European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/58 (1)

Diagnostics[1]
Diagnostic

Burkholderia caryophylli

Specific scope

This standard describes a diagnostic protocol for *Burkholderia caryophylli*.

Specific approval and amendment

Approved in 2005-09.

Introduction

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 (Jones, 1941; Gregory, 1942) and occasionally in the EPPO region (Hellmers, 1958) but a combination of plant tests, new cultivars and measures minimizing cross contamination during propagation has resulted in a carnation crop which is largely free from this pest today. There is an excellent and detailed review of the pathogen by Hellmers (1958). *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. Though *B. caryophylli* has been recorded in a number of countries around the world, including the EPPO region (EPPO/CABI, 1998), there are in many cases no recent records. There are relatively few strains deposited in collections.

The pathogen is a typical member of the genus *Burkholderia*, taxonomically distinct from all other known species.

Identity

Name: *Burkholderia caryophylli* (Burkholder) Yabuuchi *et al.*
Synonyms: *Pseudomonas caryophylli* (Burkholder) Starr & Burkholder; *Phytomonas caryophylli* Burkholder.
Taxonomic position: *Bacteria*, *Gracilicutes*.
EPPO code: PSDMCA.
Phytosanitary categorization: EPPO A2 list: no. 55; EU Annex II/A2.

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Detection

B. caryophylli can be found on plants of *Dianthus* species and hybrids, including *D. caryophyllus* (carnation), *Dianthus barbatus* and *Dianthus alwoodii*. 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 (Web Fig. 2). 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. 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). For detection of latent infection, there is no approved or widely used method. Muratore *et al.* (1986) describe an immunofluorescence technique, but give no guidance on sampling, other than macerating 100–500 stem pieces in sterile distilled water, with concentration of cells prior to immunofluoresence or direct plating and subsequent identification and confirmation.

A flow diagram for the detection and identification of *B. caryophylli* in carnation is given in Fig. 1.

Isolation from symptomatic tissue

Small pieces of discoloured vascular tissue from stems are teased out in a few drops of sterile water, and the suspension is

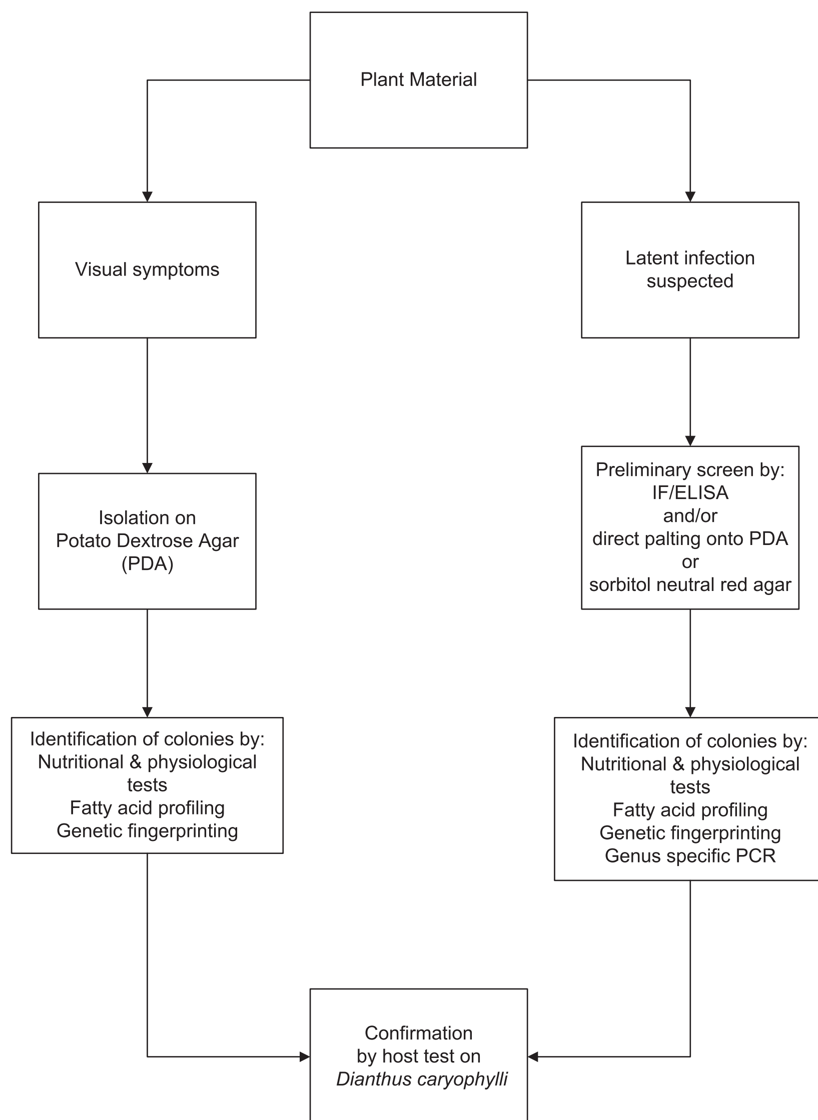

Fig. 1 Flow diagram for the diagnosis of *Burkholderia caryophylli* in carnations.

streaked out onto plates of potato dextrose agar. A Gram stain is also useful at this stage. After incubation for 48 h at 28°C, *B. caryophylli* colonies are entire, raised and are tan (brown)–coloured. After continued incubation, they often develop a purple brown colour. A presumptive diagnosis can be made at this stage.

Isolation from latent infections

Muratore *et al*. (1986) describe a procedure based on immunofluorescence, ELISA and direct plating, which is the basis for the procedure presented below. This takes into account the fact that the IF and direct plating procedure has a better detection threshold than ELISA.

Sampling

Samples should comprise between 100 and 500 carnation stems. A 1-cm long piece is cut from each, and the pieces are homogenized in 0.05 M phosphate buffer pH 7 either in a bag with a hammer or in a blender at a temperature of < 30°C. Excess blending should be avoided. The use of deflocculants and antifoam is advisable. Details are given in Lelliott & Sellar (1976), but with the antifoam content reduced to one tenth. Muratore *et al*. (1986) recommend use of a Sorvall homogenizer for 10 min at 3000 rev/min. The pulp is centrifuged at 4°C at 200 *g* and the supernatant filtered through gauze or muslin. The filtrate is centrifuged at 4°C for 15 min at 10 000 *g* to pellet the bacteria. The final pellet is resuspended in 1.5 mL sterile distilled water.

Immunofluorescence

No anti-sera are currently listed in the catalogues of major commercial suppliers. Muratore *et al*. (1986) prepared a polyclonal antiserum from 48-h-old cells of *B. caryophylli* in rabbits injected twice subcutaneously (2 mL) on days 1 and 3, followed by 4 intravenous injections of 0.5, 1.0, 1.5 and 2.0 mL,

respectively, on days 19, 21, 23, 25. They obtained an IF extinction titre of 1 : 4500 from blood taken on day 32. Muratore *et al*. (1986) used an indirect IF procedure with 1 and 1 : 10 dilutions of the carnation tissue pellet in distilled water. 0.3 mL droplets are placed on IF slide windows (positive control is a 1 : 10 dilution of a *B. caryophylli* reference strain, diluted to an optical density of 0.3 at 660 nm). A typical IF procedure is described by Muratore *et al*. (1986). IF slide wells are incubated in the dark for 30 min with 25 µL of a suitable fluorescein isothiocyanate conjugate at an appropriate dilution. The slides are washed again three times in PBS before carefully blotting dry. A drop (10 µL) of a 1 : 9 solution of glycerine in 0.05 M pH 9.0 sodium carbonate buffer is added to each well and the slide is viewed under an appropriate UV-light microscope after covering the wells with a cover slip. Cells in the test wells that have the same size, characteristics and intensity of fluorescence as the positive reference control are counted per field of view and a population per mL of carnation tissue calculated. Muratore *et al*. (1986) found a linear relationship between the IF count and the percentage of latently infected stems in the sample. A single latently infected stem in a 500-stem sample was detected on some occasions and a single latently infected stem in a 100-stem sample was always detected. A 1 : 10 dilution of the carnation tissue pellet is advisable, since chlorophyll pigments fluoresce bright red in most suspensions and can mask the IF reaction.

Direct plating

Muratore *et al*. (1986) used sorbitol neutral red agar (SNR) for isolation of *B. caryophylli* from latent infections. *B. caryophylli* does not grow well on PCAT medium which is used widely for semi selective isolation of other *Burkholderia* spp., e.g. *B. cepacia*. Samples prepared as above are plated in a dilution series onto both SNR and potato dextrose agar. Plates are incubated at 28°C for at least 48 h. Muratore *et al*. (1986) do not describe colony morphology on SNR medium. On PDA, *B. caryophylli* cells are brown becoming more purple with time. Muratore *et al*. (1986) found that IF was more sensitive than direct plating on SNR medium. The detection threshold was 10^4–10^5 cells for direct plating compared with 10^3–10^4 cells for IF.

Typical colonies on either medium can be identified by the same methods as for those derived from symptomatic tissue. A *Burkholderia*-specific PCR is also available (O'Callaghan *et al*., 1994). This will detect not only *B. caryophylli*, but also *B. andropogonis* and other *Burkholderia* spp., some of which are widespread on plant surfaces (Primer P1240-5′-CTG TTC CGA CCA TTG TAT-3′; Primer P480-5′-GGT ACC GGA AGA ATA AGC-3′).

Identification

Colonies can be identified by classical tests (Lelliott & Stead, 1987), fatty acid profiling (Stead, 1992) or a genetic fingerprint derived for example by BOX-PCR, based on comparison with reference strains. Lelliott & Stead (1987) list several other key tests to support a presumptive diagnosis from typical visual symptoms. These include no green fluorescent pigment on King's medium B, production of arginine dihydrolase, accumulation of poly-β-hydroxybutyrate and reduction of nitrates to nitrogen or nitrites. Schaad *et al*. (2001) list a range of characteristics to differentiate plant pathogenic *Burkholderia* spp. *B. caryophylli* is oxidase positive, does not grow at pH 4 or pH 8, grows at 40°C, does not grow in 3% NaCl, does not utilize starch or pectate but does utilize arginine, betaine, L-valine, adonitol, cellobiose, D-sorbitol, sucrose and D-xylose. The only other *Burkholderia* species pathogenic to *Dianthus* spp. is *B. andropogonis* (syn. *Pseudomonas woodsii*). The symptoms are different. *B. andropogonis* produces leaf spots, and is also oxidase negative and arginine dihydrolase negative.

Fatty acid profiling

Pure cultures are streaked onto trypticase soy agar and incubated for 24 h at 28°C (Stead, 1992). Profiles are obtained using the standard protocol used for the MIDI system (Stead *et al*., 1992). All plant-pathogenic *Burkholderia* spp. contain 5 hydroxy fatty acids – 14 : 0 3OH, 16 : 0 2OH, 16 : 1 2OH, 16 : 0 3OH and 18 : 1 2OH. This combination of 5 hydroxy acids is a chemotaxonomic marker for this group of *Burkholderia* species. *B. caryophylli* is readily distinguished from *B. andropogonis* by the relative amounts of 16 : 0 and 19 : 0 cyclo 11–12. *B. caryophylli* profiles always have less than 3% 19 : 0 cyclo 11–12 and more than 20% 16 : 0 (under the conditions of the test). *B. andropogonis* profiles also have more than 8% 19 : 0 cyclo 11–12 and less than 19% 16 : 0. If comparisons are made with the MIDI TSBA40 library (again under the conditions of the test), *B. caryophylli* strains will almost certainly be listed as *B. caryophylli* (first choice) with a similarity index of > 0.7.

BOX-PCR derived genetic fingerprints

B. caryophylli strains can be identified by many different genetic fingerprinting techniques. *B. caryophylli* strains have a distinct profile compared with all other *Burkholderia* species. BOX-PCR is one of the simplest and most reliable. The BOX-AIR primer sequence is 5′-CTA CGG CAA GGC CGA CGC TGA CG-3′. Under these conditions (see below), *B. caryophylli* strains are well differentiated from all known plant-pathogenic *Burkholderia* species (Richardson *et al*., 2002).

Colonies (24 h at 28°C on nutrient agar) are resuspended in 100 µL 0.05 M NaOH and boiled for 5 min prior to 1 : 10 dilution in nuclease-free micropore water. The PCR reaction mixtures contain 17.35 µL water (as above), 2.5 µL 10X buffer, 2.5 µL primer (100 pmol), 0.25 µL dNTPs (20 mM), 0.4 µL Taq polymerase (Perkin Elmer Amplitaq 50 mL) and 2.0 µL DNA extract. After an initial period of 7 min at 95°C, 30 cycles of 94°C for 1 min, 53°C for 1 min, 65°C for 8 min are followed by a final period of 65°C for 16 min, after which PCR products are separated by gel electrophoresis (2% agarose gels in

1 × TAE running butter containing 0.0005% ethidium bromide solution). Electrophoresis is performed at room temperature, at 10 V/cm for 2 h. Samples are mixed with 5 × loading buffer prior to electrophoresis. Boehringer Mannheim DNA marker VI is an appropriate marker for calculating PCR product size.

Confirmation of pathogenicity

All isolates presumptively identified as *Burkholderia caryophylli* should be confirmed by a host test. Confirmation of the diagnosis is made when typical symptoms are obtained after stem inoculation of young carnation plants. Plants should be maintained at 30–33°C for up to 10 days. Inoculum should contain 10^6–10^7 cfu mL^{-1}. Injection should preferably be made in a leaf axil. Plants should be covered with a polythene bag for 48 h after inoculation.

Reference material

NCPPB 2151 (Type strain).

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

Acknowledgements

This protocol was originally drafted by D. Stead, Central Science Laboratory, York (GB).

References

EPPO/CABI (1998) *Distribution maps of quarantine pests for Europe*, no. 251, CAB International, Wallingford (GB).

Gregory CL (1942) Carnation bacterial wilt in Indiana. *USDA Plant Disease Report* **26** (19), 413.

Hellmers E (1958) Four wilt diseases of perpetual-flowering carnations in Denmark. *Dansk Botanisk Arkiv* **18**, 13–82.

Jones LK (1941) Bacterial wilt of carnation. *Phytopathology* **31**, 199.

Lelliott RA & Sellar PW (1976) The detection of latent ring rot (*Corynebacterium sepedonicum*) in potato stocks. *Bulletin OEPP/EPPO Bulletin* **6**, 101–106.

Lelliott RA & Stead DE (1987) *Methods for the Diagnosis of Bacterial Diseases of Plants.* Blackwell, Oxford (GB).

Muratore MG, Mazzucchi U, Gasperini C & Fiori M (1986) Detection of latent infection of *Erwinia chrysanthemi* and *Pseudomonas caryophylli* in carnations. *Bulletin OEPP/EPPO Bulletin* **16**, 1–12.

O'Callaghan EM, Tanner MS & Boulnois GJ (1994) Development of a PCR probe for identifying *Pseudomonas aeruginosa* and *Pseudomonas* (*Burkholderia*) *Cepacia*. *Journal of Clinical Pathology* **47**, 222–226.

Richardson J, Stead DE, Elphinstone JG & Coutts RHA (2002) Diversity of *Burkholderia* isolates from woodland rhizosphere environments. *Journal of Microbiology* **93**, 616–630.

Schaad NW, Jones JB & Chun W (2001) *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 3rd edn. American Phytopathological Society, St Paul (US).

Stead DE (1992) Grouping of plant pathogenic and some other *Pseudomonas* spp. using cellular fatty acid profiles. *International Journal of Systematic Bacteriology* **41**, 281–295.

Stead DE, Sellwood JE, Wilson J & Viney I (1992) Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. *Journal of Applied Bacteriology* **72**, 315–321.

Appendix 1

Media

Potato dextrose agar

Glucose 20 g; peeled washed potato 200 g; Oxoid agar N°3 15 g; tap water to 1 L. Slice the potato and simmer in the water for 1 h. Strain and make up the volume to 1 L with more tap water. Adjust to pH 6.5, add the glucose and agar and mix to dissolve. Sterilize by autoclaving at 121°C for 15 min.

Sorbitol neutral red agar (SNR)

K_2HPO_4 3.0 g; Na H_2 PO_4 1.0 g; KNO_3 1.0 g; Mg SO_4 $7H_2O$ 0.3 g; neutral red (0.2% aqueous solution 69% active) 10 mL; water 950 mL; agar 15 g. Mix and sterilize by autoclaving at 121°C for 15 min, cool to 50°C and add a mixture of the following filter-sterilized solutions (per L): cycloheximide 100 mg/mL; 75% ethanol 2.0 mL; D-sorbitol (10% aqueous) 50.0 mL.

Web Fig 2 Symptoms of *Burkholderia caryophylli* on carnation after artificial inoculation (Courtesy Dr J. Németh).