

J. Phytopathology **147**, 701–706 (1999)
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 ISSN 0931-1785

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Presumptive Identification of *Pseudomonas syringae*, the Cause of Foliar Leafspots and Streaks on Pearl Millet in Zimbabwe

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With 4 figures

Received February 2, 1999; accepted May 7, 1999

Keywords: bacterial leaf spot, *Pseudomonas syringae*

Abstract

The etiology of a leafspot disease of pearl millet in Zimbabwe was investigated using Koch's postulates. *Bipolaris* sp., *Exserohilum* sp., and bacteria, were isolated from lesions but only bacterial isolates produced the original symptoms following inoculation of a susceptible pearl millet line, 852B. Biochemical and physiological tests, including LOPAT, indicated the bacterium was the fluorescent pseudomonad, *Pseudomonas syringae*. This represents the first report of *P. syringae* on pearl millet in Africa.

Zusammenfassung

Vorläufige Identifizierung von *Pseudomonas syringae* als Auslöser von Flecken und Streifen auf den Blättern von Perlhirse in Simbabwe

Die Ätiologie einer Blattpfleckenkrankheit der Perlhirse in Simbabwe wurde unter Berücksichtigung der Kochschen Postulate untersucht. Von den Läsionen wurden *Bipolaris* sp., *Exserohilum* sp. und Bakterien isoliert, doch nach Inokulation der anfälligen Perlhirselinie 852B riefen nur Bakterienisolate die ursprünglich beobachteten Symptome hervor. Biochemische und physiologische Tests, darunter LOPAT, ergaben, daß es sich bei dem Bakterium um die fluoreszente Pseudomonade *Pseudomonas syringae* handelte. Dies ist der erste Bericht über ein Auftreten von *P. syringae* an Perlhirse in Afrika.

Introduction

Pearl millet (*Pennisetum americanum*) is the principal food crop of sub-Saharan Africa, occupying almost 15 million hectares across a wide range of soils (FAO-IGG, 1990). In Zimbabwe, pearl millet is the second most important cereal after maize in terms of production area. It is grown on about 300 000 ha of marginal lands which are characterized by poor soils, frequent droughts and an

uneven rainfall distribution (Tattersfield, 1982). In these areas, many farmers grow local landrace varieties. However, about 30% of farmers have adopted PMV-2, a variety improved for drought and disease resistance and early maturity, released by the Department of Research and Specialist Services in 1991 (ICRISAT, 1996).

Diseases have not been a constraint with traditional pearl millet varieties probably because low precipitation is not favourable to disease development and local varieties have some resistance. The most prevalent diseases within the Southern African Development Committee (SADC) region are downy mildew [*Sclerospora graminicola* (Sacc, Schoet)], ergot [*Claviceps fusiformis* (Loveless)] and smut [*Tolyposporium penicillariae* (Bref)] (de Milliano, 1992; Mtisi, 1992), which are usually found sporadically and at low severities. Other diseases, such as false mildew (*Beniowskia sphaeroidea*) and leafspots of various etiology, have been noted in farmers' fields (de Milliano, 1992; Mtisi, 1992), but these do not appear to cause significant yield reduction. More recently, the increased incidence of two bacterial diseases, leaf streak (*Xanthomonas campestris* pathovar (pv) *pen-namericanum*) and leaf stripe (*Pseudomonas avenae*), was reported in pearl millet in West Africa (Quobela and Claffin, 1988; Claffin et al., 1989) but their significance to yields are unclear.

In Zimbabwe, new disease problems began to emerge in 1987, particularly as leafspots on improved pearl millet varieties. At the Henderson Research Station at Mazoe, north of Harare, IP 2696 failed to produce seed following a severe leafspot infection (Singh et al., 1990). In 1990, Singh et al. described the causal agent of the disease as a fungus, *Bipolaris urochloae*. Foliar leafspots were again noted on improved genotypes at several test locations in Zimbabwe, including Matopos and Aisleby research farms near Bulawayo, during the 1992–96 growing

seasons. Symptoms, some of which had not been reported by Singh et al. (1990), were especially severe on the improved line 852B and cultivar Okashana-1, and included foliar lesions, leaf wilting and death of the panicle. Lesions were straw-coloured with a tan margin, were variable in shape and size and were oval to circular, single or comprised of a line of 'beads' or coalesced. Chlorosis often accompanied coalesced lesions. Damage was often concentrated at leaf tips and margins. Short, wide streaks of 5 mm width by 3 cm length or thin streaks approximately 3 mm wide but up to 15 cm in length, adjacent to leaf veins, were found less frequently on some materials. There was an obvious differential reaction of genotypes in the field, e.g. Okashana-1, 852B & 863B exhibited more extreme disease reactions than SDMH 94007 and ICMB88004. Diseased germplasm included materials from the regional breeding program and commercially released varieties such as PMV1, PMV2 and Okashana-1. Disease symptoms were also observed on millets in Zambia, Tanzania and Kenya. Identification of the causal agent of this disease became a priority and results are reported herein.

Materials and Methods

Isolation of pathogens

Leaf samples from diseased plants were taken from research plots at Matopos, Lucydale and Aisleby research farms near Bulawayo, Zimbabwe. Lesions on pearl millet leaves were excised and surface sterilized in 0.5% sodium hypochlorite (2 min) and then plated onto lima bean agar (LBA) (Foudin and Calvert, 1987) for the isolation of fungal pathogens. Lesions were washed in sterile distilled water, macerated and mounted on flamed microscope slides to look for bacterial exudation. Loopfuls of exudate were streaked on nutrient agar (NA). Incubation was at 25°C for up to 10 days (fungi) or at 28°C for 24–72 h (bacteria). Single-spore isolates were made of suspect fungal pathogens (*Bipolaris* and *Exserohilum* species) and single bacterial colonies were streaked on NA.

Pathogenicity testing on pearl millet

Since saprophytic fungi and bacteria or weak parasites may colonize diseased tissue, Koch's postulates were conducted with all axenic cultures for proof of pathogenicity. After 1 week of growth on LBA, spore suspensions (10^6 spores/ml) in sterile distilled water were made of *Exserohilum prolatum* and *Bipolaris zea* fungal colonies (kept as IMI accession nos. 359357 and 359356, respectively); bacterial suspensions prepared from colonies on NA were diluted to give an absorbance of 0.15 at 660 nm. From viable plate counts this represented 10^8 CFU/ml (data not shown). Plants of the susceptible pearl millet, 852B (10–20 per pot at the five leaf stage or two per pot at the boot stage) were arranged into three replicated blocks and incubated at 25°C (fungal pathogens) or 28°C (bacterial pathogens) and 90% relative humidity (RH) for 24 h prior to inoculation in the same conditions. Millet plants were inoculated either by spraying to run-off with the test suspension (fungi and bacteria) or by direct injection

of the suspension into the leafsheath or bootsheath (bacteria only); controls were sprayed/injected with water only. Incubation at the set temperature and a high RH of 80% was continued for 1–2 weeks until the onset of symptoms. Inoculation experiments were repeated at least six times.

Tobacco hypersensitivity

Many pathogenic bacteria are able to cause a hypersensitive reaction on tobacco in addition to attacking the normal host (Lelliot and Stead, 1987). The tobacco hypersensitivity test is particularly useful for the rapid screening of a large number of test samples since the tobacco reaction is often faster than the host response to pathogenesis (Lelliot and Stead, 1987; Klement et al., 1990). Additionally, the tobacco hypersensitivity test is recommended for distinguishing between saprophytic and pathogenic fluorescent pseudomonads (Lelliot and Stead, 1987). Mesophyll injections of tobacco plants were performed using suspensions of test bacteria at 10^8 cells/ml or water (control). After 24–48 h of incubation at 28°C and 80% RH, positive reactions were evident as brown, collapsed areas of tissue. Some of the tobacco plants were pre-conditioned by previous treatment for 24 h at 18°C and high RH as per recommendations (Klement et al., 1990).

Characterization of pathogenic bacterial cultures

Pathogens were characterized by biochemical and physiological tests, host tests and following inoculation onto pearl millet; symptoms were described and compared with those seen on the original samples from field nurseries. Pathogens were re-isolated from lesions and the tests repeated to satisfy Koch's Postulates.

Biochemical and physiological tests

Bacterial isolates were tested according to methods described by Lelliot and Stead (1987) for identification of plant pathogenic bacteria. A smear of bacterial growth on nutrient dextrose agar (NDA) was tested for Gram's staining reaction and Levan, Oxidase, Potato Rot, Arginine bi-Hydrolase Tobacco Hypersensitivity (LOPAT) and subsidiary tests were initiated. Tests included fluorescence of colonies on King's B (KB) medium, levan production on 5% sucrose agar, oxidase (Kovac), potato rot, arginine di-hydrolase activity (Thornley's medium 2B), 2-keto gluconate production, lecithin hydrolysis, nitrate reduction, acid from sucrose, starch and aesculin hydrolysis and oxidation/fermentation (Hayward and Waterston, 1965; Lelliot and Dickey, 1984; Lelliot and Stead, 1987; Klement et al., 1990). Additionally, tests for Tween 80 and casein hydrolysis, growth at 4 and 41°C, growth on 0.02% and 0.1% tetrazolium chloride (TTC), growth on 0–5% sodium chloride (NaCl) medium, and pigmentation on yeast dextrose carbonate agar (YDCA) were performed (Quobela and Claflin, 1988). Control (test positive and negative) organisms, including three strains of *Pseudomonas avenae*, two strains of *Xanthomonas campestris* pv *pennamericanum* and two strains of *Pseudomonas syringae* pv *lisi* were included to validate



Fig. 1 Typical grey-brown coloured, water-soaked lesions on seedlings of pearl millet line 852B, 2 days post-inoculation



Fig. 2 Enlarged straw to tan-coloured lesions with characteristic tan-brown border on seedlings of pearl millet line 852B, 7–16 days post-inoculation

tests. All tests were performed on triplicate samples and repeated at least three times.

Host range tests

Sorghum (*Sorghum bicolor*) varieties Marupantse and Framida, maize (*Zea mays*) (varieties Cateto S3, giant sweet corn, CML 216, R201, SC501) and finger millet (*Eleusine coracana*) (varieties FM221 and FM222) were spray inoculated to run-off with cell suspensions of

10^8 CFU/ml concentration and then incubated as described in 'Pathogenicity testing on Pearl Millet' to determine host range. The pearl millet susceptible line, 852B, was included as a check for pathogenicity.

Results

Pathogenicity tests on pearl millet

Nine out of 30 bacterial isolates from pearl millet cultivars PMV2, Okashana-1, 852A, 852B, 863B and



Fig. 3 Natural leafspot infection observed on pearl millet in research plots

852 A × SDMV90003 were pathogenic to the susceptible line, 852B. Lesions were small (2–3 mm diameter), sunken, grey-brown, water-soaked and circular to oval in shape 2 days after inoculation (Fig. 1). Additionally, a few leaves per plant shrivelled from the tips downward, became limp and died. Lesions continued to develop, becoming straw-coloured with a tan margin after approximately 7 days at 28°C (Fig. 2), and strongly resembled the original leaf spot symptoms observed in the field (Fig. 3). On plants inoculated at the boot stage, the resemblance was particularly striking (Fig. 4). Occasionally, lesions developed as short, wide or long, thin streaks which are observed infrequently in the field. Injection of inoculum into the leaf-sheath resulted in elongated foliar lesions; frequently the enclosed stem rotted. When the bootsheath was inoculated, the sheath lost turgor and became brown in colour after 3–4 days. Bacteria were easily recovered from foliar lesions for biochemical testing and comparison with the original isolates.

Exserohilum prolatum and *Bipolaris zeae* were only mildly pathogenic to 852B with infection resulting in few pink-brown, oval to bar-shaped lesions, 5–8 mm in length. Lesions were not apparent until 10 days post-inoculation and did not resemble the original spots or streaks seen in the field.

Tobacco hypersensitivity tests

The nine isolates that were pathogenic to pearl millet line 852B caused necrosis 24 h after injection into mesophyll tissue. The 21 nonpathogenic isolates failed to cause necrosis. Pre-conditioning of tobacco plants at a reduced temperature of 18°C for 24 h prior to inoculation rendered the tissue very sensitive to damage and gave false positive reactions with the negative controls (water).

Biochemical and physiological tests

All nine pathogenic bacterial isolates reacted similarly in all tests performed (Table 1). Cells were rod-shaped, oxidative, Gram-negative, colonies fluoresced on KB medium and were levan positive, oxidase negative, potato rot negative and arginine-dihydrolase negative. Colonies were capable of growth at 4°C but not at 41°C. On NDA the colonies were cream-white. *Pseudomonas avenae* and *Xanthomonas campestris* pv *pennamericanum*, other known bacterial pathogens on pearl millet in West Africa, showed critical differences in reaction in some tests. For example, *P. avenae* is non-fluorescent on KB, oxidase positive and reduces nitrate to nitrite (Clafin et al., 1989); *X.c. pennamericanum* colonies have bright yellow pigmentation on YDCA, hydrolyze casein and are TTC sensitive (Quobela and Clafin, 1988). The results obtained for the nine isolates match those defined by Lelliot and Stead (1987) for a LOPAT group 1a fluorescent pseudomonad and were identical to those of *Pseudomonas syringae* pv *lisi* in the present tests.

Host range tests

Maize, sorghum and finger millet were susceptible to those bacterial isolates pathogenic to pearl millet. On sorghum, small water-soaked lesions appeared on the leaf lamina and leaf margins 2–5 days after inoculation. After 1 week, spots were straw-coloured with a red margin and infected leaf edges and tips were similarly red. Finger millet had significantly fewer lesions and the leaves shrivelled from the tips downwards with limited chlorosis. All the maize genotypes tested showed the same severe reaction: plants had few lesions but leaves became chlorotic within 5 days, wilted and died.

Discussion

Although pearl millet is an important subsistence crop in Africa, there have been few studies on bacterial diseases. Quobela and Clafin (1988) and Clafin et al. (1989) reported two new bacterial diseases, streak and stripe, of pearl millet in west Africa. They identified the causal agents as *Xanthomonas campestris* pv. *pennamericanum* (streak), and *Pseudomonas avenae* (stripe). The lack of streak or stripe symptoms and the predominance of leaf-spots suggested that neither pathogen was involved in the aetiology in Zimbabwe. All of the tests performed endorsed this hypothesis. Although it was not possible to perform host range tests (involving inoculation) with *Xanthomonas campestris* pv *pennamericanum* or *Pseudomonas avenae* for phytosanitary reasons, Quobela and Clafin (1988) and Clafin et al. (1989) reported their host ranges for comparison. *Xanthomonas campestris* pv



Fig. 4 Symptoms on pearl millet, 852B, inoculated at the boot stage of growth, closely resembled field symptoms

Table 1

Results of LOPAT and other biochemical/physiological tests for the nine pathogenic bacterial isolates from pearl millet (PM), three isolates of *Pseudomonas avenae*, two isolates of *Xanthomonas campestris* pv *pennamericanum* (*X.c. penn*) and two isolates of *Pseudomonas syringae* pv *pisi* (*P.s. pisi*)

Test	Isolates from PM	<i>P. avenae</i>	<i>X.c. penn</i>	<i>P.s. pisi</i>
Gram stain	-	-	-	-
Fluorescence on KB	+	-	NT	+
Levan (L)	+	+	NT	+
Oxidase (O)	-	+	-	-
Potato rot (P)	-	-	-	-
Arginine di-hydrolase (A)	-	-	-	-
Tobacco hypersensitivity (T)	+	NT	NT	+
2-keto gluconate	-	-	-	-
Egg yolk (lecithin)	-	NT	NT	NT
Nitrate reduction	-	+	-	-
Acid from sucrose	+	-	+	+
Starch hydrolysis	-	-	-	-
Aesculin hydrolysis	+	-	+	+
Oxidation vs. fermentation	ox	ox	ox	ox
Lipase	+	-	+	NT
Casein hydrolysis	-	-	+	NT
Growth at 4°C	+	-	-	+
Growth at 41°C	-	+	-	NT
0.02% TTC	+	+	+	+
0.1% TTC	+	+	-	+
Growth on YDCA	cream-beige colonies/brown-pink diffusable pigment	cream-beige colonies	yellow non-diffusable pigment	NT
% NaCl tolerance	4	4	2	4

+, positive reaction; -, negative reaction; NT, not tested; ox, oxidation; KB, King's B medium; TTC, tetrazolium chloride; YDCA, yeast dextrose carbonate agar.

campestris could not infect maize, sorghum or finger millet, only pearl millet; *P. avenae* was able to infect maize and sorghum, and finger millet was not tested. Although our test isolates were also pathogenic to maize, sorghum and finger millet in addition to pearl millet,

fundamental differences between the reactions of the test isolates and the two west African pathogens in biochemical tests precluded their identifications. Notable differences were that *P. avenae* isolates formed non-fluorescent colonies on KB, were oxidase positive and

reduced nitrate to nitrite and *X. campestris* pv *pennamericanum* formed bright yellow colonies on YDCA, hydrolysed casein and were TTC sensitive. The results of LOPAT tests indicated that the isolates were of LOPAT group 1a and thus represented a pathovar of *Pseudomonas syringae* since all *P. syringae* pathovars are placed in LOPAT group 1 (Lelliot and Stead, 1987). The identical reactions of isolates of the control, *Pseudomonas syringae* pv *pisi*, reinforced this conclusion.

Pathovars of *Pseudomonas syringae* are important pathogens of a wide range of plants including lilac, citrus, pea, bean, sorghum, maize and wheat (Lelliot and Stead, 1987; Gardan et al., 1991; Young, 1991). *Pseudomonas syringae* pathovar *syringae* was first described as a pathogen of pearl millet in Iowa, USA, by Kendrick in 1926 and was subsequently reported in 1975 in Nebraska and in 1979 in Texas (Odvody and Vidaver, 1980). The present research represents the first report of *P. syringae* on pearl millet in Zimbabwe or indeed anywhere else in Africa. Further characterization is necessary to confirm the pathovar of *P. syringae* represented since biochemical tests could only discriminate between species. However the literature strongly suggests that it will be pathovar *syringae* (Lelliot and Dickey, 1984; Lelliot and Stead, 1987). Full identification of the pathogen will facilitate the development of a screening method to eliminate susceptible pearl millet varieties in the early stages of the breeding programme. Continued surveillance is necessary to assess the incidence of *P. syringae* leafspots on newly released materials and those undergoing on-farm verification in order to check the spread of this disease.

Acknowledgements

D. E. Frederickson thanks the Research Board and Department of Biological Sciences of the University of Zimbabwe for funding and facilities. E. S. Monyo and S. B. King thank ICRISAT for support. We are all grateful to the International Mycological Institute, Surrey, U.K., for further confirming the identities of two representative pathogenic isolates of the nine (accession nos. 373344 and 373345), as *Pseudomonas syringae*.

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