Marginal Chlorosis, a New Disease of Strawberries Associated with a Bacteriumlike Organism

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ABSTRACT

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A new disease of strawberry (*Fragaria* × *ananassa*), marginal chlorosis, was observed in Spain in 1984 and in France in 1988. It affected all strawberry cultivars, and very high percentages of infection were recorded in some areas. Electron microscopy revealed the presence of a bacteriumlike organism (BLO) in the phloem of affected strawberry plants but not in that of asymptomatic plants. The BLO was 0.2–0.27 μm in diameter and 4 μm in length. The BLO could also be detected by staining with 4′,6-diamidino-2-phenylindole. No reaction was observed when monoclonal antibodies specific to the citrus greening BLO or DNA probes were used to test strawberry materials with symptoms of marginal chlorosis.

Since the autumn of 1988, a new type of decline, called marginal chlorosis, has been observed in strawberry (*Fragaria* × *ananassa* Duchesne) nurseries as well as production fields in all strawberry-growing areas in France. The same decline had also been observed in strawberry nurseries in Spain (Castilla Léon area) as early as 1984 (J. G. Nourrisseau, *unpublished*). The disease affects mother plants as well as F_1 and F_2 progeny plants (11) from most cultivars of both old and new selections. F_1 and F_2 progeny plants are usually infected by natural transmission of an organism, although some are infected via runners.

In 1990, symptomatic plants in affected nurseries represented 35% of the total planted, i.e., 59 cultivars in 38 nurseries totaling 1,091,000 plants. The average percentage of infected plants in production fields was 20%, but certain fields in 1991 had infection rates as high as 90%.

This paper reports symptoms of the disease, the presence of a bacteriumlike organism (BLO) in the phloem of affected plants, the use of 4′,6-diamidino-2-phenylindole (DAPI) staining for quick detection of the BLO, and attempts to characterize the BLO.

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MATERIALS AND METHODS

Samples for electron microscopy and DAPI staining were collected from plants in nurseries located in central or southern France and from plants in production fields of four farms with similar agroclimatic conditions, located in the Dordogne area of southwestern France.

Electron microscopy. Leaf midribs and petioles (15 fragments, each 2 mm long) from 16 samples of the six main cultivars grown in France (Chandler, Dover, Elsanta, Fern, Rabunda, and Selva) showing symptoms of marginal chlorosis were fixed overnight in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5), postfixed in 1% osmium tetroxide, dehydrated in a series of ethanol (25–100%), and embedded in epoxy resin (Epon 812) according to the method described by Luft (9). Longitudinal and transverse sections were cut with an ultramicrotome (LKB Ultrotome III) and observed in an electron microscope (Siemens Elmiskop 101). Sections of leaf midribs and petioles from asymptomatic strawberry plants of the same cultivars were prepared in the same manner.

DAPI staining. DAPI staining was done as described by Seemüller (13). Briefly, fresh material was cut in fragments 0.5 mm long, and longitudinal

sections 15–25 μm thick were made with a cryomicrotome (Leitz 1320). The sections were transferred into a 0.2% solution of diethyldithiocarbamate (DIECA) for 2 min, then into DAPI solution at a concentration of 1 μg/ml. The sections were observed with a Zeiss epifluorescent microscope with the filter combination BP 365/FT 395/LP397.

DAPI staining was performed on the same samples as those used for electron microscopy. Additional samples showing typical or uncharacteristic symptoms of marginal chlorosis from 159 plants comprising 50 cultivars or selections collected from October 1988 to June 1991 in the main production areas of France were also tested. The cultivars were: Addie, Aiko, Aliso, Apollo, Arking, Belle de Mai, Belrubi, Benton, Brighton, Chandler, Daribelle, Darline, Douglas, Dover, Dukat, Earliglow, Ecker, Elsanta, Favette, Fern, Fickfak, Fleuramy, Gariguette, Gea, Gorella, Hummi Gento, Maregal, Minja, Nicolla, Ostarra, Pajaro, Pandora, Rabunda, Rapella, Redgauntlet, Scott, Selva, Sequoia, Tardive de Leopold, Tioga, Tufts, Tustin, Valetta, Vista, and Dr. P. Wallbaum and the new French selections 9.278, 26, 115, 5568, and 9715.

The DAPI staining was carried out on sections of midribs, petioles, flower peduncles, roots, and runners. Asymptomatic plants of the corresponding cultivars were used as controls.

Experimental transmission of the BLO. DAPI-positive leaf petioles from strawberry cv. Dover or Selva plants affected with marginal chlorosis were grafted onto 10 plants each of *F. vesca* L. 'UC4' and 'UC5', and the grafted plants were kept under screenhouse conditions.

For dodder transmission of the BLO to periwinkle (*Catharanthus roseus* (L.) G. Don) plants, both *Cuscuta campestris* Yuncker and *C. odorata* L. were used as described by Garnier and Bové (4). The BLO-infected strawberry cultivars used to infect dodder were Selva, Elsanta, Hummi Gento, and Minja.

Cultivation of the BLO. Three media (BSR, SP4, and M1A) developed for cultivation of plant-pathogenic spiroplasmas restricted to the sieve tube (15) and two media (PW [2] and PD2 [3]) formulated for cultivation of the xylem-restricted bacterium *Xylella fastidiosa* were used for cultivation attempts. The media were inoculated with extracts from strawberry plants without symptoms or with marginal chlorosis that were prepared by chopping the petioles with a razor blade in the presence of the medium being used. Inoculated culture plates were incubated at 25 C for up to 3 mo. Bacterial growth was monitored by observation of BLO cells in one drop of inoculated medium under a dark-field microscope at a magnification of ×1,250 for liquid medium or by observation of colony development under a binocular microscope (×40) for solid medium.

Characterization of the BLO. Ten monoclonal antibodies (MAbs) produced against various strains of the BLO associated with citrus greening disease (6,7) were used to detect the BLO in infected strawberry plants by immunofluorescence on leaf midribs and petiole sections (7).

A DNA probe (In2.6) recognizing all Asian strains of the greening BLO was used for DNA-DNA dot hybridization with DNA extracted from strawberry plants without symptoms or with marginal chlorosis, according to the method of Villechanoux et al (14).

Fig. 1. Symptoms of marginal chlorosis on nursery strawberry plants: **(A)** Red coloration of leaves and **(B)** root necrosis (right); roots of unaffected plant (left).

RESULTS

Symptoms. Symptoms appeared in July in nursery plants and consisted of red discoloration of leaflets (Fig. 1A), first on the lower surface and then on the upper surface. This discoloration started from the leaf margin and progressed toward the midribs. The leaves became cup-shaped. New leaves were

greatly reduced in size (one-tenth of normal size) and showed a typical marginal chlorosis, 1–2 mm wide. No symptoms of interveinal chlorosis were observed. The disease started on the mother plant and extended to the runners, leading to infection of progeny plants. Necrosis of the root system began at the tip and progressed toward the internal crown (Fig. 1B). The size and shape of the internal crown remained normal, however.

In fruit production fields, the characteristic symptom was marginal chlorosis that began on young leaves and eventually extended to all leaves; as observed in nurseries, the infected leaves were smaller than normal and were cup-shaped (Fig. 2A). The disease appeared on individual plants in a random order in the spring and spread to form foci of infected plants in the summer. In the case of strawberry plants grown under plastic tunnels (8 m wide), the disease started generally on plants in the center rows and progressed toward the border rows. At the end of the season, all plants in the tunnel could be infected. The number of fruit was not affected, but fruit were small and deformed (Fig. 2B). At maturity, fruit were purple-red, very soft, and sour, rendering the crop unmarketable.

Etiology. Electron micoscopic observations of 16 plants of six cultivars showing marginal chlorosis and/or discoloration symptoms, from either nurseries or production fields, revealed the presence of a BLO in the phloem sieve tubes (Fig. 3). BLOs were not found in phloem tissues of asymptomatic strawberry plants of the corresponding cultivars. The BLO was 0.2–0.27 μm in diameter and up to 4 μm in length. The cell envelope (cytoplasmic membrane plus cell wall) was 250 nm thick, typical of BLOs (5). The presence of clear cell walls (Fig. 3B and D) distinguished the marginal chlorosis organism from a mycoplasmalike organism (MLO). The number of BLOs found in the phloem was low; the sieve tubes showed a major thickening of walls that led to disappearence of the lumen (Fig. 3B, C, and D).

DAPI staining was evaluated for ability to detect the BLO in the samples found positive by electron microscopy. Fluorescent DNA was observed in the phloem of infected strawberry plants (Fig. 4A), whereas no such fluorescence was present in symptomless plants (Fig. 4B). All 16 plants found positive by electron microscopy were also positive by DAPI staining.

The use of DAPI staining on 138 plants from the 50 cultivars tested allowed us to identify 124 DAPI-positive plants (90%), whereas none of the 21 plants with uncharacteristic symptoms gave positive responses to DAPI assay. No differences were observed when various tissues such as midribs, petioles, flower peduncles, roots, and runners were used as the material for DAPI staining.

Transmission of the BLO. When *F. vesca* plants were graft-inoculated with leaf petioles affected by marginal chlorosis, the grafted petioles survived for a maximum period of 15 days and then dried out. Similarly, dodder grown on affected strawberry plants decayed rapidly. None of the 40 graft-inoculated *F. vesca* plants and none of the approximately 50 periwinkle plants used in dodder transmission experiments had developed symptoms by 4 mo after inoculation. No BLO was found in these plants by DAPI staining and/or electron microscopy.

Cultivation and characterization of the BLO. No bacterial growth occurred in any of the media inoculated with extract from healthy or infected strawberry plants, except in a few cases where fast-growing contaminants were observed. The DNA probe and none of the greening BLO-specific MAbs reacted with strawberry material infected with marginal chlorosis.

DISCUSSION

Marginal chlorosis of strawberry is a new disease that recently appeared in

Fig. 2. Symptoms of marginal chlorosis on plants from strawberry production fields: **(A)** Small, cup-shaped leaves with yellow margins and **(B)** small, deformed fruit; arrow points to a normal fruit.

Spain and France. It affects more than 50 strawberry cultivars, both in nurseries and in fruit production fields, and is more prevalent in southern than in northern France. Some cultivars showing the disease in France and Spain are also grown in the United States and Canada, and symptoms of marginal chlorosis should be sought in these countries.

Symptoms of marginal chlorosis resemble those of the mild yellow edge disease (1,12) and several other viral diseases that are transmitted by aphids or by unknown means (10). Symptoms of marginal chlorosis also resemble those of mycoplasma yellows, a disease associated with an MLO present in Australia (8). Australian mycoplasma yellows inhibits fruit and flower production, whereas the marginal chlorosis disease reduces the size but not the number of fruit.

Our electron micrography showed that a BLO is associated with the marginal chlorosis disease. Another BLO disease of strawberries, named rickettsia yellows, was described in Australia (8). Because the symptoms of rickettsia yellows, which include leaf interveinal chlorosis and failure of plants to flower, differ from those of marginal chlorosis disease, we can assume that two different BLOs are involved in rickettsia yellows and marginal chlorosis. Our serological and hybridization results indicate that the BLO associated with marginal chlorosis differs from that associated with citrus greening, the only BLO for which specific reagents are available (7,14). The greening BLO MAbs have been reported to be very strain-specific (6), so it is not surprising to find that they do not react with the strawberry BLO. The greening BLO DNA probe is less specific than the MAbs and hybridized with DNA of all Asian strains. It did not react, however, with African strains of the greening BLO (14). Absence of reaction with the strawberry BLO is therefore quite likely.

The DAPI staining technique proved to be suitable for detection of BLOs in strawberry plants. Indeed, a good correlation was found based on presence of the BLO as seen by electron microscopy and by DAPI staining in the phloem. When DAPI was used to investigate the presence of the BLO in large numbers of symptomatic plants, only a few symptomatic plants (11%) had negative reactions. These negative results were probably due to uneven distribution or too low concentrations of the BLO in these plants. Because the number of organism present in the spring is low, we recommend that testing for the BLO by DAPI staining be performed from June to October. DAPI staining alone, however, is not reliable because it also stains MLO DNA in MLO-infected plants. This is particularly true for strawberries, in which MLO is a common pathogen (10).

Attempts to cultivate strawberry BLO in media formulated for growth of fastidious phloem or xylem-limited prokaryotes failed. This is not surprising, as these media were previously shown to be unsuitable for supporting the growth of the citrus greening BLO. To date, transmission of the BLO to other indicator plants has been unsuccessful. This continues to make the study of the BLO associated with marginal chlorosis quite difficult, as the amount of organisms in strawberry plants is low. Fulfillment of Koch's postulates for marginal chlorosis disease of strawberry awaits the in vitro cultivation of the strawberry BLO.

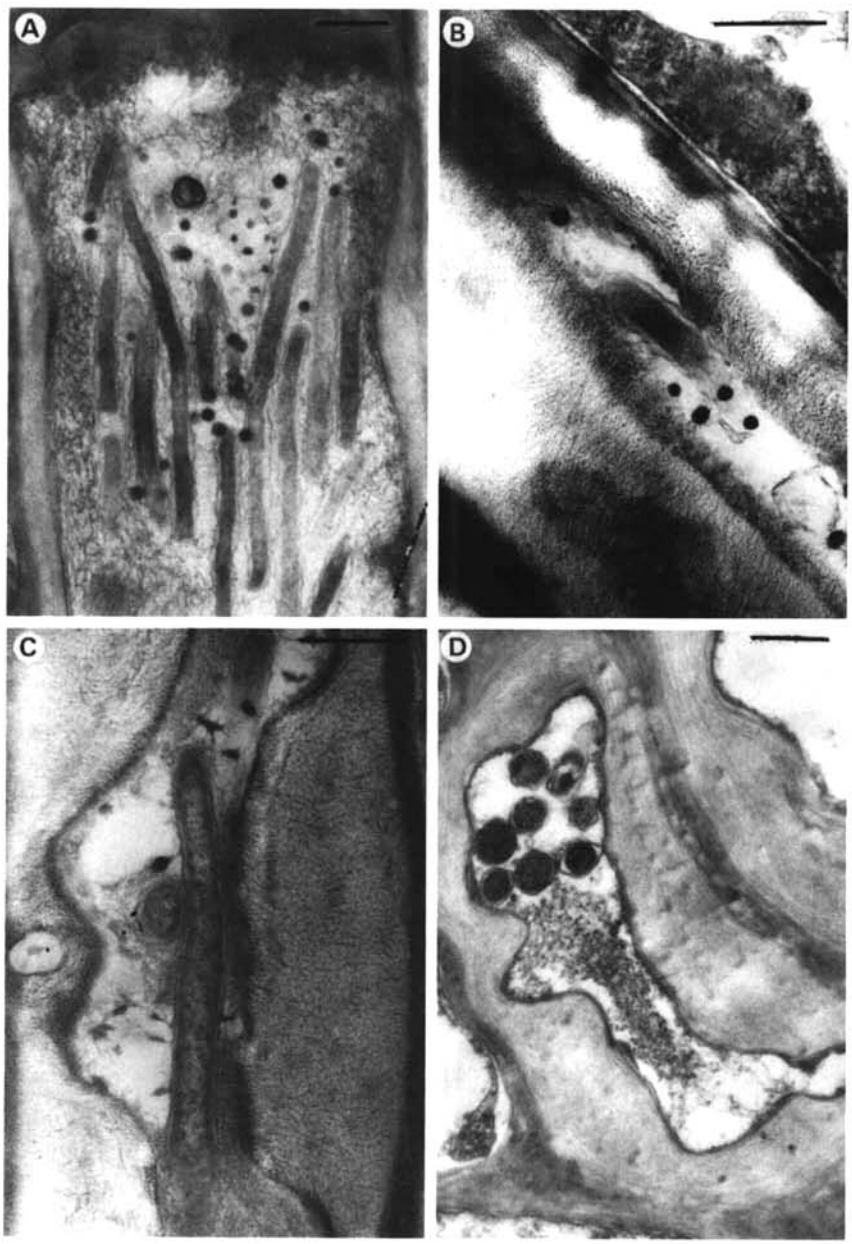

Fig. 3. Electron micrographs of phloem tissue in sections from a strawberry leaf with marginal chlorosis: **(A)** Long, filamentous bacteriumlike organisms (BLOs) in a longitudinal section and **(B, C, and D)** BLOs in sieve tubes with thickened walls in longitudinal (B and C) and transverse (D) sections; cell walls surrounding the BLOs are clearly seen in B and D. Scale bars = 0.5 μm.

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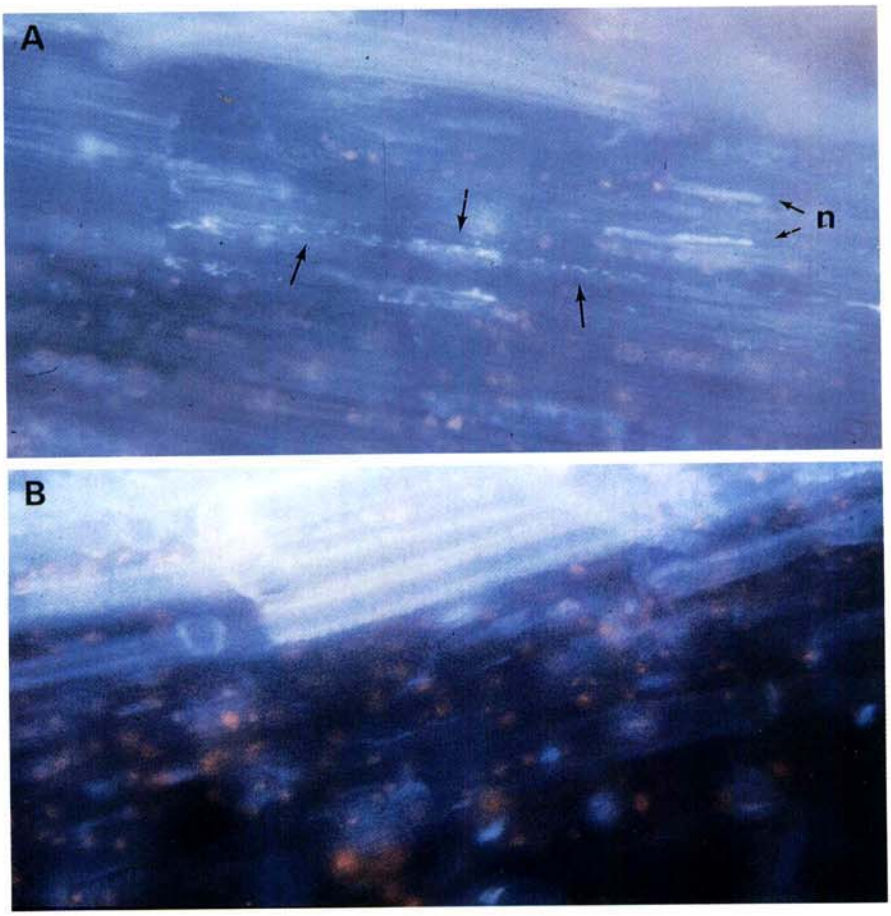

Fig. 4. DAPI staining of strawberry leaf petiole sections of **(A)** plant with marginal chlorosis and **(B)** healthy plant. In A, long arrows indicate fluorescence (dotted lines) corresponding to BLO DNA and short arrows indicate fluorescent plant nuclei (n). (×3,000)

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