

# Cultivation in vitro of Spiroplasmas from Six Plant Hosts and Two Leafhopper Vectors in Arizona

R. M. ALLEN, Professor, and C. R. DONNDELINGER, Research Assistant I, Department of Plant Pathology, Arizona Agricultural Experiment Station, University of Arizona, Tucson 85721

## ABSTRACT

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Zinnia, Aztec marigold, viola, common foxglove, wild turnip, and London rocket are naturally infected hosts of spiroplasmas of the *Spiroplasma citri* serogroup in Arizona. Symptoms and isolation procedures are described. The leafhoppers *Circulifer tenellus* and *Scaphytopius nitridus* are naturally infective. Spiroplasmas were obtained in pure culture from as few as two adult *C. tenellus* but could not be recovered from a preferred feeding and breeding host of the leafhoppers, Russian thistle. Spiroplasmas also could not be recovered from the leafhopper *Aceratagallia obscura*. Factors that appeared to influence successful isolations include season of collection, dilution of tissue extract used as inoculum, constituency of broth medium used, early microscopic examination of immature primary cultures, and maintenance of primary cultures in incubation for several weeks.

Additional key words: beet leafhopper, *Brassica tournefortii*, citrus, mycoplasma-like organisms, *Salsola kali*, *Sisymbrium irio*, stubborn disease

*Spiroplasma citri* (18,19), the causal agent of stubborn disease of citrus (4,13), also causes yellows diseases in many herbaceous cultivars and weeds as a result

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of either natural infections (2,5,16) or experimental transmissions by one or more leafhopper vectors (11,15). Reviews concerning natural and experimental hosts (6-8,14) and vectors (6,10,12) of *S. citri* and other spiroplasmas have been published.

Stubborn disease causes major losses in oranges (*Citrus sinensis* (L.) Osbeck) and grapefruit (*Citrus paradisi* Macf.) in Arizona (1). Naturally infected Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) is also widespread in the citrus-producing region of the state

(2). Thus, it was appropriate to examine other plants as possible sources from which the two vectors, the beet leafhopper (*Circulifer tenellus* (Baker)) and *Scaphytopius nitridus* DeLong, might spread spiroplasmas to citrus.

Based on results from more than 1,400 cultures from 143 plant species and three insect species, mostly made during 1976-1979, we now report successful isolations of spiroplasmas from six plant species. Four of these are common cultivars—zinnia (*Zinnia elegans* Jacq.), Aztec marigold (*Tagetes erecta* L.), viola (*Viola cornuta* L. 'Alba'), and foxglove (*Digitalis purpurea* L.). Two are weeds—wild turnip (*Brassica tournefortii* Gouan) and London rocket (*Sisymbrium irio* L.)—often found in or next to citrus groves. We also present evidence that *Circulifer tenellus* and *Scaphytopius nitridus*, but not *Aceratagallia obscura* Oman, are natural carriers of spiroplasmas.

## MATERIALS AND METHODS

**Collection and processing of plant materials.** Chlorotic and stunted as well as apparently healthy specimens of 143 plant species were collected from 15 communities in southern Arizona. Parts of plants or entire plants were placed on

ice in plastic bags immediately after collection and refrigerated at 4 C for up to 10 days. Plant tissues processed usually included two or more lots of five or six midribs with narrow bands of blade tissue (150–200 mg) cut from leaves washed for 5–10 min in running tap water or scrapings of cortex from stems disinfected in a 0.525% sodium hypochlorite solution (10% bleach) and either rinsed three times in sterile distilled water or washed for 3 min in running tap water.

Tissues were triturated in a homogenizer or mortar containing 9 ml of liquid culture medium (Table 1); each extract was then filtered at a vacuum of 10 lb/in<sup>2</sup> through a 0.45- $\mu$ m filter (HATF 04700, Millipore Corporation, Bedford, MA 01730). Quantities of filtrate (0.2–0.5 ml) were transferred aseptically with a syringe to each of four to eight glass, screw-cap vials (4-dram capacity) containing 4.5 ml of a sterile liquid medium; two or more media of differing constituency received identical quantities of each extract.

Preparations were incubated at 31 C in darkness, usually until a color change (by phenol red indicator in the medium) was noted. When the candidate plant had shown suspicious symptoms, especially stunting and yellowing, microscopic slide examinations were often made of the immature (no color change) cultures after 3–6 days. Whenever a few spiroplasmas appeared early, we transferred 0.5 ml of

the primary culture to a four-to eight-vial series of the same medium and at least one other medium. To verify the presence of spiroplasmas in positive cultures, we examined drops of the culture medium by phase or dark-field microscopy (Leitz Ortholux).

#### Collection and identification of insects.

Beet leafhoppers were collected (April–November) in lots of 200–400 adults with a sweep net and aspirator from Russian thistle (*Salsola kali* L.) in the vicinity of Tucson. Each collection was caged and propagated on healthy glasshouse-grown seedlings of sugar beet (*Beta vulgaris* L. 'USH-9B'), celery (*Apium graveolens* L. var. *dulce* Pers. 'Giant Pascal'), or Little Bright Eye periwinkle after some insects of each collection had been withdrawn for culturing, as described for plant tissue. Representative batches of insect collections were identified by M. W. Nielson, Forage Insects Research Laboratory, USDA, ARS, Tucson, AZ.

*Scaphytopius nitridus* was collected in Tucson in June 1979 from an ornamental, *Cassia didymobotrya* Fres., by M. W. Nielson. *Scaphytopius nitridus* was reared on caged celery or periwinkle seedlings grown in a leafhopper-free glasshouse. All cages of leafhoppers were maintained in insect-proof glasshouse cubicles several miles from the plant rearing site.

*A. obscura*, which has not been reported as a vector of spiroplasmas, was obtained once with beet leafhoppers when a weed mixture of Russian thistle and white bur sage or burweed (*Haplopappus tenuisectus* (Greene) Blake) was swept.

**Media.** Media used (Table 1) were modifications of the SMC medium described by Saglio et al (19). Ingredients of the basal media were heated to boiling

to dissolve them in distilled water, and the mixtures were later sterilized at 121 C for 20 min. Basal media were then cooled to 20 C, at which time 20 ml of heat-treated (56 C, 20 min) horse serum and 10 ml of serum fraction were added. Fructose for the F-2 medium was dissolved in 50 ml of water, filter-sterilized (0.45- $\mu$ m filter), and also added at this time. Horse serum was obtained from Grand Island Biological Co. (Santa Clara, CA 95050); all other organic constituents were obtained from Difco Laboratories (Detroit, MI 48232).

A sterile B-D Cornwall continuous pipetting outfit (No. 3056; Becton, Dickinson & Co., Rutherford, NJ 07072) was used to aseptically transfer 4.5 ml of each sterile complete medium into sterile vials. Filled vials were stored at room temperature for 2–3 days before being used, as a check for sterility.

Occasionally, we included in our isolation attempts media described by others for cultivation of spiroplasmas.

## RESULTS

The following sections describe symptoms on representative, naturally infected plants; when and where they were found; and deviations from usual cultural procedures that may have affected isolation of the spiroplasmas.

**Wild turnip.** Nine of 10 infected plants were severely stunted and appeared light green or yellow; one was symptomless. Cultures from midribs and blades were obtained in 10 of 23 attempts from specimens collected in Chandler, Eloy, Tempe, and Yuma in April 1977. The first isolate was recognized by color change of medium F-2 on day 9 and on day 10 in S-3 medium. Isolation from this host confirms an earlier report by Calavan et al (6).

**London rocket.** Most plants from which we obtained spiroplasmas were symptomless, although two specimens showed slight stunting, mild leaf distortion, and stem twisting. Positive cultures from midribs and blades or stem shavings were obtained in six of 35 attempts in 1977. Many of our unsuccessful attempts were made during the cooler months of January to April. Successes were restricted to collections made in May and June in Eloy, Mesa, Tempe, Tucson, and Yuma. Our first isolate from this host was recognized by color change on day 10 in a vial of MA-1 medium previously inoculated with 0.5 ml of a 10<sup>-2</sup> dilution of plant extract. A like dilution in S-3 medium yielded a positive color change on day 18, whereas 0.5 ml of undiluted extract in MA-1 required 25 days for a comparable color change.

**Zinnia.** Occasional plants were severely stunted (Fig. 1). Basal leaves first showed slight marginal chlorosis and interveinal mottling, followed rapidly by development of dry, brittle, brown necrosis; these symptoms progressed acropetally. Gray-

**Table 1.** Formulations of media used for isolation and cultivation of spiroplasmas in vitro

Ingredient <sup>a</sup>	Medium designation			
	SMC <sup>b</sup>	S-3	F-2	MA-1
PPLO broth base	34	21	21	21
Tryptone	10	10	10	10
Glucose	1	1	...	1
Fructose	1	1	10 <sup>c</sup>	1
Sucrose	10	10	...	...
Maltose	...	...	...	10
Sorbitol	70	70	70	70
L-arginine HCl	...	...	2	...
Fresh yeast extract, 25% (ml)	100	...	...	...
Yeastolate	...	2	2	2
Horse serum (ml)	200	20	20	20
Serum fraction (ml)	...	10	10	10
Sodium hydroxide, 1.0M (ml)	...	6 <sup>d</sup>	6	6
Phenol red (mg)	20	20	20	20
Water (ml)	574	1,000	950	1,000

<sup>a</sup> Amount per liter given in grams unless otherwise stated. Horse serum from Grand Island Biological Co.; all other organic ingredients from Difco.

<sup>b</sup> After Saglio et al (19).

<sup>c</sup> Fructose dissolved in 50 ml of water and filter-sterilized; added to basal medium cooled after steam-sterilization.

<sup>d</sup> Quantity of sodium hydroxide usually required to obtain pH of 7.7–7.8.



**Fig. 1.** Stunted zinnia plant from which spiroplasmas were cultured. Other symptoms shown include brown, necrotic basal leaves and wilt, adaxial leaf roll, and tip necrosis of gray-green apical leaves.

green, apical leaves had dark green tips and were rolled adaxially; these wilted, and death followed within 1 wk. Flowers were smaller and lighter in color than normal and often failed to open completely. Positive cultures from midribs and stem shavings were obtained in 11 of 18 attempts from plants collected in July and August of 1977, 1978, and 1980 at two widely separated locations in the Tucson area. Positive color change was noted on day 7 in both S-3 and F-2 media.

**Marigold.** Spiroplasmas were isolated from midribs or stem shavings in 14 of 16 attempts from plants showing one of two sets of symptoms. The "yellows" plants (Fig. 2) showed slight to severe foliar chlorosis, sometimes on individual stems only, with marked stunting and proliferation of lateral branches bearing tufts of small, yellow foliage. Individual leaflets were rolled abaxially and curved downward; leaflet margins often showed red blotches. Blooms were few and small. These plants showed little, if any, wilting before becoming browned, as if scorched, at death.

The second type we called "gray-green wilt," because symptoms resembled those seen in spiroplasma-infected periwinkle (2). These marigolds appeared normal in height and color until individual stems or the entire plant wilted rapidly with adaxial, longitudinal rolling of distinctly gray-green leaves; plants died 3–5 days after wilting.

Our first isolate from this host, from a plant showing "yellows," was recognized by microscopic examination of an immature primary culture in F-2 medium on day 3; all six vials of this medium showed color changes on day 7, whereas 10 days were required for a color change in the first of six vials of medium S-3 receiving identical inoculum, an extract from midribs and blades. Cultures from "gray-green wilt" specimens were equally successful and yielded isolates indistinguishable serologically (Ouchterlony double diffusion and deformation tests) from isolates from the "yellows" type.

Infected plants of each syndrome were collected in Tucson and in Green Valley, 20 miles south of Tucson, during August 1977, July 1978, and September 1979.

**Foxglove.** This isolate was from a midrib and blade from a single, severely stunted and chlorotic plant found in August 1977 in Green Valley. Successful isolation was indicated by color change of F-2 medium on day 13 and of MA-1 medium on day 20. We obtained no positive isolations in S-3 medium. Plants in this garden were removed before we could resample for a confirming isolation.

**Viola.** Infected plants were stunted; leaves were yellow-orange or necrotic, especially at tips and margins; and blooms usually were few and small. Stems often died, turning light brown;

surviving stems were thin, spindly, and yellow, and foliage on these stems was papery and fragmented easily. Four isolates were obtained from midrib and blade tissues in 29 attempts in May 1976. The first positive culture was recognized by microscopic examination of S-3 medium on day 7; color change occurred on day 10 in all six vials. Efforts to confirm the few isolations made in May 1976 resulted in 46 negative attempts between December 1976 and June 1977 from a new planting in the same location. On the 47th attempt in July 1977, an affected plant several miles from the first yielded an isolate in both S-3 and F-2 media after 6 days.

**Circulifer tenellus.** Spiroplasmas were isolated in May and June of 1978 and 1979 in 19 of 25 attempts from naturally infected beet leafhoppers collected from vigorous stands of Russian thistle. That large numbers of the insects carried spiroplasmas was shown in May 1978 and June 1979 when batches of two, three, three, four, and five adult leafhoppers, processed separately, yielded pure isolates. However, we failed in 23 attempts to obtain spiroplasmas from Russian thistle, a preferred feeding and breeding host of the beet leafhoppers.

**Scaphytopius nitridus.** Because the only collection of this species consisted of 40 adults from *Cassia* sp. in June 1979, no insects were processed for culture, but all were caged on healthy Giant Pascal celery to increase their numbers. Progeny were transferred to a second caged, healthy celery seedling in late August for additional propagation. In October, a batch of 25 progeny adults yielded spiroplasmas in S-3 medium after 14 days. Because these insects had been reared on healthy celery, we concluded that the original 40 adults must have been naturally infective and that the progeny became infective during feeding on the two celery seedlings. However, we were unable in 15 attempts to culture

spiroplasmas from either these or other celery plants upon which known infective *Scaphytopius* had fed.

**Aceratagallia obscura.** Cultures of two separate batches of 25 and 50 adults in July 1979 were negative in S-3 medium after 60 days. These limited results appear to support the nonvector status of these leafhoppers.

## DISCUSSION

Using different media for primary isolations of spiroplasmas is both expensive and time-consuming. This practice, however, resulted in successful culture of the foxglove isolate, whereas reliance upon a single medium probably would have resulted in failure. Each medium described in Table 1 has supported primary isolates in more than one instance, and any or all of them might well be used routinely.

After comparing variations of the SMC medium, we chose S-3 medium as our standard because it is less expensive and has been as good as or superior to SMC in supporting moderately rapid growth of numerous isolates. This medium yielded primary isolates from seven of the eight plant and insect sources reported here.

In several instances, F-2 medium changed color before S-3 medium; also, the foxglove isolate was obtained in F-2 and MA-1 media, whereas identical quantities of inoculum in S-3 medium failed to yield spiroplasmas. These results suggest continued use of the F-2 medium in primary isolations. F-2 medium has also been useful for rapid rejuvenation of cultures after long-term deep freeze. We found, however, that most spiroplasmas grow so fast in this medium that its use as a routine transfer medium is contraindicated.

It may be erroneous to assign spiroplasmas as the primary cause of the symptoms described for these naturally infected plants; spiroplasmas may simply



Fig. 2. "Yellows"-type symptoms shown by this spiroplasma-infected Aztec marigold are tufts of small, chlorotic or bronzed leaflets (some with red blotches) rolled abaxially and curved downward. The tuft of leaflets at the lower left shows necrosis as from scorching. More normal leaflets are shown at the upper right.

occur as cohabitating or, perhaps, secondary organisms. However, we do not believe the isolates reported here resulted from surface-borne contaminants, because of our washing or surface-sterilization procedures. Also, our initial isolates, except those obtained from the natural insect vectors, were generally from plants showing symptoms and the plant tissues we processed were from leaves and stems, not from flowers.

Further, all previously reported (3,9,17) isolates from surfaces or flowers are serologically or culturally distinct from the *Spiroplasma citri* serogroup. Cultural, serologic (growth inhibition, metabolic inhibition, Ouchterlony double diffusion, and deformation) tests, as well as polyacrylamide gel electrophoresis protein patterns, showed that, although there were strain differences, all of our isolates are closely related to strain C 189 (ATCC no. 27563) and to an Arizona isolate of *S. citri* from citrus (J. L. Levitt and R. M. Allen, *unpublished*) and must be considered members of the *S. citri* serogroup.

Factors that seemed to influence favorably our culture of several isolates included onset of hot weather, dilution of tissue extract used as inoculum, constituency of the medium used, early microscopic examination of immature primary cultures, and maintenance of primary cultures in incubation for several weeks. Spiroplasmas were probably not obtained from more of the 143 plant species sampled because some were processed only during cooler winter months. Also, we did not routinely examine immature cultures microscopically after 3–6 days as we now do. This practice has resulted in culture of spiropasmas from several other plants (*unpublished*). Routine use of diluted tissue extracts as primary inocula may be necessary to reduce toxic effects of the extracts on spiropasmas present in the plants.

Failure to isolate spiropasmas from a particular plant species does not prove that the plant was not infected, as is supported by our observations that Russian thistle is a preferred feeding and

breeding host of beet leafhoppers, from which spiropasmas have been cultured repeatedly. Because many leafhoppers spend most of their lives on these plants, we infer that the Russian thistle is a symptomless host and the source of spiropasmas acquired by the insects.

Spiropasmas are pathogenic and are culturable from naturally infected plants of several families, including Scrophulariaceae, a family not previously reported. Infected plants, especially weeds such as wild turnip and London rocket, may serve as reservoirs and alternate hosts of the organism causing stubborn disease of citrus. Spiropasmas may also cause hitherto unsuspected losses in important ornamental plants.

Commonality of morphological, cultural, and serologic relationships of the isolates named herein lends credence to the idea that control or avoidance of stubborn disease of citrus and the disease(s) caused in alternate hosts will be difficult.

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