

A Note on the Detection of Phytopathogenic Bacteria within the Leaf by a Differential Staining Procedure

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A simple differential staining procedure for demonstrating infection within the leaf tissue by *Pseudomonas tomato*, *Ps. lachrymans* and *Xanthomonas vesicatoria* has been developed. It is based on (1) clearing of plant tissue with a mixture of glycerol, lactic acid, phenol and water; (2) treating the leaf tissue with boiling KOH; and (3) staining with aniline blue-chloralhydrate. When observed under a light microscope, the bacteria appear dark blue, whereas the leaf tissue appears transparent and colourless.

THE METHODS USED for detection of phytopathogenic bacteria inside the host leaf tissue are very often based on transmission electron microscopy. These methods are not suitable for rapid routine work with bacterial pathogens because they are laborious, need expensive equipment and experience for the preparation of ultrathin samples (Sing & Schroth 1977; Essenberg *et al.* 1979).

Histochemical methods using dyes are commonly employed to study pathogenic fungi in leaves (Shipton & Brown 1962). These methods are, however, generally unsuitable for observing phytopathogenic bacteria in their hosts, probably because the dyes also stain the plant tissue, or are unable to penetrate the host tissue and reach the sites where the bacteria are localized.

In this report a simple, fast-staining procedure which stains only the bacteria, and turns the leaf tissue colourless and transparent is described.

Materials and Methods

Organisms, growth conditions and inoculation

Pseudomonas tomato (WT-1) causing bacterial speck of tomato, *Xanthomonas vesicatoria* causing bacterial scab of pepper and tomato, and *Pseudomonas lachrymans* causing angular leaf spot of cucumber and muskmelon isolated from diseased plants in Israel were grown in yeast peptone broth at 30°C (Rudolph & Stahmann 1966).

Tomato plants (*Lycopersicon esculentum* Mill) cv. VF-198, highly susceptible to bacterial speck (Yunis *et al.* 1980), pepper (*Capsicum annuum* L.) cv. Maor, cucumber (*Cucumis sativa* L.) cv. Alma and muskmelon (*Cucumis melo* L.) cv. Hemed were grown according to Bashan *et al.* (1978). Seeds were obtained from Hazera Co., Haifa, Israel. Growth conditions of bacterial cultures, inoculation of plants and reisolation of the pathogens were carried out according to Okon *et al.* (1978), Pohronezny *et al.* (1977) and Stall & Cook (1966), respectively.

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Leaf clearing and staining

Leaves were boiled for 5 min in a solution containing: glycerol, 31 ml; lactic acid, 16 ml; crystalline phenol, 20 g; water, 20 ml, and absolute ethanol, 125 ml (Shipton & Brown 1962). They were then washed with absolute ethanol, transferred to a boiling solution of 78 g KOH in 68 ml water (Gurr 1965), and left for 1 min for tomato and pepper and 10 s for cucumber and muskmelon. A longer treatment of these plants resulted in complete dissolution of the leaves. The leaves were washed further with absolute ethanol and then immersed for 4 min in a mixture of: chloralhydrate, 30 g; lactic acid, 20 ml; absolute ethanol, 5 ml; 1% aniline blue solution, 50 ml (Skiip & Samborski 1974). The stained samples were finally washed with absolute ethanol.

Results and Discussion

Two main obstacles have so far prevented the direct observation of phytopathogenic bacteria inside the plant tissue by conventional light microscopy; (1) the opacity of the plant tissue due, among other factors, to its chlorophyll and starch content, and (2) non-specificity of the dyes used because of their affinity to both bacterial and plant cell components.

To overcome these difficulties, the following procedure was finally adopted: leaves were washed under a water stream for 30 min, to remove saprophytic phyllosphere microflora that otherwise interfered with microscopic observations. They were cleared as described above, rinsed with absolute ethanol, immersed in boiling KOH solution and washed in absolute ethanol. The leaves were then immersed in a boiling solution of aniline blue in chloralhydrate (which stained the bacteria very poorly at room temperature). The greasy dye solution was then removed by washing thoroughly in a large volume of absolute ethanol. When observed under the light microscope, the bacteria were stained dark blue and were easily seen within the colourless and transparent plant tissue.

Microcolonies of *Ps. tomato* inside tomato leaves, *X. vesicatoria* inside pepper leaves, and *Ps. lachrymans* inside cucumber and muskmelon leaves were observed. No bacterial cells could be seen in uninoculated controls. Suspensions of the bacteria were prepared from 48 h old cultures grown in yeast peptone broth, filtered through 0.45 μ Millipore filters and stained on the filters by the same procedure. Microscopically, they looked similar to the bacteria stained inside the leaf tissue. The presence of the pathogens within tested leaf tissue was further confirmed by development of typical symptoms of bacterial speck (*Ps. tomato*), bacterial scab (*X. vesicatoria*) and angular leaf spots (*Ps. lachrymans*) on tomato, pepper, cucumber and muskmelon leaves, respectively, and by isolating the pathogens from the diseased tissue on growth media.

Aniline blue stains the chitin cell wall components of many fungi (Gurr 1965). The partial alkaline hydrolysis of the bacterial cell probably resulted in making bacterial cell wall components (*e.g.* peptidoglycans) more available to this dye. This hypothesis, however, requires verification.

The staining procedure described above is simple and may be used successfully for observing the development of phytopathogenic bacteria inside the leaves of their hosts.

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