mg/L of cycloheximide), using an L-shaped glass rod. NBY or YPGA can also be used.

Maize

Pantoea stewartii subsp. *stewartii*

The plates are incubated at 25–27°C, and examined after 2–3 days for colonies of *P.* s. *stewartii*. 5–7 colonies with typical morphology are streaked on King's B slants or plates for further analysis. Colonies are lemon to orange-yellow or pale-yellow, flat to convex, transparent, with entire edges, slow to medium growing. The cells are Gram-negative straight, short rods (0.4–0.7 × 0.9–1.7 µm).

Maize

Pantoea stewartii subsp. *stewartii*

Identification:

The isolates incubated on King's B slants are checked for motility, Gram-staining, tobacco HR and oxidase reaction. Non-motile, Gram, HR and oxidase negative isolates are further examined by agglutination, ELISA, IF or directly checked by the confirmatory tests listed below. Serological tests can be performed with the suspected isolates without a preliminary motility check, Gram staining, HR or oxidase reaction

Maize

Pantoea stewartii subsp. *stewartii*

PCR test:

This test is performed following the protocol of Coplin & Majerczak (2002). The primers are designed from the 16S-23S rRNA/ITS region, forward primer ES16: 5′-GCG AAC TTG GCA GAG AT-3′, reverse primer ESIG2c: 5′-GCG CTT GCG TGT TAT GAG-3′. A colony from the suspected culture is suspended in 100 µL of sterile, 50 mm phosphate buffer (pH 7.0) in a microvial. The closed vials are heated at 95°C for 10 min, and the suspension cooled on ice

Maize

Pantoea stewartii subsp. *stewartii*

Alkalilysis may be used to improve DNA isolation from the bacterial cells (the sample of 100 µL is treated with 50 µL 0.25 N NaOH, heated for 10 min at 95°C, put on ice for 2 min, treated with 50 µL 0.25 N HCl and 25 µL 0.5 m Tris-HCl (pH 8.0) with 0.1% Tween-20, heated for 10 min at 95°C and put on ice for 2 min).

Pantoea stewartii subsp. *stewartii*

PCR is performed in 25-µL volumes using 3 µL template DNA, 1 U PLATINIUM Taq DNA polymerase (Invitrogen), 25 pmole of each primer and 200 µm dNTPs in 1.5 mm $MgCl_2$. The source and quality of the Taq polymerase is critical.

Maize

Pantoea stewartii subsp. *stewartii*

The conditions of PCR amplification are: 1 cycle for 1 min at 94°C, 25 cycles of 15 s at 94°C, 15 s at 55°C, 30 s at 72°C. The PCR product is separated on 1.5% agarose gel at 1.5 V/cm. The DNA fragment is stained in ethidium bromide solution (0.5 µg/mL in TAE buffer) for 30 min. The size of the expected amplicon is: 0.92 kb.

Maize

Pantoea stewartii subsp. *stewartii*

Alternatively, primers designed from the *hrpS* region (HRP1d: GCA CTC ATT CCG ACC AC and HRP3c: GCG GCA TAC CTA ACT CC) or primers from the sequence of the *cpsD*-region (CPSL1: CCT GTC AGT CTC GAA CC, and CPSR2c: ATC TCG AAC CGG TAA CC) can be used, applying the same amplification conditions as above. Amplicon sizes are 0.9 kb and 1.1 kb, respectively.

Maize

Pantoea stewartii subsp. *stewartii*

Fatty acid profiling.

Pathogenicity test:

The pathogenicity test is carried out on 5–10 plants by stem inoculation of 8–14 days old (1–2 leaf stage) plants of a susceptible cultivar of maize (e.g. 'Jubilee', 'Meritosa') grown in the glasshouse. Plants are inoculated by syringe with 10^7–10^8 cell mL^{-1} bacterial suspension prepared in sterile distilled water from suspected isolates, incubated for 48 h at 25°C on King B.

Maize

Pantoea stewartii subsp. *stewartii*

After inoculation, the plants are kept in a humid chamber for 24–48 h at 25–27°C. First disease symptoms (streaking) may appear after 3–5 days, but for the more typical symptom appearance (water soaking, yellow pockets of ooze in the vascular tissues) 7 or more days are necessary. Wilt symptoms generally appear after 14 days. In some cases, the yellow bacterial ooze forms in the vascular system. Many bacteria other than *P. s. stewartii* can cause water-soaked symptoms around wounds if stem-inoculated into maize (e.g. *Pantoea agglomerans*) but without ooze formation.

Maize

Pantoea stewartii subsp. *stewartii*

Reference strain:

NCPPB 2295 (= CFBP 3167) NCPPB 449 (= CFBP 3168). The suggested reference strain of *P. s. indologenes* is NCPPB 2280 and of *P. agglomerans* LMG 1286.

Rice

Xanthomonas oryzae

The species *Xanthomonas oryzae* includes two pathovars, namely, *oryzae* and *oryzicola*

X. oryzae pv. *oryzae* enters either through wounds or hydathodes, multiplies in the epitheme and moves to the xylem vessels where active multiplication results in blight on the leaves. The symptoms of the disease include leaf blight, wilting and pale yellow leaves.

Rice

Xanthomonas oryzae

Leaf blight is characterized by wavy elongated lesions, which develop along the leaf margins. They start as small water-soaked stripes from the tips where water pores are found and rapidly enlarge in length and width, forming a yellow lesion with a wavy margin along the lead edges. Later on, diseased areas turn white to grey. These lesions can develop on one or both sides of the leaf and occasionally along the midribs, and leaf blight symptoms generally occur from maximum tillering stage and onwards. In young lesions, drops of bacterial ooze can be observed early in the morning.

Potato

Clavibacter michiganensis ssp. *sepedonicus*

Potato ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* is a damaging disease of potato.

EPPO protocol allows the detection and identification of latent infections of *C. m. sepedonicus* of 10^3–10^4 cells per mL of resuspended pellet. The diagnostic procedures comprise isolation from infected tissue and detection of latent infections, including presumptive diagnosis with a rapid test, identification of presumptive isolates and determination of pathogenicity.

Potato

Clavibacter michiganensis ssp. *sepedonicus*

C. m. sepedonicus is renowned for the difficulties encountered with its detection. The main problems are in detecting latent infection and in obtaining cultures even from symptomatic material, since it is slow growing on agar media and is easily overgrown by saprophytes. Ring rot is a vascular disease in both aerial stems and tubers.

Potato

Clavibacter michiganensis ssp. *sepedonicus*

Potato tubers:

Tuber symptoms are not unlike those of brown rot caused by *Ralstonia solanacearum.* The infected vascular bundles break down releasing bacteria into the adjacent cortical tissues which then rot partly due to activity of cellulase enzymes. This rot extends around the vascular ring as the rots from adjacent infected bundles merge. Although not frequently mentioned in the literature, a common feature in many of the specimens seen is the progression of the rot from the vascular tissues of the heel end to the central cortex of the tuber.

Potato

Clavibacter michiganensis ssp. *sepedonicus*

In the early stages of disease, the rotted tissues usually remain creamy white and not brownish as in brown rot. Eventually however, rots do become discoloured as secondary invaders become established. Early rots have a cream cheese-like consistency and are distinct from the more slimy, ooze-like rots of brown rot.

Potato

Clavibacter michiganensis ssp. *sepedonicus*

Potato plants

Under European climatic conditions, symptoms are rarely found in the field and often only at the end of the season. Moreover the symptoms are frequently masked by or confused with other diseases, senescence or mechanical damage, so symptoms may easily be missed in field inspections. Wilting symptoms in stems are not like those of other diseases, and are very different from those of brown rot. Wilting is usually slow, initially limited to the leaf margins. Young infected leaves often continue to expand, though less so in the infected zones.

Potato

Clavibacter michiganensis ssp. *sepedonicus*

This creates odd-shaped leaves. Leaves affected by xylem blockages further down the stem often develop chlorotic, yellow to orange, interveinal areas. Infected leaflets, leaves and even stems may eventually die. Wilting symptoms are often absent, leaves and tubers being simply reduced in size. Occasionally plants are stunted.

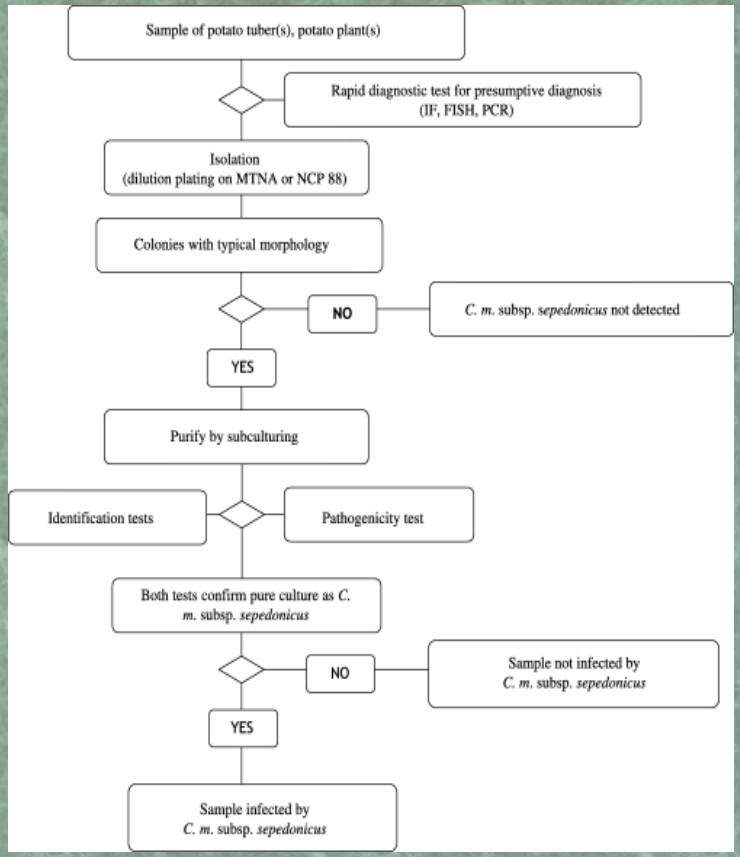
Potato

Clavibacter michiganensis ssp. *sepedonicus*

Wilt symptoms caused by *C. m. sepedonicus* may be confused with those caused by other systemic pathogens, e.g. *R. solanacearum*, *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica*, *Erwinia chrysanthemi*, *Phoma exigua* var. *foveata*, as well as large populations of saprophytic bacteria. In particular, *E. chrysanthemi* can cause leaf symptoms and wilt that is very similar to the symptoms of *C. m. sepedonicus.* The only difference is blackening of the stems in *E. chrysanthemi* infections. Other wilts can be distinguished from those caused by *C. m. sepedonicus* since whole leaves or whole plants wilt rapidly.

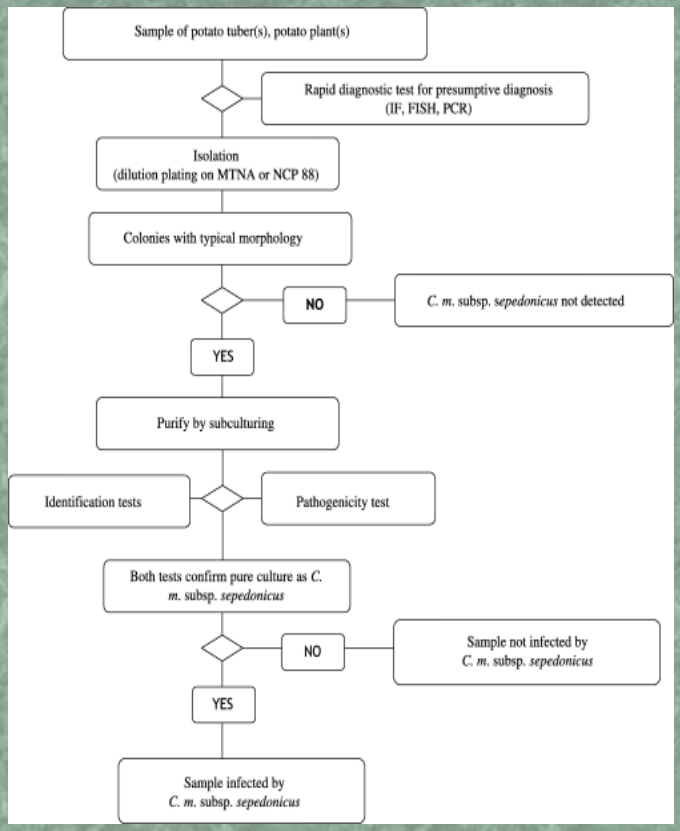
Potato

Clavibacter michiganensis ssp. *sepedonicus*



Potato

Clavibacter michiganensis ssp. *sepedonicus*



Potato

Pseudomonas (Ralstonia) solanacearum

Bacterial wilt caused by *Ralstonia solanacearum*

Race 1 occurs in tropical areas all over the world and attacks tobacco, many other solanaceous crops and many hosts in other plant families. It has a high temperature optimum (35°C, as do race 2, 4and 5). Race 2 occurs mainly in tropical areas of South America and attacks bananas and *Heliconia* (causing so called Moko disease), but also in the Philippines (causing so-called bugtok disease on plantains). Race 3, occurring at higher altitudes in the tropics and in subtropical and temperate areas attacks potato, tomato, occasionally *Pelargonium zonale* , aubergine and capsicum, some solanaceous weeds like *Solanum nigrum* and *Solanum dulcamara*.

Potato

Pseudomonas (Ralstonia) solanacearum

A number of non-solanaceous weed hosts have also been found to harbour race 3 infections, often asymptomatically (Pradhanang *et al*., 2000; Strider *et al.* ., 1981; Wenneker *et al.* ., 1999; Janse *et al.* ., 2004). This race has a lower temperature optimum (27 ° C) and appears to be mostly biovar 2 A RFLP group 26 with a worldwide distribution (Cook & Sequeira, 1994), biovar 2 A RFLP group 27 (found in Chile and Colombia), or biovar 2T (sometimes also called 2 N, found in tropical areas in South America). Race 4 is particularly aggressive on ginger, race 5 (biovar 5) is specialized on *Morus*.

Potato

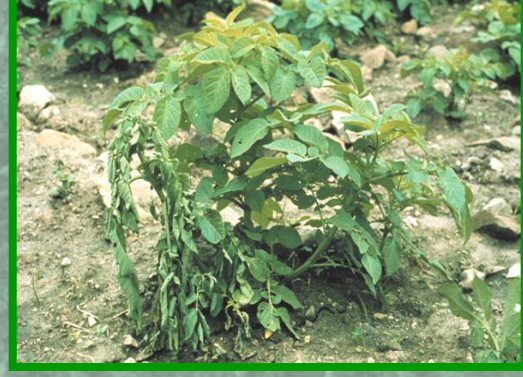
Pseudomonas (Ralstonia) solanacearum

Disease symptoms

Potato

Foliage symptoms include rapid wilting of leaves and stems, usually first visible at the warmest time of day.

Eventually, plants fail to recover, become yellow and brown necrotic and die. As the disease develops, a streaky brown discoloration of the stem may be observed on stems above the soil line, and the leaves may have a bronze tint. Epinasty of the petioles may occur. A white, slimy mass of bacteria exudes from vascular bundles, when broken or cut.



Potato

Pseudomonas (Ralstonia) solanacearum

This slime oozes spontaneously from the cut surface of a potato stem in the form of threads, when suspended in water. Such threads are not formed by other bacterial pathogens of potato. This test is of presumptive diagnostic value in the field. Under cool growing conditions, wilting and other foliar symptoms may not occur.

On tubers, external symptoms may or may not be visible, depending on the state of development of the disease.

Potato

Pseudomonas (Ralstonia) solanacearum

Symptoms may be confused with those of ring rot due to *Clavibacter michiganensis* subsp. *sepedonicus* (EPPO/CABI, 1997). *R. solanacearum* can be distinguished by the bacterial ooze that often emerges from the eyes and stolon-end attachment of infected tubers. Soil may adhere to the tubers at the eyes Cutting the diseased tuber will reveal a browning and eventual necrosis of the vascular ring and immediately surrounding tissues. A creamy fluid exudate usually appears spontaneously on
the vascular ring of the cut surface a few minutes after cutting.

Potato

Pseudomonas (Ralstonia) solanacearum

In the case of ring rot, the tuber has to be squeezed in order to press out a mass of yellowish macerated vascular tissue and bacterial slime. Atypical symptoms have been described on potato (necrotic spots on the epidermis), possibly caused after lenticel infection. Plants with foliar symptoms caused by *R. solanacearum* may bear healthy and diseased tubers, while plants that show no signs of the disease may sometimes produce diseased tubers.

Tomato

Pseudomonas (Ralstonia) solanacearum

The youngest leaves are the first to be affected and have a flaccid appearance, usually at the warmest time of day. Wilting of the whole plant may follow rapidly if environmental conditions are favorable for the pathogen. Under less favorable conditions, the disease develops less rapidly, stunting may occur and large numbers of adventitious roots are produced on the stem. The vascular tissues of the stem show a brown discoloration and, if the stem is cut crosswise, drops of white or yellowish bacterial ooze may be visible.

Pelargonium

Pseudomonas (Ralstonia) solanacearum

First symptoms are wilting and subsequent chlorosis (often sectorial yellowing) of leaves. Stems may blacken and eventually become necrotic. Internal vascular browning is often visible. In a later stage, leaves become brown necrotic and the whole plant desiccates and dies. In final stages, plants collapse totally.

Tobacco

Pseudomonas (Ralstonia) solanacearum

One of the main symptoms is unilateral wilting and premature yellowing. Leaves on one side of the plant or even a half leaf may show wilting symptoms. In severe cases, leaves wilt without changing color and stay attached to the stem. As in tomato, the vascular tissues show a brown discoloration when cut open. The primary and secondary roots may become brown to black.

Banana

Pseudomonas (Ralstonia) solanacearum

Moko disease, caused by *R. solanacearum* , is easily confused with Panama disease caused by *Fusarium oxysporum* f.sp. *cubense* . A clear distinction is possible when fruits are affected: brown dry rot is seen only in the case of Moko disease. On young and fast-growing plants, the youngest leaves turn pale green or yellow and collapse. Within a week, all leaves may collapse. Young suckers may be blackened, stunted or twisted. The pseudo stems show brown vascular discoloration.

Pseudomonas (Ralstonia) solanacearum

Solanum dulcamara

Thank you for your attention

TWINNING PROJECT BA/12/IB/AG01

“ Act. 3.3 „Train laboratory staff on laboratory methods for diagnosing harmful organisms”

Methods for determination of quarantine bacteria.

MSc. Anna Kołodziejska

Main Inspectorate of Plant Health and Seed Inspection

Central Laboratory – bacteriology section, Toruń, Poland.

Methods for determination of quarantine bacteria.

Symptoms can also be caused in a number of cases by fungi, mycoplasmas, viruses or nematodes. Visual judgement in most cases is insufficient to make a reliable diagnosis.

Methods for determination of quarantine bacteria.

1. Assessment of symptoms:

- different stages of the disease (young or old);
- different plant parts (roots, bulbs, stems or leaves);
- present of the border line between heslthy and diseased tissue;
- in case of latently infected plants samples should be representative

Methods for determination of quarantine bacteria.

Five types of symptoms:

a) **leaf spot** (pseudomonads, xanthomonads) – invasion of intercellular spaces and necrotization of parenchymatous tissues. Water–soaked spots; bacterial exudate or ooze; angular leaf spots; halo blight; chlorosis of young leaves; leaf blight; fruit spots.

b) **canker and dieback** diseases (*P. s. pv. syringue* group)- invasion of bark and woody tissues through wounds, buds, leaf, scars, young leaves and blossoms. Bark necrosis, canker- gummosis, dieback, shoot blight, shot-hole on leaves, bud blast, bark necrosis, canker –bacterial ooze, dieback, blossom and twig blight.

Methods for determination of quarantine bacteria.

c) **vascular wilts** (coryneform bacteria, *R.solanacearum,* a few xanthomonads and erwinias)- invasion of vascular elements. Wilting, dwarfing, browning of vascular tissues, ring rot of potato, bird's-eye-spots on tomato fruits.

d) **soft rot** diseases (soft rotting erwinias, a few pseudomonas) – maceration of middle lamella and primary cell wall. Soft rot of tubers and onions and others, black-leg disease.

e) **proliferation and tumour** (agrobacteria, *P.s.pv. savastanoi*)- stimulation of plant cells and tissues to grow abnormal. Crown gall, twig gall, hairy root, fasciation.

Methods for determination of quarantine bacteria.

Equipment of a standard phytobacterological laboratory:

1- Petri dishes (usually 10 cm diameter)

2- Test-tubes and bottles in several sizes (erlenmeyer flasks, glass or plastic beakers, plastic or glass measuring cylinders(

3- automatic pipettes, Pasterur pipettes, micro slides and cover glasses, metal or plastic caps, Drigalski spatula, loop needle,

4- Bunsen burners, vortex, magnetic stirrer with heating plate, tips, pH-meter, sterile filtration set, colony counter, laboratory precision balance,

5- stomacher, centrifuges (with cooling system),

Methods for determination of quarantine bacteria.

6- incubators,
7- water baths,
8- refrigerator, deep freeze, autoclaves, safety cabinets, liophilizator.
9- Microscopes

Methods for determination of quarantine bacteria.

-serological methods (IF, test Elisa, agglutination)

-isolation (different media) – to exclude non-pathogenic bacteria or to enhance the recognition of target bacteria (semiselective)

-molecular methods (PCR, FISH, TaqMan)

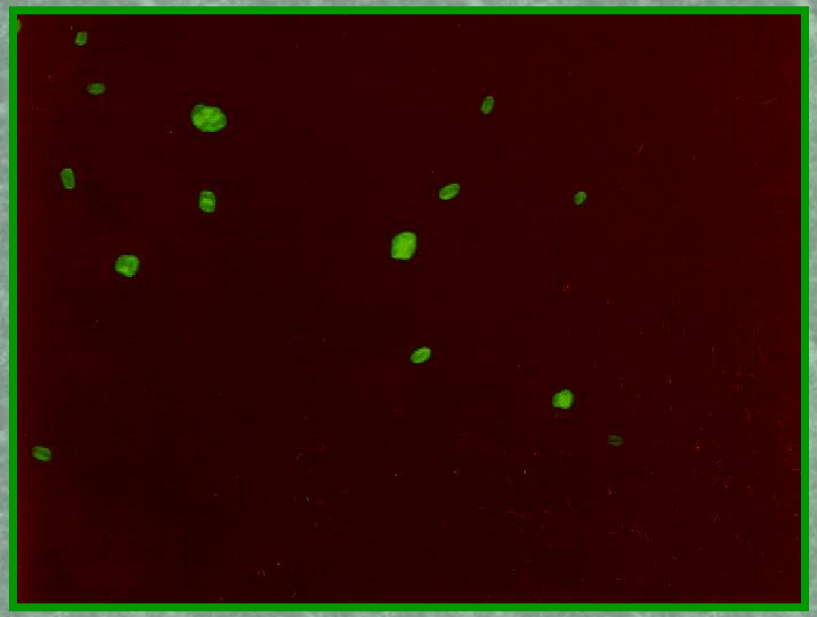
-biological tests

Methods for determination of quarantine bacteria.

Serological methods

1. IF test (monoclonal antibodies – reactivity of monoclonal sera is usually lower than that of polyclonal sera but the specificity is higher)
2. ELISA, DASI-ELISA test – sensitivity about 10^5 cfu /ml
3. Agglutination tests („field tests”) – a lot of cross reactions

Methods for determination of quarantine bacteria.

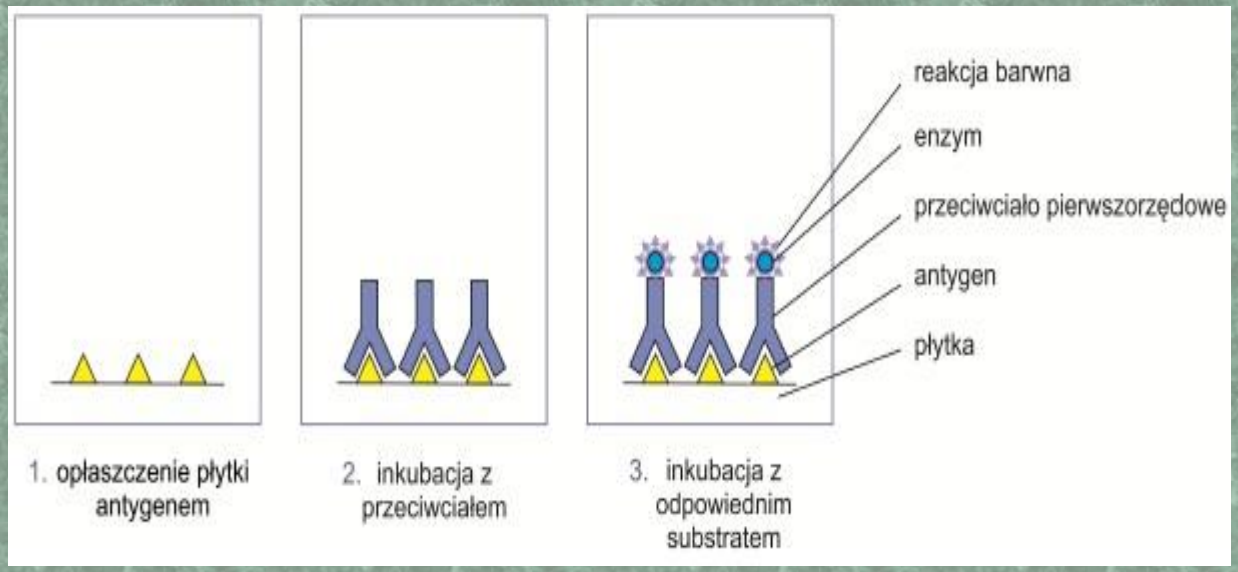


Bacteria cells IF-stained

Methods for determination of quarantine bacteria.

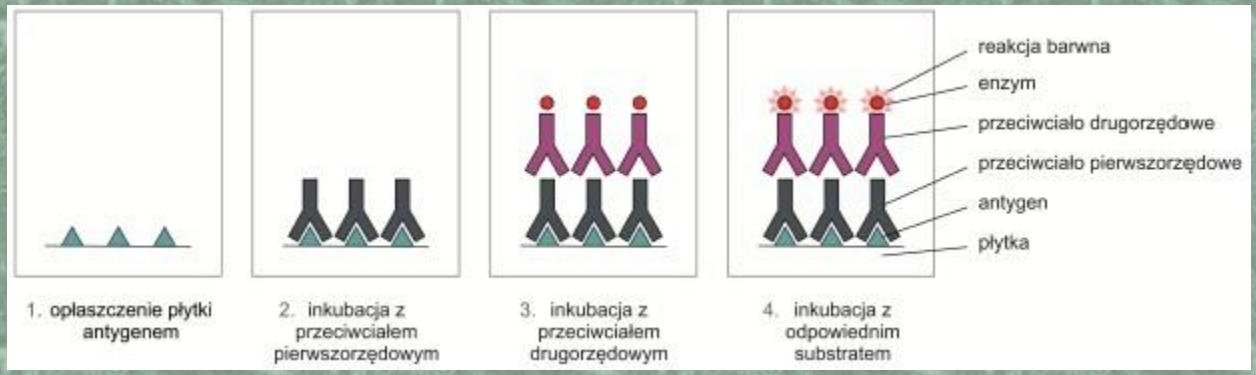
Direct ELISA

Wells are coated with antibodies and after incubation with plant extract or bacterial suspension a second serum labelled with an enzyme is used. After incubation of this second antiserum positive reaction between antibodies and antigens is made visible through enzyme-substrate reaction (yellow colour).



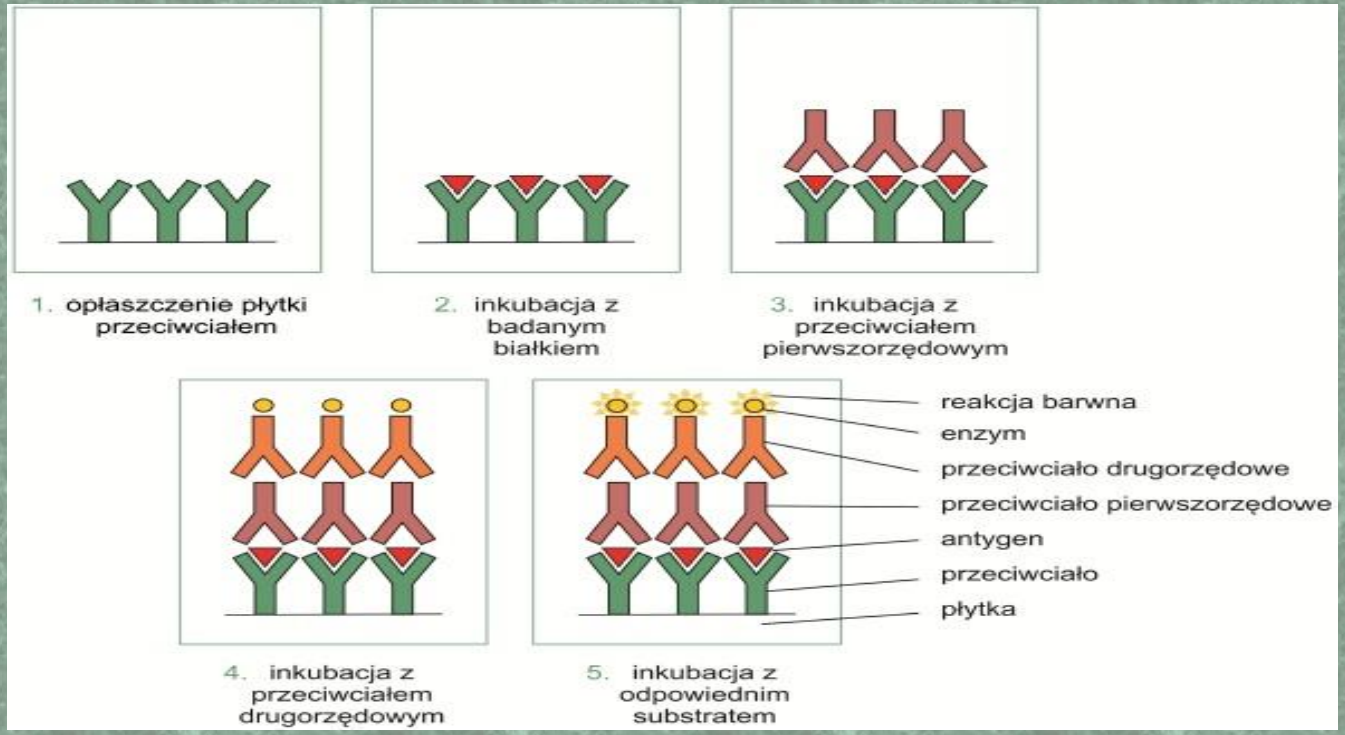
Methods for determination of quarantine bacteria.

Indirect ELISA



Methods for determination of quarantine bacteria.

Sandwich ELISA



Methods for determination of quarantine bacteria.

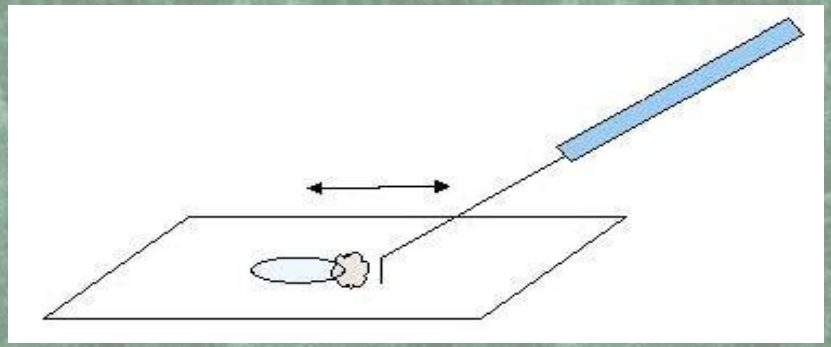
The disadvantage of ELISA is that in many cases only products of bacteria and not the entire cells are detected. Cells are washed out due to insufficient binding capacity of coating antibodies. Moreover there is no information on morphology of cells and the sensitivity is lower than that of IF (c.10^5 cfu /ml).

Often, sera are not specific enough. They cross-react with (non-) pathogenic bacteria possessing some of the antigenic determinants of the target bacterium in common, especially at lower serum dilutions.

Methods for determination of quarantine bacteria.

Agglutination test

I. On microscopic slide put a drop of sera.
II. Using a sterile loop mix the drop of sera with the drop of bacteria suspension.

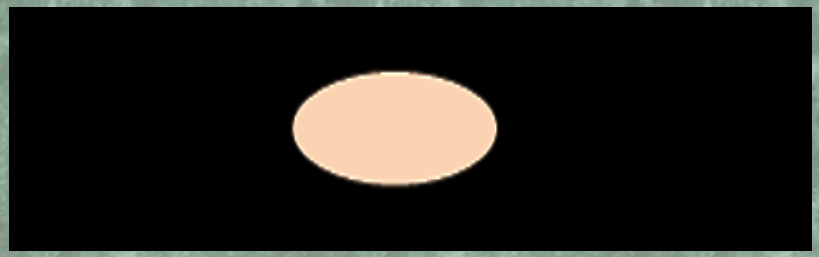


Methods for determination of quarantine bacteria.

Agglutination test

III. Mix it for **10 – 30 sec. (max. 1 minute)**.

Agglutination „+++”



No Agglutination

If antibodies (in antiserum) and a suspension of bacteria era mixed in certain concentration they will clump together and an agglutination reaction takes place.

Methods for determination of quarantine bacteria.

Molecular methods

1.PCR (polymerase chain reaction) and the RFLP – restriction enzyme analysis

2. Real-time PCR- quantitative, can measure fluorescence that is emitted during the PCR cycle

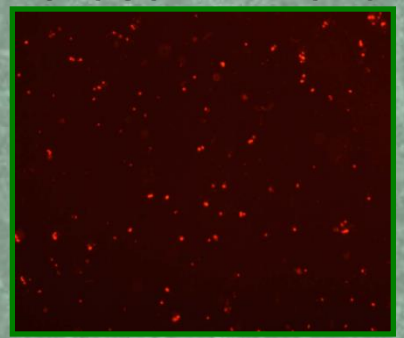
3. Fingerprinting method – uses repetitive sequences (mostly of unknow function, that are interspersed throughout the DNA) present in the genomic DNA of bacteria

4. FISH- short oligonucleotide probes against 16S or 23S rRNA/DNA are used

Methods for determination of quarantine bacteria.

Test FISH – Fluorescent *in situ* Hybridization

1.Możliwość przenikania sond oligonukleotydowych przez ściany komórkowe mikroorganizmów utrwalonych na szkiełkach mikroskopowych.
2.W przypadku bakterii Gram-dodatnich konieczność użycia lizozymu (zniszczenie warstwy peptydoglikanu w ścianie komórkowej.
3.Czułość uzależniona od aktywności metabolicznej komórki - czasami martwe komórki dają wynik pozytywny przez pewien czas.



Methods for determination of quarantine bacteria.

PCR - positves

1. **quick**
2. **Sensitive(theoretically one copy of target DNA in a sampl can be detected**
3. **available**

Methods for determination of quarantine bacteria.

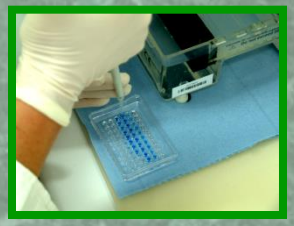
PCR - negatives

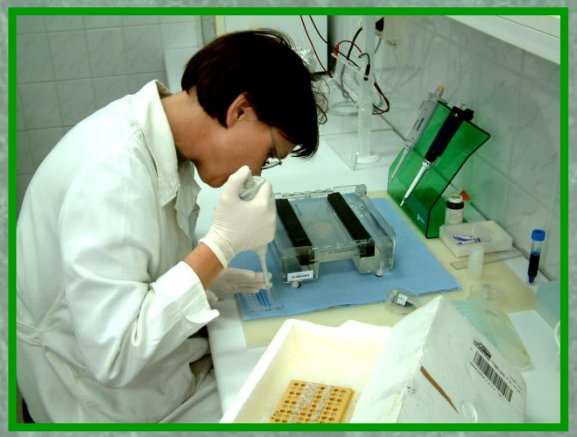
1.Not possible to different live and death cells of bacteria

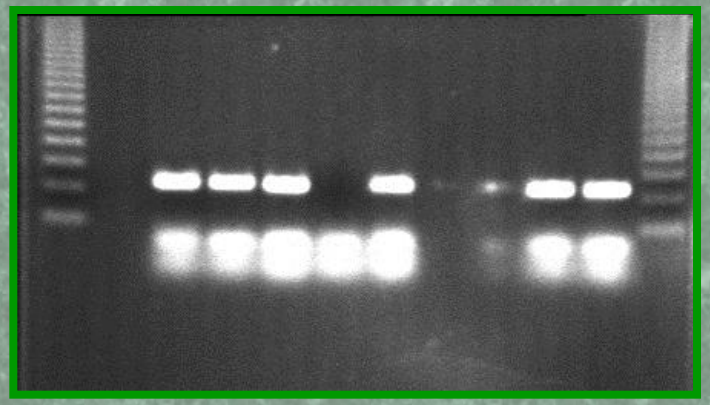
2.False positive and false negative results

3.Contamination is possible

Methods for determination of quarantine bacteria.

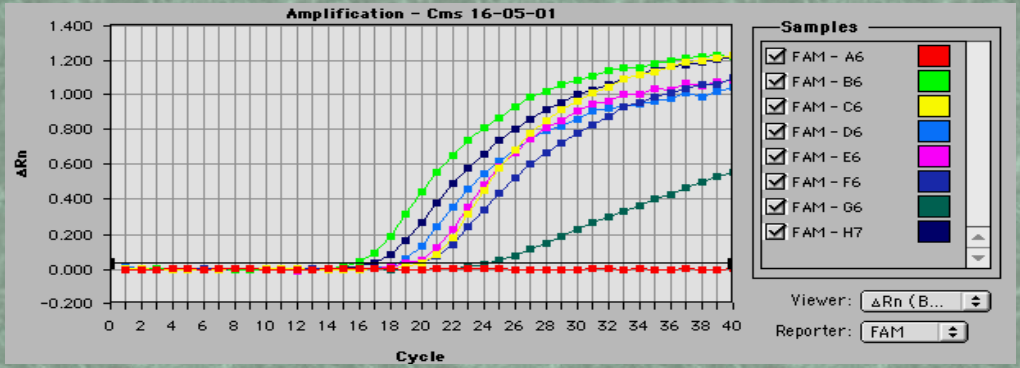






Methods for determination of quarantine bacteria.

Real-time PCR



Key to traces:

A6 negative control
H7 positive control

B6 sample A pastrik extraction
C6 sample B pastrik extraction
D6 sample C pastrik extraction

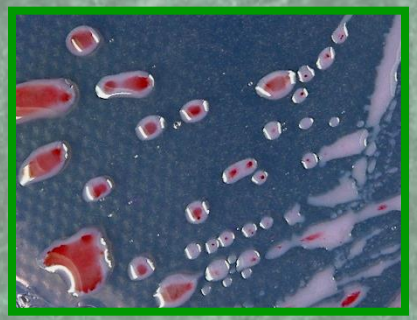
E6 sample A alkali boiling
F6 sample B alkali boiling
G6 sample C alkali boiling

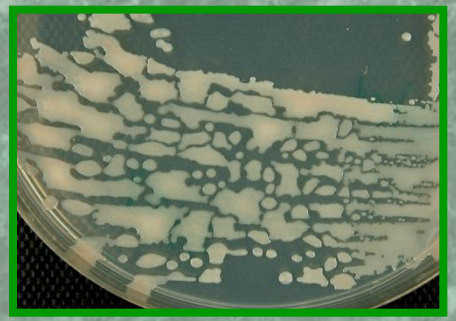
Methods for determination of quarantine bacteria.

Metoda hodowlana

Rodzaje podłoży hodowlanych:

1. Nie selektywne (peptony, ekstrakty drożdżowe, mięsne, sole nieorganiczne, związki organiczne) odpowiednie dla wzrostu wielu gatunków bakterii
2. Pół – selektywne (substancje umożliwiające produkcję pigmentu lub substancje wykorzystywane tylko przez określone bakterie lub zawierają inhibitory w postaci antybiotyków)
3. Selektywne (zaw. składniki które ze względu na jakość lub ilość umożliwiają wzrost tylko określonych bakterii)





Methods for determination of quarantine bacteria.

Metoda hodowlana

Pożywki stosowane do namnażania bakterii powinny:

-zawierać odpowiednie składniki pokarmowe,

-mieć odpowiednią wilgotność,

-mieć odpowiednie pH,

-mieć odpowiednie ciśnienie osmotyczne,

-być jałowe

Methods for determination of quarantine bacteria.

Metoda hodowlana

Ekstrakty mięsne – wodne wyciągi z tkanki mięsnej, serca, wątroby - bogate źródło azotu, węgla i witamin

Ekstrakty z drożdży - dobre źródło azotu, węgla, bogate źródło witamin z grupy B

Peptony – produkty częściowej, enzymatycznej hydrolizy białek - bogate źródło azotu, węgla

Hydrolizaty enzymatyczne lub kwasowe kazeiny, białek soi, białek mięsa - bogate źródło wolnych aminokwasów (azotu), węgla

Białka – kazeina, żelatyna, białka jaja, surowicy krwi - bogate źródło azotu, węgla oraz mikroelementów

Methods for determination of quarantine bacteria.

Metoda hodowlana

Podłoża mogą być dzielone na wiele sposobów.

I Dzieli się je ze względu na stan na:

1.Stałe

2.Półpłynne

3.Płynne (zwykły bulion odżywczy).

II Ze względu na pochodzenie na:

1.Naturalne

2.Półsyntetyczne

3.Sztuczne.

Methods for determination of quarantine bacteria.

Metoda hodowlana

Podłoża proste lub podstawowe: np. nutrient agar, nutrient broth:

Składniki: pepton, wyciąg mięsny, chlorek sodu i woda.

Używany do oczyszczania kolonii, obserwacji morfologii kolonii, pigmentacji oraz biochemicznych testów identyfikacyjnych

Methods for determination of quarantine bacteria.

Metoda hodowlana

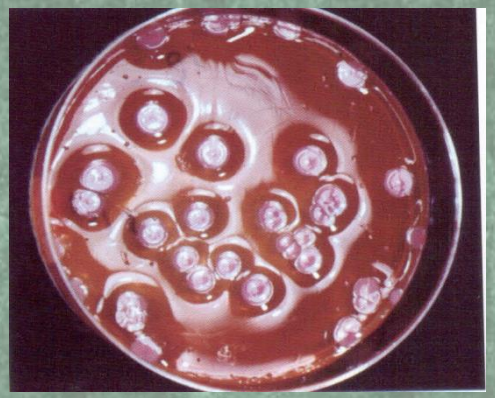
Podłoża złożone: szeroko używane do hodowli bakterii w celach diagnostycznych.

Podłoża półsyntetyczne i syntetyczne: wykonane z czystych składników chemicznych, ich skład jest określony, używane do badań specjalnych np. sprawdzanie metabolizmu bakterii.

Methods for determination of quarantine bacteria.

Metoda hodowlana

Hydroliza pektyn wywołana przez enzymy pektolityczne bakterii *Erwinia carotovora* na podłożu dwuwarstwowym Perombelon po 48 godzinach wzrostu. Górna warstwa składa się z czystych pektyn.



Ryc. „Phytobacteriology principles and practice" J.D.Janse

Methods for determination of quarantine bacteria.

Metoda hodowlana

Bezpośrednio przed pobraniem fragmentów tkanki do izolacji porażone części roślin odkażamy używając podchlorynu sodu w stężeniu ok. 0,5 % lub alkoholu etylowego w stężeniu 70%.

Nie zaleca się używania do powierzchniowego odkażania organów roślinnych silnie działających środków chemicznych ponieważ dyfundują one w głąb tkanek niszcząc komórki bakteryjne.

Methods for determination of quarantine bacteria.

Metoda hodowlana

Do izolacji pobrać mały wycinek chorej tkanki z brzegu typowego uszkodzenia, na granicy ze zdrową tkanką lub z systemu naczyniowego (w zależności od objawów choroby).

Fragmenty tkanki umieścić w małej objętości sterylnej wody lub w roztworze fizjologicznym (0,85% wodny roztwór NaCl).

Materiał rozdrobnić, pozostawić na 5-10 minut.

Wykonać posiew zawiesiny na płytki Petriego z podłożem wzrostowym

Methods for determination of quarantine bacteria.

Metoda hodowlana

1.Posiew za pomocą ezy: pobrać zawiesinę i nanieść na skraj podłoża wzrostowego w postaci linii, następnie wysterylizowaną w płomieniu ezą dotknąć końca wykonanej linii i ciągnąć wężykowatą linię pokrywającą powierzchnię podłoża zgodnie ze schematem poniżej.

2.W ostatnim etapie posiewu nieliczne, przylegające do oczka ezy komórki bakterii zostają pojedynczo rozprowadzone po powierzchni podłoża i dają pojedyncze kolonie bakterii.

Methods for determination of quarantine bacteria.

Metoda hodowlana

Zawiesinę bakterii można rozprowadzić po powierzchni podłoża za pomocą głaszczki lub bagietki. W tym przypadku należy umieścić po środku płytki Petriego ok. 100 µl zawiesiny bakteryjnej i rozprowadzić sterylną głaszczką po całej powierzchni podłoża.

Methods for determination of quarantine bacteria.

Metoda hodowlana

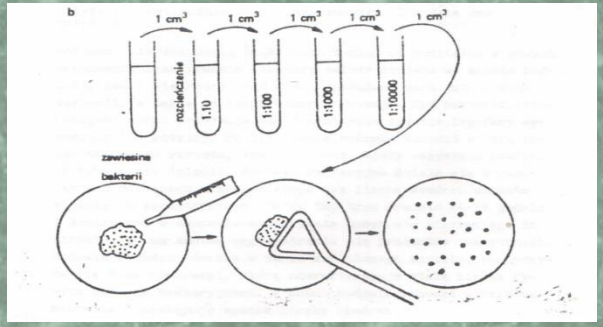
Posiew można wykonać stosując także ilościowe rozcieńczenia zawiesiny.

Jeżeli uzyskana zawiesina zawiera dużą liczbę komórek bakteryjnych stosuje się rozcieńczenia od 10^1 do 10^5 . Do sporządzenia rozcieńczeń używa się sterylnej wody wodociągowej lub roztworu fizjologicznego, który nalewamy do probówek po 9 cm^3 . Do pierwszej probówki wprowadzamy 1 cm^3 wyjściowej zawiesiny i po wymieszaniu przenosimy 1 cm^3 do kolejnej probówki, po czym czynność tę powtarzamy z następnymi probówkami. Z trzech kolejnych ostatnich rozcieńczeń wykonujemy

Methods for determination of quarantine bacteria.

Metoda hodowlana

posiewy na płytki przy użyciu sterylnej głaszczki. W ten sposób możemy uzyskać nie tylko pojedyncze kolonie, ale również określić liczbę bakterii w zawiesinie. Płytki z wykonanymi posiewami umieszczamy w inkubatorach w warunkach optymalnych dla wzrostu poszczególnych bakterii (temperatura , czas inkubacji).



Methods for determination of quarantine bacteria.

Metoda hodowlana

Antybiotyki
Stosuje się do izolacji w celu zapobieżenia przerastania przez inne bakterie.

Roztwór wyjściowy musi być przechowywany w lodówce w ciemnym miejscu po wcześniejszej sterylizacji z użyciem filtrów (np. Millipore) nie dłużej niż 1 miesiąc

Antybiotyki dodaje się do pożywki o temperaturze ok. 40-50^{o}C. Wyższa temperatura wpływa na denaturację większości antybiotyków.

Methods for determination of quarantine bacteria.

Metoda hodowlana

Np.

1.Chloramphenicol (SMSA) - inhibitor bakterii Gram dodatnich i Gram ujemnych

2. Penicillin G (SMSA) - inhibitor bakterii Gram dodatnich.

3. Siarczan Polymixin B - inhibitor bakterii Gram ujemnych.

4.Trimethoprim (MTNA) - inhibitor bakterii Gram dodatnich i Gram ujemnych

5. Kwas nalidyksowy (MTNA) - inhibitor przede wszystkim bakterii Gram ujemnych w mniejszym stopniu Gram dodatnich

6. Amphoterycyna B inhibitor grzybów.

Methods for determination of quarantine bacteria.

Metoda hodowlana

Kolonie bakteryjne badamy po ich posiewie i hodowli na podłożu agarowym.

Sprawdza się:

wielkość kolonii – duże, średnie, małe, drobne lub podaje się średnicę w mm;

typ wzrostu kolonii na podłożu – powierzchniowy, podpowierzchniowy; wgłębny;

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kształt kolonii – okrągła, okrągła z pomarszczonym brzegiem, okrągła z wałem brzeżnym, rozgałęziona, soczewkowata, nitkowata, strzępiasta, amebowata, korzonkowata, pofałdowana, nieregularna, koncentryczna, złożona;

powierzchnię kolonii – gładka, lśniąca, matowa, pomarszczona, pofałdowana, krzaczkowata;

brzeg kolonii – gładki, falisty, ząbkowany, z wyżłobieniami, rozgałęziony, nitkowaty

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szczyt kolonii (wzniesienie kolonii) – kolonia płaska, lekko wypukła, wzniesiona;

przejrzystość kolonii i jej otoczenia – przejrzysta, półprzejrzysta, opalizująca, nieprzejrzysta,

występowanie lub brak fluorescencji;

barwa kolonii i jej otoczenia – podaje się rodzaj zabarwienia lub jego brak, ewentualnie stwierdza się, czy barwnik dyfunduje do podłoża;

konsystencję kolonii – sprawdza się za pomocą ezy – sucha, krucha, ziarnista, skórzasta, ciągnąca się, mazista, śluzowata;

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Pathogenicity test

Thank you for your attention