Isolation of *Spiroplasma citri* from Flowers and Seeds Collected from Infected Periwinkles

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ABSTRACT

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Isolations were attempted from flowers, stems, and seeds from six *Spiroplasma citri*-infected periwinkles and from flowers and stems from two healthy plants. Each flower was separated into four parts: petals, sepals, pistils, and corolla tubes. A total of 140 seeds from infected plants were germinated, with a germination rate of 59%. Two and three months after germination, 25 plants from seeds were sampled for the isolation of *S. citri*. The average spiroplasma cells g⁻¹ of diseased pistils, sepals, petals, corolla tubes, or stems was 1.87×10^8 , 1.9×10^7 , 1.89×10^6 , 3.7×10^6 , or 2.35×10^6 , respectively. The number of cells isolated from pistils was significantly higher than that from stems, whereas the numbers from sepals, corolla tubes, and petals were comparable to those from stems. No spiroplasma was isolated from flower parts of two healthy plants, from seeds, or from samples collected from 25 plants grown from seeds. Results indicated that flowers were good sources for the isolation of *S. citri* and confirmed that there is no seed transmission of the *S. citri* infection in periwinkles.

The diseased tissues commonly used for the isolation of spiroplasmas are young stems, leaves, petioles, or roots, even though citrus seeds had been mentioned (1,5). Bove et al. (1) mentioned that aborted seeds associated with the stubborn disease are a good source for isolation of *Spiroplasma citri*. Flowers are rarely used as the source for the isolation of phytopathogenic spiroplasmas, even though flowers were widely used for the isolation of certain epiphytic microorganisms, including some flower spiroplasmas and achleplasmas (4,8). Due to the fact that *S. citri*-infected periwinkles continue to produce smaller flowers until severely wilted (3), and no reports exist describing the use of infected flowers as the source of *S. citri* isolation, we conducted a project to investigate the possibility of isolating *S. citri* from infected flowers, seeds, and seedlings germinated from the seeds collected from infected plants. For as long as the phytoplasmas remain uncultivable (7), a search for proper medium ingredients and source of tissues for primary isolation is essential to this difficult task. Results of this project may provide important information for the eventual cultivation of phytoplasmas.

MATERIALS AND METHODS

Isolation of *S. citri* from flowers and stems. Six *S. citri*-infected periwinkles and two healthy plants of the same age were used as the source of test flowers and stems. All six periwinkles were infected by graft-transmission, using one *S. citri*-infected scion per each healthy periwinkle, on 22 August 1996. Samples were collected from these eight plants on 26 May 1997 and used for isolation in R₂ medium (2). A total of 10 flowers and 2 stems were randomly picked from each plant. Each flower was carefully separated into four parts: petals, sepals, pistils, and corolla tubes. Tender stems of each plant were used for comparison. The amounts of pistils and sepals used for isolation from each plant were either 0.001 g or 0.01 g, whereas those of petals, corolla tubes, and stems were 0.1 g. The isolation was conducted by using a razor blade to mince the individual parts finely in 1 ml of R₂ medium, followed by a Millipore (0.45 µm) filtration. A 10-fold serial dilution, starting with adding 0.1 ml of the filtrate into 0.9 ml of fresh R₂ medium, was performed through 1×10^{-7} . The culture tubes were incubated at 30°C. The color-changing units signifying growth of *S. citri* were recorded for 20 to 22 days after initial isolation. The last culture tube in the dilution serial that had changed color was used to calculate the color-changing units per 1 ml of the final filtrate, which were then converted to the total cells per gram of respective tissues. The dark-field microscopic observation of the presence of *S. citri* cells was carried out for each tube in which the medium had changed color from

red to yellow. Analysis of variance was applied to the spiroplasma cells isolated from various tissues with the GLM procedure (SAS Institute, Cary, NC). The least significant difference test was applied when the analysis of variance showed statistical significance ($P < 0.05$).

Isolation of *S. citri* from seeds and seedlings germinated from the collected seeds. Seeds collected from the six diseased plants were pooled together for isolations and germinations. Isolation of *S. citri* was done by grinding 20 seeds (approximately 0.02 g) in 1 ml of R₂ medium (two sets) or grinding 30 seeds (approximately 0.04 g) in 2 ml of R₂ medium (two sets) followed by a Millipore filtration (0.45 µm) of the suspension. The filtrate (1/10 ml) was pipetted into 2.5 ml of fresh R₂ medium, which was incubated at 30°C for up to 30 days before a conclusion was drawn. Of the collected seeds, a total of 140 seeds were germinated and the rate of germination was recorded. Each seedling was transferred into a 5-in. pot 1 month after germination for symptom development, if any. In all, 25 plants were sampled for the isolation of *S. citri* 1 and 2 months after they were transferred into individual pots following the method mentioned above, except that the ratio between plant tissues and R₂ medium was set at 1:3 (g:ml).

RESULTS AND DISCUSSION

Isolation of *S. citri* from flowers and stems. None of the 70 culture tubes used for the isolation of *S. citri* from two healthy periwinkles changed color from red to yellow, indicating that no spiroplasma cells were found in all four parts of flowers and stems. Of the 210 culture tubes that were used for the isolation of *S. citri* from six diseased plants, some started to change culture medium color from red to yellow as early as 6 days and some as late as 18 days after initial isolation. The final color-changing units were recorded 22 days after initial isolation. The average number of spiroplasma cells per gram of tissue (Table 1) for each plant was calculated based on the color-changing units and the amount of tissues used in the isolation. The average number of spiroplasma cells per gram of pistils was significantly higher than per gram of stems, whereas the numbers for corolla tubes, sepals, and petals were comparable to the number for stems. These results seemed to indicate that the

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flowers were good sources for the isolation of *S. citri*.

Liao et al. (6) reported that both corn and periwinkle stem extracts suppressed the growth of *S. citri*. For corn stem extracts, the inhibitory action was detected at a dilution of up to 1/160 (corn stem extract/C-3G medium), whereas the action was detected at a dilution of 1/40 for periwinkle extracts. A study was carried out to compare the inhibitory action of the periwinkle stem extracts and flower extracts. The same amount of stem or whole-flower tissues collected from diseased periwinkles was minced in fresh R₂ medium at a ratio of 1:3 (g:ml). The sap was filtered through 0.45-µm Millipore filters. The filtrates, labeled stem extracts or flower extracts, were used as the inocula for *S. citri*. A 10-fold serial dilution, starting by adding 0.1 ml of each filtrate to 0.9 ml of fresh R₂ medium for up to 1×10^{-6} , as performed at 0, 12, 24, 48, 72, and 96 h after the initial filtration. A total of three stem tissues and three flower tissues from three different periwinkles were evaluated. *S. citri* survived in stem extracts for up to 48 h and in flower extracts for up to 96 h. The average cells/ml in the stem filtrates after 48 h were 10³-fold fewer than the number found in the filtrate at 0 h after filtration. The average cells/ml in the flower filtrates after 96 h remained about the same as the number found in the filtrate at 0 h after filtration. (C. J. Chang, *unpublished*). Data seemed to suggest that flower extracts were not as inhibitory to the growth of *S. citri* as stem extracts. Virescent flowers may be worth trying for the isolation of phytoplasmas.

Table 1. Numbers of *Spiroplasma citri* cells (cell log₁₀/gram of tissue) in petals, sepals, pistils, corolla tubes, and stems of diseased periwinkles^x

Plant part	Cell log ₁₀ (CCU/g of tissue) ^y						Mean ^z
	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	
Pistil	9	7	7	8	6	6	7.17 a
Sepal	8	6	6	7	6	6	6.50 ab
Corolla tube	7	7	5	6	6	5	6.00 bc
Stem	6	5	7	6	6	6	6.00 bc
Petal	7	5	5	6	4	5	5.33 c

^x No *S. citri* was isolated from petals, sepals, pistils, corolla tubes, or stems of two healthy plants.

^y CCU = color-changing unit.

^z Means followed by the same letter within a row are not significantly different (least significant difference $P < 0.05$) according to the GLM procedure in SAS (SAS Institute, Cary, NC).

Isolation of *S. citri* from seeds and seedlings germinated from collected seeds. Four different sets of isolations of *S. citri* from seeds collected from diseased periwinkles were negative. The germination rate of the seeds collected from diseased periwinkles was 59% (82/140). Of the 25 seedlings used for the isolation of *S. citri* 2 to 3 months after germination, the isolation was negative. The fact that no symptom was observed in any of the seedlings 5 months after germination confirmed that there was no seed transmission of *S. citri* in periwinkles.

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