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Isolation of Peirce's disease bacteria from grapevines in Europe

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Abstract

Xylella fastidiosa, the causal agent of Pierce's disease (PD) of grape, was isolated from diseased grapevines grown in Kosova, Yugoslavia. The Kosova isolate was a rod-shaped bacterium which showed a typically rippled cell wall under electron microscopy. ELISA comparisons indicated that the Kosova isolate was closely related to the U.S. PD strains and to several other strains of *X. fastidiosa*. When DNA extracted from diseased grapevines collected from Kosova was used as template in PCR with primer sets specific for *X. fastidiosa*, a band of about 730 bp diagnostic for PD bacteria was detected. DNA from the isolated Kosova bacteria and the type strain of PD yielded the same length of DNA fragment in PCR assay. The Kosova isolate was inoculated into young healthy grapevines through the roots with negative pressure applied to the shoots. Typical scald and scorch symptoms appeared on the leaves of the inoculated grapevines 40–80 days after inoculation. The same bacteria were reisolated from these inoculated diseased plants and used to reinoculate young grapevines. The reinoculated grapevines produced the same symptoms, thereby fulfilling Koch's postulates. This is the first confirmation that PD of grapes occurs in Europe.

Introduction

Pierce's disease (PD), a lethal disease of grapevines, is found in almost all the major grape growing regions in the Gulf Coastal Plains of the United States and in California (Goheen and Hopkins, 1988). The disease was first observed in Southern California in 1892 and was found later to be endemic in the southeastern United States where it limited the commercial grape production (Hopkins, 1989). PD was subsequently reported to occur in Costa Rica, Mexico and Venezuela (Goheen et al., 1979; Goheen and Hopkins, 1988). The disease, however, has not been reported outside the Americas (Goheen and Hopkins, 1988; Hopkins, 1989).

PD is caused by a strain of fastidious gram-negative xylem-limited bacterium, *Xylella fastidiosa*, Wells et al. In nature, *X. fastidiosa* is transmitted by the xylem-feeding suctorial insect vectors (Purcell and Finlay, 1979), such as genera of sharpshooter leafhopper (*Cicadellidae*) and spittlebugs (*Cercopidae*). In 1978, Davis and coworkers successfully isolated and cultured the PD bacterium in a complex medium and subsequently established its disease etiology (Davis et al., 1978). To date, *X. fastidiosa* has been reported to be associated with diseases of many economically important plants, including grapevine, alfalfa, peach, plum, almond, elm, sycamore, oak, maple, citrus, pears, coffee and Oleander (Beretta et al., 1996; Chang and Walker, 1988; Chang et al., 1993; French and Kitajima, 1978; Goheen et al., 1973; Hartung et al., 1994; Hearon et al., 1980; Hopkins, 1982; Hopkins and Mollenhauer, 1973; Hopkins et al., 1973; Kitajima et al, 1975; Kostka et al., 1986; Leu and Su, 1993; Mircetish et al., 1976; Purcell, pers. comm. 1996; Raju et al., 1982; Sherald et al., 1983; Sherald et al., 1987; Wells et al., 1983). It has also been shown that strains of *X. fastidiosa* associated with PD of grapevine can be transmitted to a very wide range of host plants including at least 28 families of monocotyledons and dicotyledons.

Although PD is important in North and South America, it has not been observed in grape-growing regions in Europe. One explanation is that severe winter climates in these areas may limit the range of the disease (Hopkins, 1989; Purcell, 1980). In the mid 1980s, symptoms resembling those from PD-infected grapevines had been observed in vineyards of cermjan region in Kosova, Yugoslavia (B. Berisha, unpubl.). The disease symptoms were attributed to the infection of various fungi although none of them had been proved to be the causal organism. We report here the isolation and cultivation of *X. fastidiosa* from grapevines collected in Kosova, Yugoslavia, and the completion of Koch's postulates with the isolated bacterium. We also present characterization data of our isolate in comparison with three PD isolates of the United States and other *X. fastidiosa* strains. Our study is the first confirmation that PD of grapevines occurs in Europe.

Materials and methods

Plant materials

Grapevine twigs (with leaves attached) from symptomatic and symptomless plants were collected from vineyards in Kosova, Yugoslavia, and kept at 4 °C. Healthy grapevines were either generated from White Riesling seeds or cultivar Blue Vernon Seedless obtained from Horticulture Products, Lindenhurst, New York; they were maintained in an insect-controlled greenhouse.

Culture media and bacterial isolation

Two different media, PD3 (Davis et al., 1980) and CS20 (Chang and Walker, 1988) were prepared for the primary isolation of bacteria from the diseased grapevine samples. Small twigs and petioles were surface sterilized in 10% Clorox (0.525% sodium hypochloride) for 10 min, rinsed in four changes of sterile distilled water, and air dried in a laminar flow hood. At other times, the twigs and petioles were flamed after dipping in 95% ethanol. Of the several isolation methods tried, the following two methods were found to be satisfactory. After surface sterilization, the grapevine twigs were sliced tangentially with a sharp knife. The slices were then directly placed on the agar medium in culture plates. The plates were incubated at 28 °C for 7 to 10 days. After incubation, the twig slices were carefully lifted and bacterial colonies, if any, were observed, identified and sub-cultured. The second isolation method was a modification of the vacuum infiltration method described by French et al. (1977). Twigs of grapevine were cut to 5–6 cm lengths. One end was placed in a small container, such as a microcentrifuge tube or a small culture tube containing 0.5 to 1 ml liquid PD3 medium. To the other end of the twig, a sterilized Tygon tube (Fisher Scientific, Springfield, NJ) was attached and sealed with parafilm. Negative vacuum pressure was applied to the tube to allow the liquid culture medium in the tube to pass through the xylem tissues, and be collected in the Tygon tube. The collected liquid was centrifuged for 3 min at 12,000 g. The pellet was resuspended in 100 μl of PD3 liquid medium and then immediately inoculated onto agar culture plates. The plates were observed for bacterial colony growth after incubation at 28 °C for 7 to 10 days. Colonies of the slow growing bacteria were selected, identified, and subcultured on fresh plates. Several single colonies picked from these subcultures were triply cloned on PD3 and were used for cellular microscopy, serological assays, molecular comparisons and plant inoculation studies. The bacterial cultures were stored in liquid PD3 medium with 20% glycerol at −70 °C.

Cellular microscopy

The primary isolate was first observed with a dark field microscope for tentative identification before subcultures were made. Cultures of the Kosova isolate were harvested from culture plates into 0.01 M phosphate buffered saline, pH7 (PBS). The bacterial suspension was centrifuged at 12,000 g and the pellet was resuspended and fixed with 3% glutaraldehyde for 3 h at 4 °C. The suspension was centrifuged at 12,000 g for 3 min with a microcentrifuge. The supernatant was aspirated. The pellet was rinsed twice with PBS and post-fixed with 1% osmium tetroxide (OsO_4) in PBS at 4 °C for 1 h. The pellet was dehydrated in a cold graded ethanol series and finally transferred to propylene oxide. The pellet was embedded in Spurr's medium (Ladd Research Industries, Inc., Burlington, VT) and polymerized in an oven at 70 °C over night.

Ultrathin sections (60–80 nm) were cut with a diamond knife. Specimens were double stained with 2.5% uranyl acetate in 50% ethanol for 6 min and 2% lead citrate for 2 min and observed with a Philips 301 electron microscope.

Inoculation and reisolation

The Kosova isolate was cultured on PD3 medium and inoculated into young healthy grapevines (Cultivar Blue Vernon Seedless). Bacteria from an 8-day old culture were harvested and suspended in PBS. Young grapevines were removed from the pots and the roots were thoroughly washed with water to remove soil particles. The roots were trimmed and immediately immersed into the bacterial suspension. The stem was trimmed and fitted to a Tygon tube connected to a vacuum, negative pressure was applied for 90 min. Control grapevines were treated in the same manner except that PBS was used instead of the bacterial suspension.

Reisolation of bacteria was carried out following the same procedures as described above for bacterial isolation.

Antisera production and serological assays

An eight-day old log-phase culture of the Kosova isolate was used to immunize Swiss Webster mice. The bacteria were prepared by washing twice with PBS and resuspending in the same buffer. The bacterial suspensions were then sonicated with a Fisher Sonic Dismembrator, (Model 300 Fisher Scientific, Springfield, NJ) for 4 times of 30-50 sec each duration. The immunization scheme and the production of polyclonal antisera were performed as described by Lin and Chen (1985, 1986). The mouse antisera were collected on day 41 after the initial injection and then pooled.

Indirect ELISA (Lin and Chen, 1986) was performed to compare the Kosova isolate with known strains of *X. fastidiosa* from the U.S.. *X. fastidiosa* PD strain 116 was kindly provided by Dr. C.J. Chang, University of Georgia. Other *X. fastidiosa* strains: PD strains, PCE-GG (ATCC# 35877) and PCE-RR (ATCC# 35879), Plum strain PLM-G83 (ATCC# 35871), Periwinkle strain PWT-22 (ATCC# 35878), Ragweed strain RGW (ATCC# 35876), Elm strain ELM-2 (ATCC# 35872), Oak strain OAK, Mulberry strain MUL (ATCC# 35868), and Sycamore strain SYC were kindly provided by Dr. J.M. Wells, USDA. *Xanthomonas campestris* pv. *campestris* (ATCC# 33913) was used as negative control. Specific antiserum to PD 116 was received from Dr. C.J. Chang and antisera to PCE-RR, PCE-GG, PLM-G83, Elm-2 strains of *X. fastidiosa* were received from Dr. J.M. Wells.

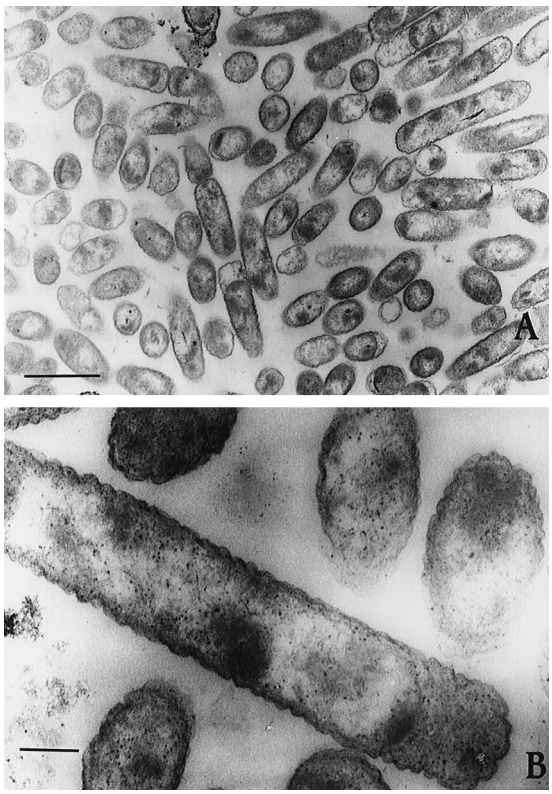

Figure 1. Electron microscopy of the bacterial isolate from diseased grapevines grown in Kosova, Yugoslavia. The bacterium has a characteristic rippled wall. Scale bars, 1 μm in (A), and 0.2 μm in (B).

Polymerase Chain Reaction (PCR) amplification

DNA from Kosova bacterial isolate, as well as DNA from *X. fastidiosa* strains of grapevine PD 116, PCE-GG, PCE-RR, and PLM-G83 were extracted for PCR amplification. DNA of *X. campestris* pv. *campestris* was used as a negative control.

The oligonucleotide primers, RST31 and RST33, were selected according to the report of Minsavage et al. (1994). The PCR amplification was performed in a mixture containing approximately 10^4 bacterial cells, 0.5 mM each primer of a pair, 0.2 mM each of dNTP, 10mM Tris HCl(pH 8.9), 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, and 0.5 U of *Tag* DNA polymerase (Promega Corp., Madison, WI). PCR was carried out for 35 cycles in a Hybrid Thermo Reactor (National Labnet Co., South Plainfield, NJ) with the initial denaturation for 3 min at 94 °C. Each cycle consisted of denaturation for 40 sec at 94 °C, primer annealing for 1 min 20 sec at 52 °C and extension for 1 min 30 sec at 72 °C. After 35 cycles, the reaction was completed by incubating for 5 min at 72 °C. PCR

products were analyzed by electrophoresis in a 1.2% agarose gel containing 0.05 μg/ml ethidium bromide and visualized under UV light.

In addition, total DNA from diseased plant samples from Kosova, infected plants from inoculated grapevines with the Kosova bacterial isolate, and healthy grapevine were also prepared for PCR studies. The large veins of the grape leaves were excised and DNA was extracted from them following the method described by Rowhani et al. (1993) with some modifications. Briefly, 1.0 g of leaf veins were ground to powder in liquid nitrogen, transferred directly into 10 ml of 65 °C preheated extraction buffer (100 mM Tris-HCl, 20 mM EDTA, 500 mM NaCl, 1.25% SDS, pH 8.0) containing 0.1 g PVP, mixed thoroughly and incubated at 65 °C in a water bath for 20 min. After incubation, 4 ml of 5M potassium acetate were added and placed in ice for at least 20 min before centrifugation at 12,000 g for 10 min (4 °C). To the supernatant, 1 ml of 5% CTAB (hexadecyltrimethylammonium bromide) was added followed by 10 ml of chloroform. The mixture was centrifuged at 12,000 g for 10 min (4 °C); 2/3 volume of isopropanol was added to the supernatant to precipitate DNA. The same procedure was used to isolate DNA from cultured bacteria except that liquid nitrogen, PVP and CTAB treatments were omitted.

Results

Isolation of bacteria

Two isolations were made initially from stems and petioles of the grapevine samples collected from Kosova. Both PD3 and CS20 media were successful in obtaining primary isolations; thus, PD3 was used exclusively in all the later tests. In every isolation attempt, obvious bacterial contaminants were observed, but they were easily separated from the slow growing Kosova isolate. The colonies of Kosova isolate usually appeared on agar medium after incubation for 7–10 days at 28 °C. The Kosova bacterium was never isolated from healthy control grapevines.

We have found that using negative vacuum pressure to flush out bacteria with liquid medium through the xylem tissues of twigs worked well. The bacteria collected from this technique had relatively less contaminants and the procedure could be carried out efficiently.

Morphology of Kosova isolate

Under dark field microscopy, the Kosova isolate was a rod shaped bacterium smaller than most of the rod-shaped contaminants in the isolation. Electron microscopy revealed that the bacterial isolate had the same morphology as that reported previously for *X. fastidiosa* associated with PD. The Kosova bacterium had a characteristic rippled wall and measured 0.4 μm $\times$ 2–3 μm (Figure 1).

Inoculation and reisolation

Four of the four plants inoculated with the Kosova bacterial isolate developed disease symptoms on leaf blades in 40–80 days. Small necrotic spots appeared first along the major veins. They gradually enlarged and coalesced. Scald and scorch symptoms became apparent along the edge and in the outer regions of the leaves. Some of the infected leaves withered quickly while others stayed on the vines through the season. Plants inoculated with PBS without the Kosova isolate remained healthy.

The Kosova bacterium was reisolated from these symptomatic grapevines. They were further identified by serology and PCR assays before being re-used to inoculate two healthy young grapevines. The symptoms appearing on the reinoculated plants were similar to the first group of plants infected with the primary Kosova isolate, except that the coalesced spots did not spread as extensively on the leaf blades. In all cases, PCR assays showed the same length of DNA fragment as was amplified from the primary isolate. The same microorganism was thus recovered from the reinoculated plants.

ELISA comparison between Kosova isolate and strains of X. fastidiosa

Strains of *X. fastidiosa* were used as antigens to compare with the Kosova isolate for indirect ELISA. Antibodies prepared against specific strains of *X. fastidiosa* were used to compare with the antibody to the Kosova isolate in reciprocal serological tests (Table 1). Antigens assayed at 4 mg/ml protein (200 ng per-well) showed that the Kosova isolate was serologically related to various strains of *X. fastidiosa* and unrelated to *X. campestris*. The antiserum to the Kosova isolate and the antisera against various other PD strains, cross-reacted similarly to each other, but reacted weakly with the oak, sycamore and ragweed strains. The antisera to plum and elm strains showed slightly different

Table 1. ELISA showing serological relatedness between the Kosova isolate and strains of *Xylella fastidiosa*

Strains[1]	Antisera to					Serum from unimmunized mouse
	PCE116	PCE-RR	PLM-G83	ELM-2	Kosova	
PCE116	0.416[3]	0.540	0.789	0.450	0.385	0.014
PCE-RR (35879)[2]	0.464	0.760	0.428	0.773	0.779	0.017
PCE-GG (35877)	0.655	0.645	0.388	0.605	0.572	0.030
PLM-G83 (35871)	0.452	0.601	0.415	0.544	0.518	0.005
PWT-22 (35878)	0.380	0.436	0.233	0.437	0.281	0.001
RGW (35876)	0.301	0.313	0.100	0.287	0.213	0.004
ELM-2 (35872)	0.392	0.574	0.266	0.620	0.539	0.037
Oak (35874)	0.330	0.366	0.143	0.341	0.276	0.019
SYC	0.301	0.321	0.152	0.286	0.211	0.020
MUL (35868)	0.721	0.829	0.466	0.363	0.818	0.038
Kosova	0.480	0.524	0.593	0.414	0.412	0.023
X.c.c. (33913)	0.012	0.067	0.045	0.363	0.057	0.033

[1] PCE = Pierce disease of grape; PLM = Plum leaf scorch; PWT = Periwinkle wilt; RGW = Ragweed stunt; ELM = Elm leaf scorch; OAK = Oak leaf scorch; SYC = Sycamore leaf scorch; Kosova = Bacterial isolate from Kosova, Yugoslavia; *X.c.c.* = *Xanthomonas campestris* pv. *campestris*; MUL = Mulberry leaf scorch.
[2] Number in parenthesis represents the ATCC number of the *X. fastidiosa* and *X. campestris* pv. *campestris* strains.
[3] OD_{414}.

reactivities to different strains from those of the PD and Kosova isolate antisera, but the results still indicated that all the tested *X. fastidiosa* strains and the Kosova isolate were serologically related.

PCR detection

The oligonucleotide primer pair (RST31/RST33) used in this study was developed by Minsavage and coworkers (1994). They reported that this primer set amplified a 733 bp DNA fragment of *X. fastidiosa*. In addition, when the PCR product was digested with restriction endonuclease *Rsa* I, the PD strain differed from several other strains of *X. fastidiosa*. In our study, the Kosova isolate, the strains of PD, and the plum strain of *X. fastidiosa*, after PCR amplification, yielded a product of about 730 bp DNA fragment, thus indicating that all of them share the same specific genome fragment of *X. fastidiosa*. The RFLP analysis with *Rsa* I digestion clearly differentiated the plum strain from the PD strain; this result placed the Kosova isolate in the same pathotype as the PD strain (Figure 2).

Gel electrophoresis of the PCR amplification products of total DNA extracted from grapevine samples collected from the Kosova vineyards, and from grapevines inoculated with the Kosova isolate, is shown in Figure 3. As with the cultured *X. fastidiosa* strains, the DNA of both the Kosova and the inoculated grapevines were amplified and yielded the 730 bp fragment specific for *X. fastidiosa*. No PCR amplification of DNA from healthy grapevine samples or of DNA from bacteria other than *X. fastidiosa* was detected.

Discussion

The infected grapevines, from which we isolated the PD bacterium, were collected from Cermjan,

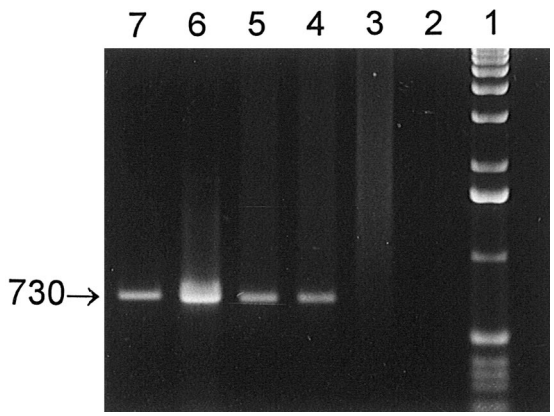

Figure 2. Gel electrophoresis of PCR products of DNA from diseased grapevine collected from Kosova, Yugoslavia and DNA extracted from grapevine inoculated with the Kosova bacterial isolate. Lane 1, 1 kb DNA marker (GIBCO BRL, Gaithersburg, MD); Lane 2, water; Lane 3, healthy grapevine; Lane 4, diseased grapevine from Kosova, Yugoslavia; Lane 5, Young grapevine inoculated with Kosova isolate; Lane 6, PCE-116; Lane 7, Kosova isolate.

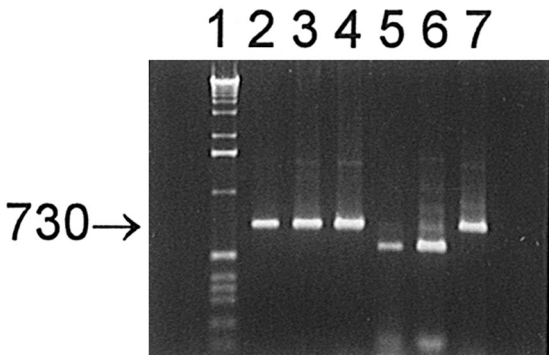

Figure 3. Comparison of gel electrophoresis of *Rsa*I restriction digestion fragments of PCR products between DNA of Kosova isolate and other strains of *Xylella fastidiosa.* Lane 1, 1 kb DNA marker (GIBCO BRL, Gaithersburg, MD); Lane 2, PCE-GG; Lane 3, PCE-RR; Lane 4, Kosova isolate, Lane 5, PLM-G84; Lane 6, PLM-G83; Lane 7, PCE-116.

Gjakova, Kosova, Yugoslavia. According to the statistics released by the Federal Agriculture Committee of Yugoslavia (1990), decline or die-back disease of grapevines occurred as a result of many phytopathogenic fungal infections. Chief among those isolated were *Phomopsis viticola*, *Monscheatia viticola*, *Botryodipledia* sp., *Phiolophora parasitica*, *Aplosporella fabaeformis* and *Stereum* sp. [B. Berisha, unpubl.] The death of grapevines throughout the country ranged from 5–50% with about 30% loss occurring in Kosova. The disease was more frequently manifested in older vines. There were no effective control measures because the causal agent(s) had not been clearly identified.

In the mid 1980's, it was first suspected that some of the grapevines growing in Cermjan, Kosova, Yugoslavia might be infected with pathogens other than the die-back fungi. Among the diseased grapevines, a small number of them (less than 1%) developed chlorotic spots and enlarged discoloration and necroses on leaf blades during the dry summer period. Burning or scald and scorch appeared later and spreaded rapidly in the inner areas. When high temperature was followed by rainfall, the leaves shriveled and died (B. Berisha, unpubl.). Fungi were generally suspected to be responsible for the disease, but these particular symptoms were different from those of die-backs. They resembled the symptoms described for PD of grapes in the United States. Since PD has never been reported in Europe, we therefore decided to determine whether PD might be a source of grape disease in Kosova.

Our EM study of the Kosova bacteria showed they were morphologically identical to the *X. fastidiosa* strains isolated from various plants in the U.S. Antiserum prepared against the Kosova strain gave strong reactions with type PD strains. Serological comparisons with other strains of *X. fastidiosa* have indicated that the Kosova isolate was serologically related to these strains. In addition, specific oligo primers for *X. fastidiosa* used in PCR have amplified a DNA band of about 730 bp from DNA of both the Kosova strain and the type PD strain. PCR detection of *X. fastidiosa* from total DNA extracted from diseased grapevines also resulted in the same band as that from the cultivated bacterial strains. Moreover, RFLP analysis has placed the Kosova isolate and the PD strain in the same pathotype (Figure 2). All of these results suggest that grapevines collected from Kosova showing PD-like symptoms were infected with a PD strain of *X. fastidiosa*.

The results of our inoculation and reinoculation studies with the isolated bacterium to healthy young grapevines have further established that the Kosova isolate of *X. fastidiosa* was the etiological agent of the disease. In both isolation and inoculation experiments we have found that the vacuum method drew in or flushed the bacterium out of the xylem elements with equal efficiency. Young grapevines inoculated with this method developed typical scald or scorch symptoms on leaf blades in 40–80 days. Therefore, Koch's postulates have been fulfilled with the Kosova isolate of *X. fastidiosa*. This is the first confirmation that PD of grapes does occur in Europe.

At present, there are many questions which are still unclear. For example, how was *X. fastidiosa* of PD transferred to Kosova, Yugoslavia? When did PD first appear in Kosova? What insect vectors are there to transmit the PD agent in nature? How widely spread is the disease? It is possible that the disease source was originally introduced to Kosova from North America by American grapevine cultivars or root stocks. The activities of the insect vectors and the winter conditions in Kosova may determine the range of the disease dissemination. Since PD is an important disease of grapevines and endemic in parts of U.S., incidence of PD in Kosova can be viewed as a potential threat to the European grape and wine industries. Concerted efforts to study this grape disease are urgently needed because of potential plant quarantines.

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