Detection of Mycoplasmalike Organisms in Peach and Chokecherry with X-Disease by Fluorescence Microscopy

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ABSTRACT

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Colonization of peach (*Prunus persica*) and chokecherry (*P. virginiana*) by the mycoplasmalike organism associated with X-disease (XMLO) was examined throughout two growing seasons in relation to external symptom development. Leaf midveins and petioles were sampled biweekly in 1983 and weekly in 1984. Peach trees were visually rated for symptom severity using a rating scale 0–4, where 0, 10, 50, 90, and 100% of the canopy exhibited X-disease symptoms. Samples were fixed in glutaraldehyde and sectioned with a cryostat. Tissue sections were stained with the DNA-specific fluorochrome 4′-6-diamidino-2-phenylindole (DAPI) and examined in blind tests. Presence or absence and relative abundance of fluorescent particles (DAPI-stained DNA of XMLO) varied with sampling date, date of initial symptom expression, overall symptom severity, and host species. Observations made by fluorescence microscopy were verified by electron microscopy. XMLO were found in all peach and chokecherry samples from trees that became symptomatic, but none were detected in samples from trees that remained apparently healthy. XMLO-invasion of sieve tubes was more extensive in severely than in mildly symptomatic peach and was often evident up to 6 wk before external symptoms were expressed. In comparison, XMLO-invasion of symptomatic chokecherry was always earlier and more extensive than that of peach. XMLO were consistently detected before abnormalities developed in the phloem.

The association of a mycoplasmalike organism with X-disease (XMLO) of stone fruits was first reported in 1971 (6). Additional lines of evidence have subsequently supported the hypothesis that an XMLO is the etiologic agent, including: further electron microscopy (8,20), symptom remission after tetracycline chemotherapy (12,14), budding, grafting, and leafhopper transmissions (5,13,20), and eradication of the pathogen in budwood by heat treatment (23).

Because attempts to culture XMLO have been unsuccessful, identification and detection of X-disease is usually based on graft and leafhopper transmissions or on electron microscopy. Although these techniques have usually been successful, the irregular distribution of XMLO in peach and other woody hosts has led occasionally to inconsistent successes with bud transmissions (7). Schneider (17) associated necrosis and the buildup of wound gum in phloem of peach and chokecherry with symptoms of western X-disease and concluded, based on the variation in the level of observed phloem necrosis, that the X-disease agent was not equally active or distributed in affected tissue. Jones et al (8) reported that XMLO were found by electron microscopy in only one of 34 symptomatic peach samples from 10 orchards, although they obtained positive graft transmissions from nine out of the same 10 orchards. They also observed XMLO more consistently in chokecherry than in peach with the electron microscope.

Rosenberger and Jones (13) conducted additional graft transmission studies with X-diseased peach and chokecherry and reported the infectivity of peach buds was highest from late June to mid-August and declined during late summer to fall. Transmission of XMLO was more consistent for chokecherry than for peach throughout their test period and reaffirmed previous observations of the more irregular distribution and lower XMLO populations in peach compared with chokecherry.

A number of staining techniques have been tested to improve detection and diagnosis of MLO in plants and to reduce dependency on electron microscopy and various grafting techniques for identification of yellows diseases. The most promising technique uses the DNA-specific fluorochrome 4′-6-diamidino-2-phenylindole (DAPI), which stains specific components of both host and pathogen, whereas many other stains are limited to changes in staining patterns in host tissue.

The objectives of this study were to determine the reliability of DAPI stain to detect XMLO in woody hosts with X-disease and to determine if XMLO can be detected before symptoms are expressed or if there is a relationship between the extent of XMLO-invasion and severity of X-disease symptoms as they develop during the growing season.

MATERIALS AND METHODS

X-Diseased and healthy peach trees (*Prunus persica* (L.) Batsch (cvs. Washington and Loring)) located in a commercial Connecticut orchard and chokecherry bushes (*P. virginiana* L.) at the periphery of the orchard were studied during 1983 and 1984. Peach trees were rated visually for symptom severity using the following scale: 0, 1, 2, 3, and 4 corresponding to 0, 10, 50, 90, and 100% of canopy expressing symptoms of X-disease, respectively. Trees with a rating of 0 throughout the season were considered healthy. Biweekly (1983) and weekly (1984) observations of symptom expression were recorded and leaves were sampled from May through September. Observations on symptom development on individual limbs were more detailed in 1984 than in 1983. Three peach trees were sampled in each severity rating category. Samples consisted of four leaves from each of two limbs per tree on each sampling date. Limbs with symptoms were selected and marked the growing season before sampling to ensure that leaf samples taken before symptom expression were from limbs that were symptomatic the previous year. Four leaves were also sampled from each of three healthy and three X-diseased chokecherry bushes on each sampling date. The X-diseased chokecherry were selected and tagged the growing season before sampling.

Petioles and midveins were dissected from leaf lamina under fixative to minimize fixation artifacts (24). Tissue was sectioned into 2-mm pieces, fixed in 5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7. All tissue pieces were cross sectionally divided in two and processed for both fluorescence and electron microscopy. For fluorescence microscopy, 8–10 μm cryostat tissue sections were processed according to a slight modification of the method reported by Seemüller (18). Tissue sections were stained for approximately 20 min with the fluorochrome DAPI (1 μg ml^{-1} in

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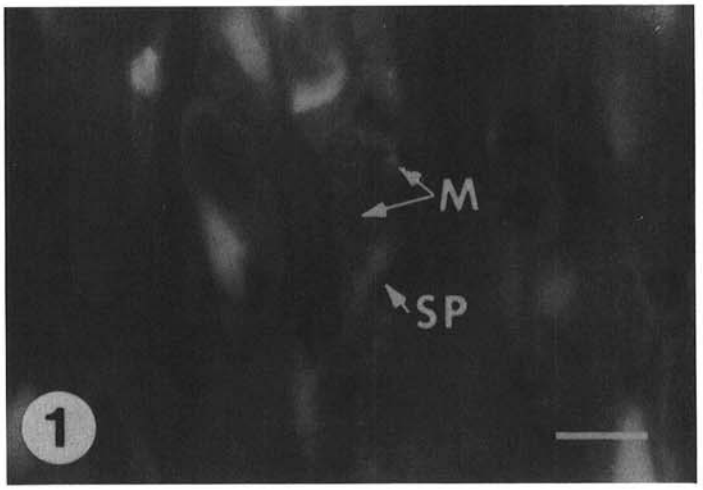

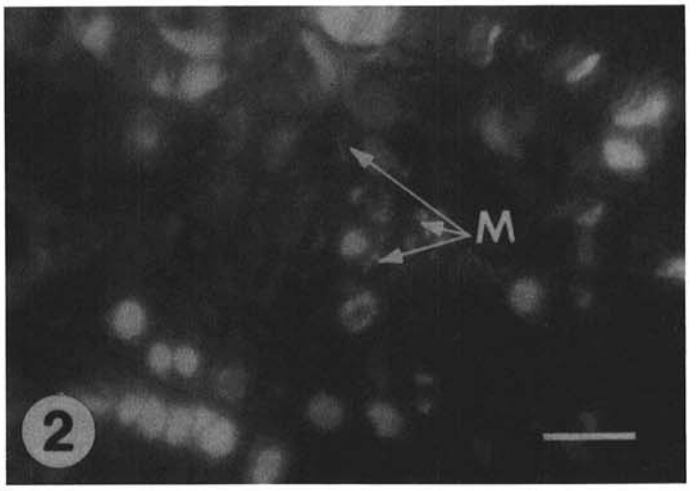

Figs. 1 and 2. Fluorescence micrographs of X-diseased chokecherry tissue stained with DAPI. SP = sieve plate, M = particulate fluorescence of mycoplasmalike organisms (XMLO). Each bar = 10 μm. **1,** Longitudinal section through the phloem of a petiole containing XMLO in a sieve tube. **2,** Cross section through the phloem of a midvein containing sieve elements, which are packed with XMLO.

0.1 M sodium phosphate buffer, pH 7) and washed three times in buffer. Five sections from each of three separate leaf pieces for each tree (regardless of severity rating) and sampling date were examined in blind tests using a Zeiss microscope equipped for epifluorescence with an HBO 50W mercury source and a Zeiss filter set 02, including an exciter filter at 365 nm, a dichromatic beam splitter at 395 nm, and a barrier filter at 420 nm. A sample was rated as DAPI-positive if fluorescent particles were present in at least three tissue sections.

Observations made with fluorescence microscopy were verified with electron microscopy. Tissue for electron microscopy was postfixed with osmium tetroxide, dehydrated in a graded acetone series, and embedded in Spurr's resin (21). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM-09 electron microscope.

Morphological characteristics of XMLO were noted in both peach and chokecherry for comparison with previous reports (6,8,20). Changes in the condition of phloem and the extent of XMLO-invasion was rated from low to high throughout the sampling periods. The relationship of symptom expression to the presence or absence of XMLO was determined by regression analysis.

RESULTS

Fluorescence and electron microscopy. After staining with DAPI, fluorescent particles (DAPI-stained DNA of XMLO) were observed in all peach and chokecherry samples from trees that became symptomatic, but none were detected in samples from trees that remained symptomless. Fluorescent particles were limited to sieve tube elements and were readily distinguished from other sources of fluorescence, including host nuclei, mitochondria, and secondary xylem thickenings (Figs. 1 and 2).

With electron microscopy, XMLO were consistently observed in samples whose counterparts contained fluorescent particles, but none were observed in samples whose counterparts were not DAPI-positive (Fig. 3). Structural and morphological characteristics of XMLO were similar to those reported by Sinha and Chiykowski (20). XMLO were bounded by single unit membranes and usually contained electron-dense ribosome-like granules. They occasionally had central nuclear areas of presumed DNA-like fibrils (Fig. 3). The morphology of the XMLO did not appear to vary from peach to chokecherry. However, XMLO-invasion of chokecherry was always more extensive and XMLO were more regularly distributed than in peach.

Sieve elements in all stages of maturity contained variable numbers of XMLO, but mature elements were most frequently invaded. XMLO were never found in surrounding companion or parenchyma cells. Although XMLO were present in many sieve elements before any irregularity or necrosis of the phloem, such changes were common in the later stages of symptom expression.

Relationship between XMLO-invasion and symptom expression. Characteristically, initial symptoms of X-disease developed quite suddenly and were apparent in 50% of the peach trees in the selected site by 19 July 1983 and 10 July 1984. These were used as reference dates for reporting when XMLO were first detected in tissue samples. Symptoms developed fairly synchronously in all trees, regardless of cultivar or severity rating (i.e., percentage of canopy with X-disease symptoms), and symptomatic portions of these canopies developed X-symptoms at approximately the same time. Fluorescent particles were not

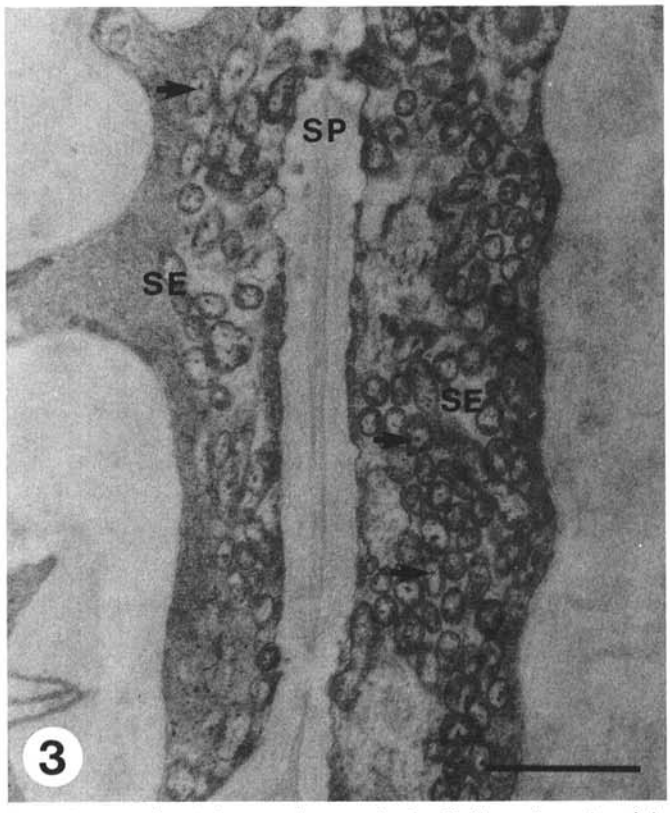

Fig. 3. Cross-sectional electron micrograph of an X-diseased peach petiole with sieve elements (SE) packed with pleomorphic mycoplasmalike organisms, which have occasional central nuclear areas of DNA-like fibrils (arrows). SP = sieve plate. Bar = 700 nm.

detected in samples from trees rated 0 but were consistently detected in samples from trees rated 1–4. Particles were detected in some leaf samples up to 8 wk before symptom expression.

The relationship between sampling date, symptom severity, and appearance of detectable levels of XMLO in peach is summarized for 1983 and 1984 in Figure 4. The vertical bar represents the 1983 and 1984 reference dates for initial X-disease symptoms as previously described. The relationship between XMLO-invasion and symptom expression was very similar for both years, although these events occurred approximately 7–10 days earlier in 1984. XMLO were present at an earlier date and with greater frequency in samples from severely symptomatic peach trees when compared with those samples from mildly symptomatic trees. Samples from trees in rating categories 1 and 2 (with $\leqslant 50\%$ of the canopy exhibiting symptoms) did not contain detectable levels of fluorescent particles until approximately 2 wk after or at the same time as the reference date (i.e., date by which initial symptoms of disease were apparent in the canopy). In contrast, fluorescent particles were observed an average of 2 and 6 wk before symptom expression in samples from trees with ratings 3 and 4 and with 90 and 100% of the canopy symptomatic, respectively.

A Detection Index (DI) was calculated as the difference in weeks between the time XMLO were first detected and the reference date (i.e., date by which initial symptoms were apparent in the canopy) in 1984 (Fig. 5). In general, the DI was proportional to the percentage of the canopy that developed X-disease symptoms. The relationship between XMLO-invasion and symptom expression in peach was described well ($r^2 = 0.96$) by the equation, $DI = -0.3150 - 0.0256\ X + 0.0008\ X^2$, where X is the percentage of the canopy showing X-disease symptoms.

Symptoms of X-disease developed earlier and more gradually in chokecherry than in peach. Foliage of diseased chokecherry bushes exhibited yellow-red fall coloration by mid-June. Leaf samples from chokecherry bushes that remained nonsymptomatic throughout the season were DAPI-negative, whereas samples from symptomatic bushes contained detectable levels of fluorescent particles approximately 2 wk into the sampling period (Fig. 4). XMLO were detected in chokecherry 2 wk before they were observed in samples from the most severely X-diseased peach.

Once detected, the extent and relative frequency of XMLO-invasion usually increased steadily throughout the sampling period in both woody hosts, regardless of symptom severity. However, by mid- to late-August, symptomatic branches of peach often exhibited a flush of growth from newly formed terminal buds after symptomatic leaves began to abscise. X-disease symptoms usually developed on these new leaves but varied from mild to severe. When samples from these leaves were examined by fluorescence and electron microscopy, XMLO were not always observed, even in samples from severely symptomatic leaves. This contrasted sharply with results from leaf samples taken earlier in the season from the same limbs in which samples from symptomatic leaves were DAPI-positive.

DISCUSSION

The fluorochrome DAPI reliably determined the extent of XMLO-invasion of peach and chokecherry throughout the growing season. Fluorescent particles (XMLO) were observed in samples from trees that became symptomatic, but were not observed in samples from trees that remained symptomless. Previous reports of the irregular distribution of XMLO in peach and the more uniform colonization of chokecherry (8) were also confirmed; when samples were not prescreened with DAPI, XMLO were more difficult to locate in both hosts with electron microscopy. The positive relationship between XMLO detection by fluorescence and electron microscopy in the present study is consistent with previous reports correlating MLO fluorescence and positive MLO transmission by budding. DAPI-staining has been used to reliably detect MLO associated with several yellows diseases, including lethal disease of coconut (4), apple proliferation and pear decline (16,19), and blueberry stunt (15).

Sizes and structures of XMLO observed with electron microscopy in both symptomatic peach and chokecherry were consistent with previous reports (6,8,20). Considerable numbers of XMLO were found in sieve elements before any evidence of necrosis or formation of excessive replacement phloem, although the amount of phloem that was invaded was variable. Braun and Sinclair (1,2) also reported that necrosis was not necessarily associated with the presence of MLO in sieve elements of elms affected by elm phloem necrosis. They observed that sieve elements containing MLO often appeared normal and not outwardly different than uninvaded elements. Parthasarathy (10) reported similar observations in palms affected by lethal yellowing.

Season-long sampling of peach trees related X-disease symptom

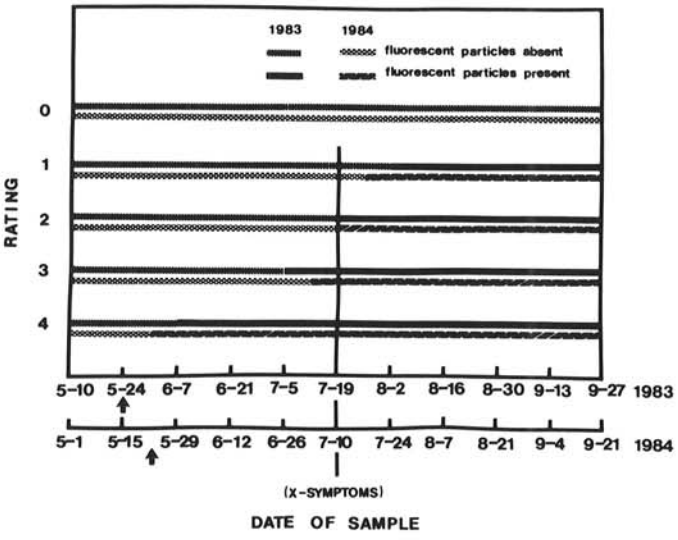

Fig. 4. Relationship of 1983 and 1984 sampling dates and symptom severity rating to appearance of detectable levels of the mycoplasmalike organism associated with X-disease (XMLO). XMLO were observed as fluorescent particles after staining with DAPI. Severity ratings 0 – 4 = 0, 10, 50, 90, and 100% of peach canopy exhibiting symptoms. X-disease symptoms developed by 19 July 1983 and by 10 July 1984. Each bar is a mean of three (1983) or four (1984) trees. The arrows indicate when XMLO were first observed in symptomatic chokecherry for 1983 and 1984.

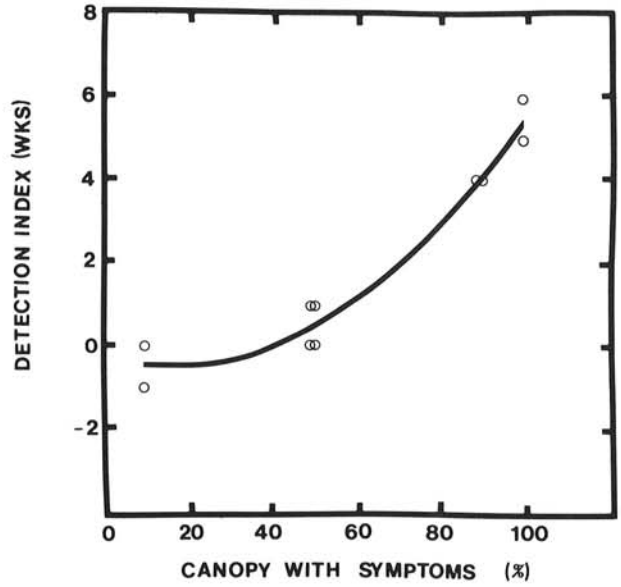

Fig. 5. Relationship between the Detection Index (DI) and the percentage of a peach canopy exhibiting symptoms of X-disease. DI was calculated as the week in the sampling period that mycoplasmalike organisms were detected with DAPI minus the week initial X-symptoms were expressed in individual limbs that were sampled in 1984. Correlation significant at $P = 0.05$.

severity and XMLO-detection in that the greater the percentage of the canopy showing symptoms (as determined by the severity rating) by the reference date, the earlier XMLO populations were detected. A similar relationship has been reported for several herbaceous hosts of *Spiroplasma citri* where severity of symptoms differed among hosts and was correlated with number of spiroplasmas (3).

Considerable differences were also observed in colonization of the two woody hosts by XMLO. In chokecherry, XMLO were present very early in the season (by mid-May), and invasion was always more extensive than that of peach. This agrees with results from budding experiments reported by Rosenberger and Jones (13), where buds taken from symptomatic chokecherries in late May-early June transmitted XMLO in all budding trials. The high levels of XMLO that were detected in chokecherry early in the growing season reinforce the importance of this reservoir host to the epidemiology of X-disease; high numbers of XMLO increase the chances for vector acquisition and subsequent spread of disease from chokecherry to peach, especially when they correspond to the first major leafhopper vector peak (9).

In peach, XMLO were usually present before symptom expression in samples from severely X-diseased trees, and appeared to increase steadily throughout the season. These observations were generally consistent with results of peach budding experiments by Rosenberger and Jones (13), except that they reported a decrease in successful transmissions by the end of the growing season. Severely X-diseased peach trees examined in the present study contained unexpectedly high levels of XMLO by July, and might therefore contribute more to transmission and spread of disease than previously thought, because spread of X-disease in the apparent absence of nearby symptomatic chokecherries has been observed by Rosenberger (11) and Douglas (*unpublished*).

The apparent time lag between bud-break and detection of XMLO reported in this study could be interpreted as the time required for multiplication and/or translocation of the pathogen. This information, in conjunction with previous reports, outlines a theoretical yearly colonization pattern for XMLO. XMLO overwinter in roots (22) and in some buds (13); each spring, XMLO present in buds begin to multiply, whereas XMLO in roots multiply and are translocated to aboveground parts; the time lag before XMLO are detected reflects rates of multiplication and movement that might be associated with physical and physiological differences of host species and might also contribute to patterns of symptom expression in different woody hosts.

Although data from this study strongly support a relationship between the percentage of the canopy involved in symptom expression and date when XMLO are first detected, the onset of initial X-disease symptoms was sudden and occurred at approximately the same time (±2 wk) in peach trees of all severity rating categories. This implies that symptom expression is probably not solely dependent upon XMLO populations, an idea supported by observations of symptom expression before, or concurrent with, XMLO detection by DAPI. Additional evidence supporting this notion is supplied by observations of rat-tails on terminals of symptomatic limbs at the end of the season: XMLO were not always found in these symptomatic leaves. Furthermore, the extent of necrosis and/or formation of excessive replacement phloem, the number of observed XMLO, and the relative frequency of invaded sieve elements do not appear extensive enough to account for either the sudden onset or the severity of symptoms that are associated with X-disease. The suddenness and synchronicity with which peach trees developed disease symptoms, regardless of XMLO level, suggest several additional hypotheses to account for symptom expression. One hypothesis might be based on an environmental trigger (e.g., an accumulation of heat units or a critical threshold temperature) to initiate the onset of symptoms by affecting the host and/or the pathogen. Another hypothesis suggests that symptom expression, while probably only indirectly related to XMLO population, might be related to complex interactions involving metabolically active compounds such as toxins, and/or a variety of host- and pathogen-produced by-products.

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