Distribution and Multiplication of Western Aster Yellows Mycoplasmalike Organisms in *Catharanthus roseus* as Determined by DNA Hybridization Analysis

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ABSTRACT

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Mycoplasmalike organism (MLO) specific DNA probes derived from chromosomal or plasmid DNA of the severe strain of western aster yellows MLO (SAY) were used to monitor the distribution and multiplication of MLOs in periwinkle plants infected with the SAY or dwarf strain (DAY) of the western AY MLO. Plants were graft-inoculated, and DNA was extracted from different regions of the inoculated plants over a 10-wk period. DNA samples were applied to nitrocellulose membranes and hybridized to cloned, ^{32}P-labeled, MLO-specific DNA probes. Relative concentration and distribution of MLOs were determined by measuring the amount of hybridized probe. Colonization patterns for the two AY-MLO strains were similar. The MLOs were first detected in grafted shoots about 2 wk before symptoms appeared. From the grafted shoots, the MLOs moved into ungrafted shoots, and then systemically throughout the plant. Distribution and concentration of MLOs correlated directly with expression of virescence and proliferation symptoms in aerial portions of the plants. MLO concentrations were highest in symptomatic, actively growing shoots and generally lowest in roots.

Plant pathogenic mycoplasmalike organisms (MLOs) infect phloem sieve elements and occasionally the adjacent parenchyma cells (20,24) of plants. Because of the relatively small amount of phloem tissue present in most plants and the difficulty in quantifying MLOs by microscopic techniques, the manner by which MLOs systemically colonize plants and the relative concentration of MLOs in various plant tissues is poorly understood. Sensitive detection of some plant pathogenic mollicutes has been accomplished by DNA hybridization assays (4,10,14–16,21) and serological assays such as enzyme-linked immunosorbent assay (ELISA) (1,3,7,9). Fluorescent staining of MLO DNA has also been used to follow the colonization of MLOs in certain tree species (6,18,22,23). Fragments of chromosomal and plasmid DNA of the severe strain of western aster yellows MLO (SAY-MLO) (8) have been cloned (13,15) and these, as well as other

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DNA probes specific for AY-MLO (16), have been used to differentiate AY-MLOs from other MLOs.

Characterization of the movement, distribution, and multiplication of MLOs in plants is essential for understanding the interaction between the MLO and its host plant. The objectives of this study were to monitor the movement and multiplication of two virescence-inducing MLOs within plants and to correlate the presence and relative titers of the pathogens with symptom expression.

MATERIALS AND METHODS

Test plants. Periwinkle (*Catharanthus roseus* (L.) G. Don) plants were grown from cuttings to a height of approximately 12 cm. The terminal bud was then removed to induce the growth of two symmetric, terminal shoots. When plants reached a height of approximately 25 cm, the two terminal shoots were large enough to be grafted, and several axillary shoots had developed below the two terminal shoots (see Fig. 1). Flowers were removed from the terminal shoots of 15 plants that were then graft-inoculated with scions infected with either the dwarf (DAY-MLO) or severe (SAY-MLO) strains of aster yellows MLO (8). Scions were taken from one symptomatic plant that had been inoculated with either MLO by using *Macrosteles fascifrons* (Stål) leafhoppers. Each scion consisted of a 5-cm segment of infected periwinkle shoot exhibiting symptoms characteristic of each MLO strain (8). Both terminal shoots of each test plant were wedge-grafted with symptomatic scion material, and the graft union was wrapped with latex bandage (Sealtex, Sealtex Company, Clearwater, FL). Grafted shoots were covered with plastic bags and incubated in growth chambers for 10 days, while graft unions healed. After 10 days, all of the scions were successfully established on the grafted plants. The plastic bags were removed, and plants were grown in growth chambers for the duration of the experiments. Growth chamber conditions were 28 C (day) and 21 C (night), 60–80% relative humidity, and a 14-h photoperiod.

Plant samples and DNA preparations. Test plants were sampled at 10, 17, 27, 30, 40, 50, and 70 days after graft inoculation. At the first two sampling times, tissue samples were removed from the following regions of the plant: the grafted shoot below the graft union, the ungrafted shoots that originated from the main stem below the grafted shoots, the main stem, and a mixture of feeder and primary roots (Fig. 1). When side shoots began to emerge from the grafted shoots, these were also sampled (at 27 days and afterward; Fig. 1).

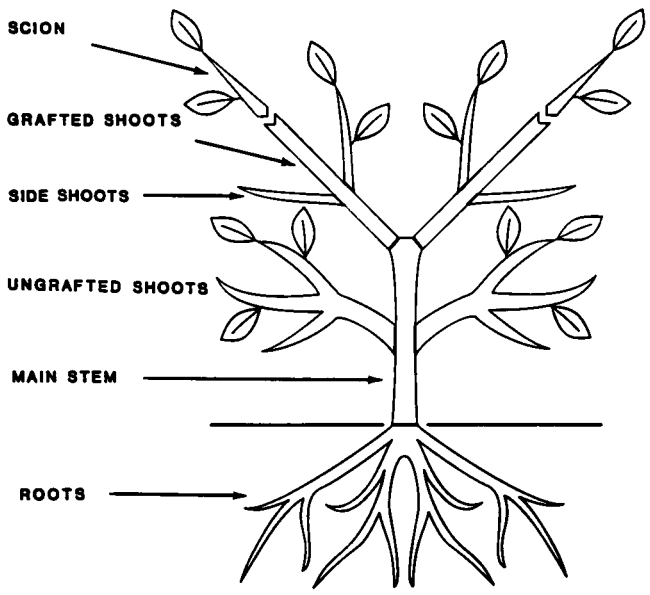

Fig. 1. Diagram of a periwinkle plant illustrating regions that were sampled for the presence of western aster yellows mycoplasmalike organisms (MLOs). Young plants were pruned to develop two primary shoots that were subsequently grafted with MLO-infected scions.

One plant infected with each AY-MLO strain was removed from the growth chamber 10, 17, 40, 50, and 70 days after being grafted, and two replicate samples were taken from each region of the plant. When symptoms first became apparent, 27–30 days after inoculation, two samples from each region of two test plants infected with each strain were sampled (e.g., four replicate samples of each plant region from plants infected with each MLO strain).

Two 1.5-g samples, consisting principally of stems with some leaf petioles at stem apices, were taken from the above-ground regions. Two 3.0-g root samples, consisting of both primary and feeder roots, were also taken. Tissues were ground in an osmotically supplemented buffer by using a mortar and pestle. An MLO-enriched fraction was obtained by differential centrifugation, and DNA was extracted from this fraction as previously described (10). After digestion with RNase A, DNA was electrophoresed in 1% agarose gels, stained with ethidium bromide, and the concentration of DNA in each sample was estimated relative to standards (17). DNA concentrations of all samples were then standardized. DNA extracted from ungrafted, healthy periwinkle was used as an experimental control.

Dot blot hybridization. The proportion of MLO DNA in DNA preparations extracted from infected plants was initially unknown. For this reason, a dilution series was made from each DNA preparation to obtain a range of concentrations for hybridization analysis. Sample DNAs were diluted to final applied concentrations of 120, 80, 40, 20, 10, 5, 1, and 0.5 ng in 6× saline sodium citrate (SSC, 1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) (17). Each DNA sample was denatured by heating at 98 C for 6 min, and one-half of the sample was applied to each of two nitrocellulose membranes (BA45, Schleicher and Schuell, Keene, NH) with a dot blot manifold (Bethesda Research Laboratory, Gaithersburg, MD). Identical, duplicate dot blots were thus prepared for each DNA sample. Membranes were air-dried and baked under vacuum at 80 C for 2 h.

Each of the replicate nitrocellulose membranes were hybridized with either an SAY-MLO chromosomal- or plasmid-specific DNA probe. The chromosomal DNA probe was a 4.1-kb, gel-purified fragment isolated from the recombinant plasmid pAYC4 (15). The SAY-MLO plasmid probe was a 4.9-kb, gel-purified fragment isolated from the recombinant plasmid pPSA45 (14). The extrachromosomal DNA in pPSA45 is thought to be of plasmid rather than viral origin, because no virions were observed in plants infected with SAY-MLO (13). These SAY-MLO chromosomal and plasmid DNA probes also cross-hybridize with DAY-MLO chromosomal and plasmid DNA, respectively (13–15).

The concentration of the two SAY-MLO DNA fragments used as hybridization probes was determined spectrophotometrically (17). Standard curves were generated for both probes by applying known amounts (2.0, 1.2 ng; 800, 400, 200, 100, 50, 25, 10, 5, 1, 0.5 pg) of each DNA fragment to nitrocellulose membranes and hybridizing with the homologous ^{32}P-labeled probe. Standard curves were replicated six times. Probes were radioactively labeled with ^{32}P-dATP by using random oligoprimers (Multiprime Kit, Amersham Corp., Arlington Heights, IL). Triplicate labeling reactions were performed for each probe to determine the range of ^{32}P-dATP label incorporation. The prehybridization, hybridization, and moderately stringent wash conditions used for this experiment have been previously described (10,13).

Quantification of hybridized probe. After being exposed to X-ray film, the 120-ng DNA sample was removed from each blot with a hole punch, placed in 3 ml of scintillation fluid (Scintiverse, Fisher Scientific, Pittsburg, PA), and counted for 5 min in a liquid scintillation counter (Model LS 3801, Beckman Instruments, Fullerton, CA). All of the DNA samples from four replicate dilution series of both the plasmid and chromosomal probes were counted in a similar manner to generate standard curves. Five blank nitrocellulose samples were also counted to determine background hybridization levels. Twenty of the 120-ng plant DNA samples were counted twice to determine variability between counts over the 24-h time period necessary to count all the samples.

Statistical analysis. Analysis of variance of the scintillation count data was done by using the General Linear Models procedure of the Statistical Analysis System package (SAS, SAS Institute, Cary, NC). For analysis, the model used counts per minute as a function of probe identity, plant region sampled, MLO strain, time, and sample replication. No significant differences were found between sample replications.

RESULTS

Detection of AY-MLO in presymptomatic plants. The SAY-MLO plasmid and chromosomal probes (Figs. 2,3) detected MLO DNA 10 days after plants were grafted (the first sampling time) in grafted shoots of plants infected with SAY-MLO. At this time, the graft unions had just healed. MLOs were first detected in grafted shoots in as little as 10 ng of extracted DNA. After 17 days, positive hybridization signals were detected in 0.5 ng of sample DNA from the grafted shoots (data not shown). At this time, the SAY-MLO was also detected in the main stem and ungrafted shoots. The plasmid DNA probe first detected the DAY-MLO in grafted shoots 17 days after plants were grafted. Initial detection of both MLO strains occurred at least 2 wk before development of visible symptoms. In similar experiments, SAY-MLO was detected in presymptomatic celery, aster, and *Nicotiana rustica* (L.) plants that had been inoculated by using *M. fascifrons* leafhoppers (data not shown).

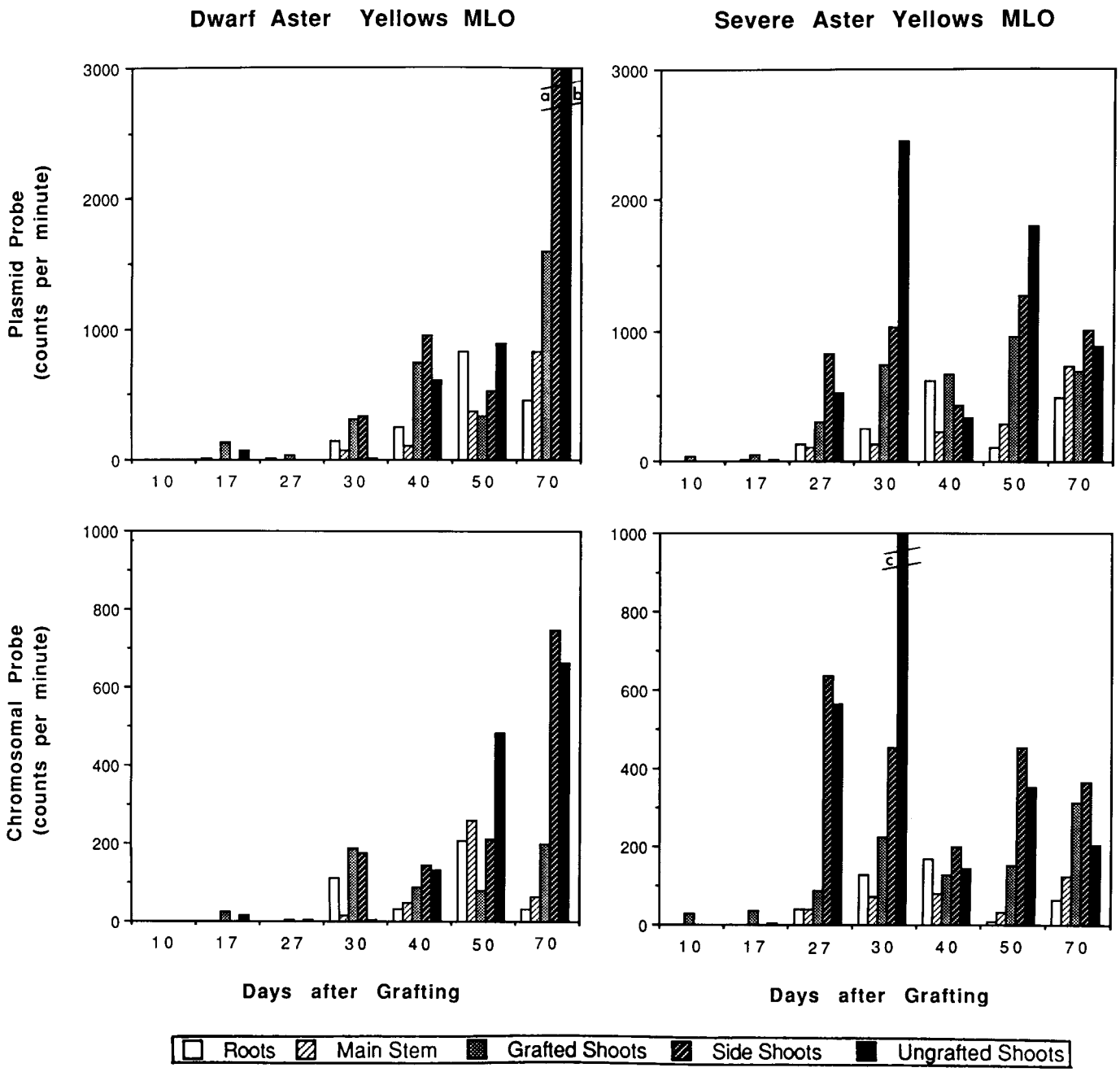

Fig. 2. Distribution of western aster yellows (AY) mycoplasmalike organisms (MLOs) in different regions of periwinkle plants at various times after graft inoculation. Relative MLO concentrations were estimated by quantifying the amount of ^{32}P-labeled SAY-MLO chromosomal or plasmid DNA probes that hybridized with 120 ng of DNA extracted from MLO-enriched fractions. For each AY-MLO strain, two samples from each region on a single plant were collected at 10, 17, 40, 50, and 70 days after inoculation. Two samples from each region on two plants were collected at 27 and 30 days after inoculation. Each value on the graph represents the average counts per minute of hybridized probe with either two samples per region (10, 17, 40, 50, and 70 days after inoculation) or four samples per region (27 and 30 days after inoculation). Count-per-minute values that exceeded the graph scales were (a) 3,673, (b) 3,714, and (c) 1,223. Note differences in the scales of the Y axes of the plasmid vs. chromosomal probe graphs.

Analysis of variance of the scintillation count values indicated that the SAY-MLO plasmid probe provided more sensitive detection of both MLO strains than the chromosomal probe (F test using the type I sum of squares for probe identity, $P = 0.0001$). The SAY-MLO plasmid probe was, on average, three to four times more sensitive than the chromosomal probe in detecting both SAY- and DAY-MLOs in infected plants (note scale of Y axes in Fig. 2). Incorporation of the ^{32}P-dATP label and the standard curves of the plasmid and chromosomal probes were similar (data not shown).

Symptom development. Symptoms first appeared in plants that were grafted with scions infected with SAY-MLO. All flowers that developed after grafting were either partially or completely virescent depending on tissue location. Twenty-seven days after the plants were grafted, five of the remaining 13 plants developed virescent flowers on new shoots that emerged from the grafted shoots (i.e., side shoots; Fig. 1). By 30 days after plants were grafted, all plants inoculated with SAY-MLO developed virescent flowers on side shoots and ungrafted shoots. Between 30 and 40 days after plants were grafted, the shoots of plants infected with SAY-MLO became proliferated and etiolated, and they had small, chlorotic leaves, reddish stems, and virescent flowers. Virescent flowers were present in about half of the remaining plants infected with DAY-MLO 30 days after plants were grafted. After 40 days, all plants infected with DAY-MLO had virescent flowers and proliferated, dwarfed shoots, but they maintained the deep green color characteristic of this AY-MLO strain (8).

Symptoms developed in a relatively uniform manner among all test plants infected with each MLO strain. Symptoms caused by both strains were most severe in young, expanding foliage of the side shoots, ungrafted shoots, and scion tissue. Symptoms were more severe and progressed more rapidly in plants infected with SAY-MLO than in those infected with DAY-MLO. Plants grew rapidly for approximately 40 days, after which time the roots became pot-bound, and the plants grew more slowly. Seventy days after plants were grafted, all above-ground tissues of the plants infected with SAY-MLO were completely symptomatic. At this time, the plants infected with DAY-MLO were symptomatic in most but not all shoots, however, they had not declined as much as the plants infected with SAY-MLO.

Distribution and multiplication of AY-MLO in periwinkle. Both the DAY- and SAY-MLOs moved from the grafted shoots into ungrafted shoots approximately 1 wk after the graft unions had healed (day 17; Fig. 2). As symptoms developed, MLOs could be detected in all parts of the plant, and pathogen titers increased throughout the plant (F test using the type I sum of squares for time, $P = 0.0001$) (Fig. 2). During the first 30 days after plants were grafted, SAY-MLO colonized the plants faster than DAY-MLO (F test using the type I sum of squares for MLO strains, $P = 0.0015$). Maximum SAY-MLO titers occurred 30 days after inoculation and declined afterwards, but DAY-MLO titers continued to increase in most plant regions throughout the 70-day experiment (Fig. 2).

The hybridization probes detected different concentrations of both MLO strains in the plant regions that were sampled (F test of the type I sum of squares of plant regions sampled, $P = 0.0001$). Generally, there was a direct correlation between MLO titer and symptom severity. MLO concentrations were highest in the actively growing, meristematic regions, such as the ungrafted shoots and the side shoots that emerged from the grafted shoot (Fig. 2). MLO concentrations were generally lower in the roots compared to the shoots. DNA was extracted from twice as much root tissue (3.0 g) as stem and shoot tissue (1.5 g) to optimize potential pathogen detection in the roots and to make sure the apparent low concentration was not caused by erratic distribution of the MLO in the root system. Comparatively low concentrations of MLOs were detected in the main stem, which became woody after several weeks.

DISCUSSION

The results obtained in this study indicate that MLO-specific DNA hybridization assays can be used to quantify the multiplication and distribution of MLOs in plants. Statistically significant differences were found in the distribution of MLOs in various plant regions, in the relative sensitivity of MLO plasmid- or chromosomal-specific DNA probes, and in initial colonization rates of DAY- vs. SAY-MLO strains after graft inoculation. Because the number and size of the plants used in this study were limited by growth chamber space, we did not statistically address potential interactions between the test variables. For example, we did not determine if the different strains colonize plant regions differently, if MLO plasmid copy number fluctuates during the infection process, or if one type of hybridization probe provides better MLO detection than the other in certain plant regions.

Comparative sensitivity of AY-MLO chromosomal and plasmid hybridization probes. Hybridization assays using cloned SAY-MLO DNA fragments as probes detected AY-MLOs in periwinkle plants at least 2 wk before symptom expression. In general, the SAY-MLO plasmid probe provided three to four times more sensitive detection of either MLO than did the chromosomal probe. This difference is probably due to a higher copy number of the plasmids compared to that of the MLO chromosomes.

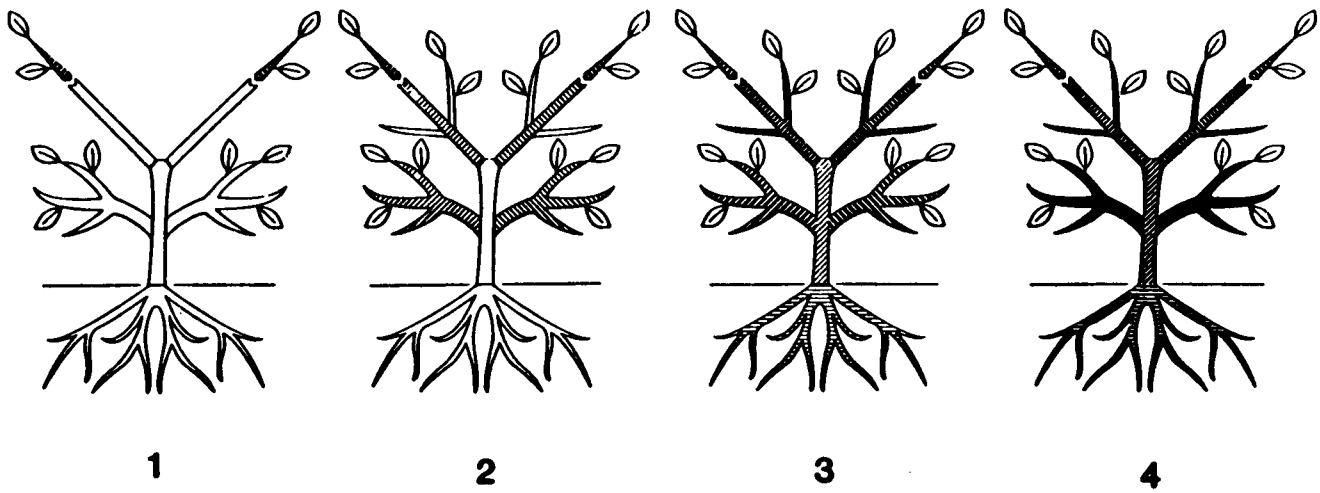

Fig. 3. Diagram illustrating the multiplication and relative concentration of the dwarf strain of western aster yellows (DAY) mycoplasmalike organisms (MLO) in various regions of periwinkle plants after graft inoculation. See Figure 1 for the identity of plant regions sampled. From left to right, drawings 1, 2, 3, and 4 = 10, 17, 40, and 70 days after graft inoculation, respectively. Clear region on drawing = 0–10 cpm, light stripe = 30–150 cpm, dark stripe = 300–2,000 cpm, and black = 3,000–4,000 cpm.

However, both the plasmid and chromosomal SAY-MLO DNA probes used in this study hybridize to multiple DNA restriction fragments in Southern blot analyses. The plasmid probe hybridizes to four AY-MLO plasmids (13,14), and the chromosomal probe hybridizes to three or four *Eco*RI fragments in SAY- or DAY-MLOs, respectively (15). The increase in detection sensitivity of MLO extrachromosomal DNA probes was similar for the maize bushy stunt (4) and *Oenothera* (21) MLOs. However, it is also possible that MLO plasmid copy number could fluctuate during the infection process. Such fluctuation might explain why the ratio of the counts per minute obtained with the plasmid vs. the chromosomal probe was not the same at every time interval for each tissue tested.

Colonization of periwinkle by AY-MLOs. The DAY- and SAY-MLO strains differ in the symptoms they produce in infected host plants (8), but their colonization pattern and distribution in periwinkle were generally similar over a 10-wk period (Figs. 2,3). SAY-MLO colonized periwinkle more rapidly than DAY-MLO, and SAY-MLO was detected about 1 wk earlier than DAY-MLO. Similarly, symptoms were evident in plants infected with SAY-MLO about 1 wk before plants infected with DAY-MLO.

Differences in colonization rates of DAY- and SAY-MLOs could be the result of several factors. Differences in the relative virulence of SAY-MLO compared to DAY-MLO could explain the changes observed in pathogen titers over time. By 4 wk after plants were grafted, the titer of SAY-MLO was higher than that of DAY-MLO, and symptoms caused by SAY-MLO were more pronounced. However, SAY-MLO titers declined after 4 wk, and by 10 wk after inoculation the concentration of DAY-MLO was higher than SAY-MLO (Fig. 3), and the plants infected with DAY-MLO were not as symptomatic as the plants infected with SAY-MLO. If DAY-MLO infection is not as damaging to the plant as SAY-MLO infection, then plants infected with DAY-MLO could support a larger MLO population than plants infected with SAY-MLO.

It is also possible that the apparent difference in colonization rates between these two AY-MLO strains could be due to different concentrations of MLO present in the scions that were used to initiate infection. Although we attempted to use uniform scion material, the initial MLO concentrations in the scions were unknown, and scion tissue infected with SAY-MLO was taken from a somewhat younger, more rapidly growing plant than scion tissue infected with DAY-MLO. Thus, it is possible that the SAY scions may have contained a higher initial concentration of MLOs, or they may have contained more MLO-colonized phloem elements.

Systemic movement in plants has been described for eastern and western X-diseases, clover phyllody (CP), apple proliferation (AP), and pear decline (PD) MLOs, which infect both woody and herbaceous hosts (3,6,9,18,19,22,23). Seemüller et al (18,22,23) followed the seasonal movement of AP- and PD-MLOs in their respective woody hosts by using DAPI staining. Throughout the year, MLOs were most consistently detected in the roots and trunks of trees, but high pathogen titers were also present in symptomatic foliage. Seasonal movement of the MLOs correlated with phloem production and degeneration. Douglas (6) also postulated that the X-MLO overwinters in the roots and some buds, and from these sites the pathogen multiplies and systemically infects the host.

Movement of the plant pathogenic spiroplasmas, *Spiroplasma citri* Saglio (1,3,7,11) and *S. kunkelii* Whitcomb (5,11), has been monitored by microscopy (5), ELISA (1,3,7), and direct plating onto media (11). The movement of *S. citri* in graft-inoculated periwinkle plants was similar to that described for AY-MLO; the spiroplasma was first detected in shoot tips growing from grafted stems, followed by ungrafted stems and roots (1). *S. citri* was first detected in the roots of turnip 4 days after inoculation by *Circulifer tenellus*. After 8 days, the spiroplasma was detected in young uninoculated leaves, inoculated leaves, and in roots, but seldom in old leaves (7).

Relationship between AY-MLO titer and symptom expression. The highest concentrations of both AY-MLO strains were consistently detected in the expanding, symptomatic shoots of infected periwinkle plants. AY-MLO titer was usually lowest in the roots. A similar pathogen distribution was observed in strawberry plants infected with CP-MLO, in which CP-MLO titers were highest in symptomatic pedicels and receptacles, followed by sepals, petals, and leaves (3). The CP-MLO was not detected in strawberry roots. In fully symptomatic plants, concentrations of *S. citri* in celery and orange plants and *S. kunkelii* in maize plants were higher in petioles and/or midveins than in roots as measured by dilution plating (11). However, in maize with early symptoms of corn stunt disease, much higher populations of *S. kunkelii* were observed in roots than in leaves (5). In turnip, *S. citri* was first detected in roots, but multiplied to highest concentrations in the youngest, fully expanded leaves (7). Similarly, the western X-MLO was first detected in the roots of leafhopper-inoculated celery plants, but in fully symptomatic plants, X-MLO titers were highest in the shoot apex, followed by the youngest, symptomatic leaves (9). The X-MLO was also present, but in much lower concentration in the older, completely chlorotic leaves. Similar studies in which the multiplication of X-MLO in cherry trees was monitored by ELISA found that MLO titers were highest in fruit peduncles followed by symptomatic leaf petioles and midribs (9). Douglas (6) also reported a direct correlation between pathogen titers and symptom severity in peach and chokecherry infected with the eastern X-MLO.

Colonization patterns of plant pathogenic MLOs and the phloem-limited spiroplasmas vary with the particular pathogen and its plant host. However, in all of the above cases, pathogen movement in the plant was directed toward meristematic regions of the plant such as the expanding shoots in periwinkle and turnip, the apical crown region of celery, and the expanding fruits of strawberry and cherry. These meristematic regions function as metabolic sinks, to which nutrients are directed via the phloem. MLOs and spiroplasmas move, or are moved, to these nutrient-rich metabolic sinks, where they are able to multiply to high concentrations.

The highest concentrations of AY-MLO occurred in the regions of the plant where symptoms were most severe, which suggests that the MLO may be synthesizing (or utilizing) metabolic substances that induce localized plant symptoms, rather than translocating symptom-inducing metabolites from other colonized parts of the plant. Symptoms of virescence and phyllody caused by AY-MLOs resemble plant growth responses produced by elevated levels of cytokinins, relative to auxins. Certain plant pathogenic bacteria produce cytokinins and auxins that are responsible for symptoms in their host plants (2), and the possibility exists that AY-MLOs produce similar phytoactive compounds (12). The direct correlation between MLO titer and the location and severity of plant symptoms supports this hypothesis, but the identity of these possible metabolites or their mode of action is currently unknown.

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