Natural Transmission of *Spiroplasma citri* to Periwinkles in Morocco

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In California, extensive natural spread of citrus stubborn disease was reported by Calavan *et al.* (1974, 1976), and three leafhopper vectors of *Spiroplasma citri*, the causal agent of the disease, have been identified (Kaloostian *et al.*, 1975, 1979; Oldfield *et al.*, 1976; 1977*a*, *b*). Symptoms of stubborn have been seen in Morocco on seedling and nucellar trees and it was assumed that natural transmission of the stubborn pathogen occurred. This assumption was supported by discovery of an ornamental periwinkle from a flower bed in the city of Rabat infected with *S. citri* (Bové *et al.*, 1978). Natural infection of periwinkles in Arizona and California with spiroplasmas presumed to be *S. citri* has also been reported (Allen, 1975; Granett *et al.*, 1976).

Periwinkle is an excellent indicator plant for *S. citri* (Markham and Townsend, 1974; Calavan and Oldfield, 1979). Here we report a survey for disease of ornamental periwinkles in Morocco, and the use of experimental periwinkles to demonstrate natural transmission of *S. citri* in the Tadla region of Morocco.

MATERIALS AND METHODS

Surveys of periwinkles grown outdoors as ornamentals in various regions of Morocco were conducted in November and December, 1977 (Bové *et al.*, 1977*b*).

To obtain evidence for natural spread of *S. citri*, periwinkles were sown on May 6, 1978, and 1 month later, 200 uniform seedlings were selected. Two beds of 100 plants each were planted 50 m apart in a large, bare field, and 25 m from the eastern border of a stubborn-affected Valencia late sweet orange orchard (UP 3006), near the town of Beni Mellal, in the Tadla region of Morocco. Each periwinkle bed contained an insect trap made of a vertical piece of yellow cardboard (50 x 50 cm) covered with glue. On one bed, the trap was oriented north-south, facing the orchard, and on the other bed the trap was oriented east-west at right angle with the orchard. The field on which the two periwinkle beds were planted was previously planted to sugar beets, and had just been plowed. It was kept free of vegetation. No insecticide treatments were applied to the Valencia late sweet orange orchard after the spring of 1977 and the periwinkles also were left untreated.

Enzyme-linked immunosorbent assays (ELISA) were carried out as described by Saillard *et al.* (1978, 1980). Protein analysis of polyacrylamide gel electrophoresis was done according to Mouches *et al.* (1979, 1980). Electron microscopy of ultrathin sections through phloem tissue of periwinkle leaves was described previously (Garnier and Bové, 1977). Culture of *S. citri* was also attempted (Bové *et al.*, 1978).

RESULTS

Survey for diseases of ornamental periwinkles in Morocco. Ornamental periwinkles are grown in many regions of Morocco in parks, and around homes and hotels. They overwinter and are kept for 2-3 years before being replaced. The survey revealed that while some were apparently symptomless, many showed typical phyllody-virescense symptoms, a few had flower dwarfing-stolbur aspects, one resembled *S. citri*-infected periwinkles, and still others had dark green islands on the leaves, suggesting virus infection. Shoots were collected on plants showing these symptoms in various parts of Morocco (table 1). They were carried to Bordeaux and used for the following experiments:

1) graft inoculation of healthy periwinkles;

2) mechanical inoculation of periwinkles, *Chenopodium quinoa, C. amaranticolor*, cowpea and tobacco;

3) electron microscopy for the detection of mycoplasma-like organisms (MLO) and spiroplasmas;

4) culturing for the presence of *S. citri.*

Table 1 indicates that the various symptoms observed on the periwinkles in Morocco could be graft-transmitted to healthy periwinkles. Periwinkles showing phyllody-virescence (fig. 1 B and C) or stolbur symptoms (fig. 1 E) contained numerous MLO's in their sieve tubes. One periwinkle (Rabat, M4) was infected with *S. citri* (fig. 1 E) (Bové *et al.*, 1978; Bové and Saillard, 1979). Mechanical transmission was obtained only in the case of periwinkle leaves showing dark green islands. In one case (Marrakech periwinkle, RP1) the virus involved was identified as cucumber mosaic virus (CMV) by the following criteria.

The virus, purified from mechanically inoculated tobacco leaves by the technique of Lot *et al.* (1972), was isometric with a particle diameter of approximately 27 nm. It reacted serologically in double immunodiffusion tests with antiserum against CMV, serogroup DTL (Devergne and Cardin, 1973). Strains of this serogroup are known to multiply at relatively high temperatures, such as those prevalent in Morocco. Four viral RNA's were identified by polyacrylamide gel electrophoresis and their electrophoretic mobilities were very close to those of CMV-RNA's (strain TL). No RNA 5 was detected. Mechanically inoculated Xanthi tobacco plants showed severe mosaic symptoms, but no necrotic local lesions formed on the inoculated leaves. Such reactions are characteristic of symptom group C (Marrou *et al.*, 1974).

From these studies (table 1, fig. 1), it appears that the most widespread diseases of periwinkles in Morocco are those associated with MLO's. Natural transmission of these diseases by some of the many leafhoppers species present in Morocco (Bové *et al.*, 1979*a*; Moutous *et al.*, 1980) must take place. In the course of the survey, only one periwinkle infected with *S. citri* was encountered. The very low proportion of *S. citri*-infected plants to MLO-infected ones was probably because the periwinkles observed were all grown for ornamental purposes in urban areas away from citrus orchards.

Natural transmission of *S. citri* and MLO's to experimental periwinkles grown close to a stubborn-affected Valencia sweet orange orchard.

The two periwinkle beds with 100 plants each established in the Tadla region were exposed from June to October 1978 to natural infection. They were examined on October 8 and 9 for symptoms and shoots of some of the plants were taken to Bordeaux for electron microscopy, graft transmission, ELISA, culture of *S. citri* and protein analysis by polyacrylamide gel electrophoresis.

As shown in table 2, 165 plants were still alive at the end of the experiment. Of these, 33 per cent were symptomless, 27 per cent had typical flower dwarfing-stolbur symptoms, 19 per cent were affected by strong phyllody-virescence symptoms, 16 per cent showed various degrees of wilting (fig. 2) and 5 per cent had undetermined symptoms. Even though phyllody or stolbur symptoms were severe, these diseases were not destructive. In contrast, the disease affecting the wilted plants was clearly lethal. Dead periwinkles were not included in the final tabulation. Quite a few plants probably died of the lethal wilting and, hence, the total number of plants affected by wilting was probably higher than 16 per cent. The wilting symptoms were very similar to those observed on *S. citri*-infected periwinkles grown in the glasshouse at about 30°C.

To confirm *S. citri* infection of the wilted periwinkles, some of the plants

TABLE 1

DISEASES AND AGENTS OF NATURALLY INFECTED ORNAMENTAL PERIWINKLES IN VARIOUS REGIONS OF MOROCCO

Origin	Symptoms obtained by graft inoculation of healthy periwinkles	MLO or spiroplasma present in sieve tubes	Mechanically transmissible	Type of virus present
Rabat*				
Periwinkle M1	Phyllody-virescence	MLO	No	None
Periwinkle M2	Phyllody-virescence	MLO	No	None
Periwinkle M3	None	None	No	None
Periwinkle M4	Leaf dwarfing, stunting	Spiroplasma†	No	None
Kenitra				
Periwinkle 1	Dark green mottle	None	Yes	Not determined
Periwinkle 2	Phyllody-virescence	MLO	No	None
Beni Mellal‡				
Periwinkle PLM 1	Phyllody-virescence	MLO	No	None
Periwinkle PLM 2	None	None	No	None
Periwinkle PLM 3	None	None	No	None
Periwinkle PLM 4	Flower dwarfing-stolbur	MLO	No	None
Marrakech				
Periwinkle RP 1	Dark green mottle	None	Yes	CMV
Periwinkle RP 2	None	None	No	None
Periwinkle HES	None	None	No	None
Periwinkle PM	Phyllody-virescence	MLO	No	None

* All 4 plants were from the same periwinkle bed.

† A spiroplasma was cultured from periwinkle M4 and shown to be *S. citri* (Bové *et al.*, 1978; Bové and Saillard, 1979).

‡ All 4 plants were from the same periwinkle bed.

TABLE 2
SYMPTOM EXPRESSION OF PERIWINKLES EXPOSED TO NATURAL INFECTION IN THE TADLA REGION OF MOROCCO

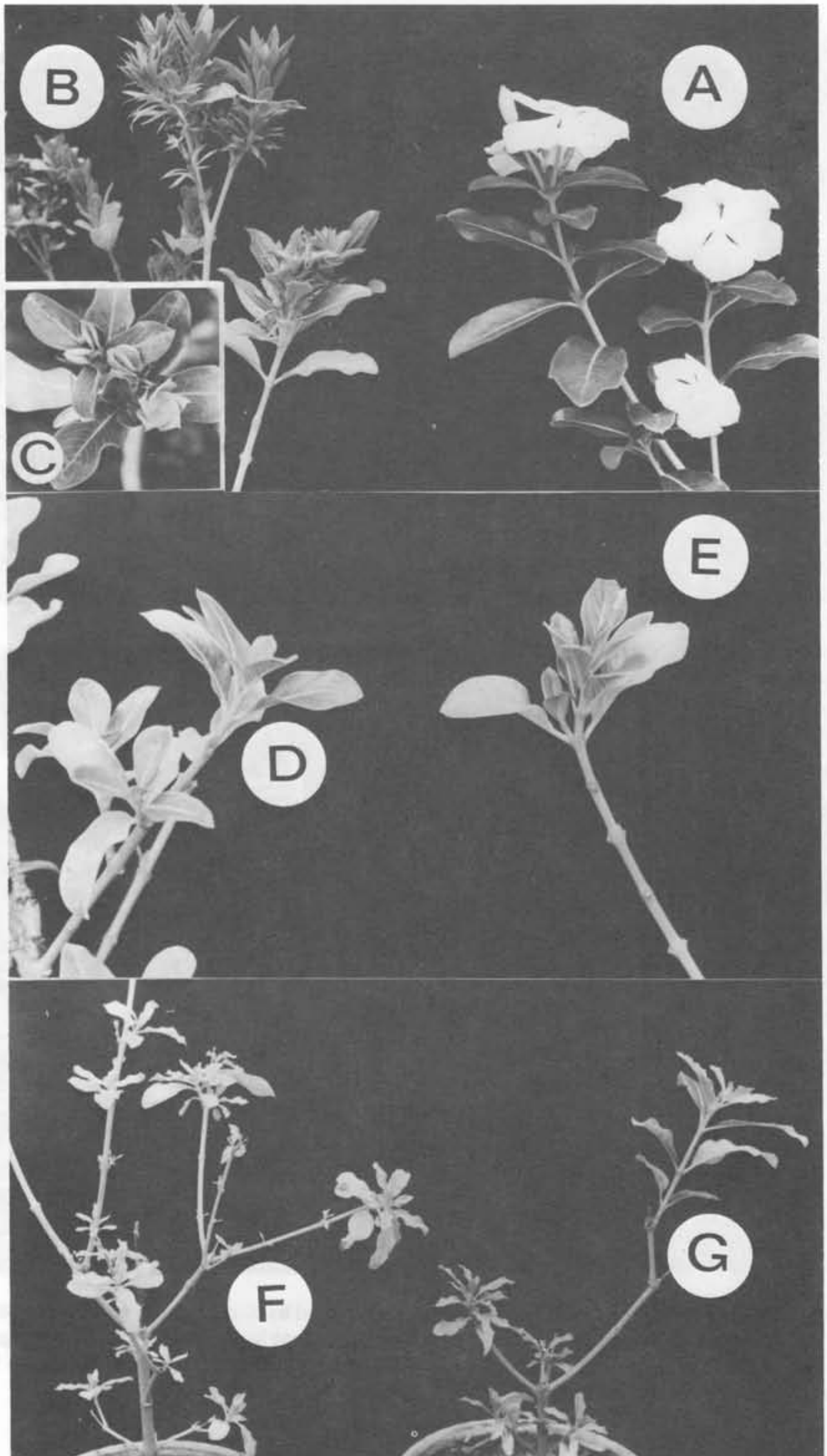
Bed number	Total number of plants	Number of plants: No symptoms	Stolbur-like	Phyllody	Wilting	Undetermined symptoms
1	73	21	28	10	10	4
2	92	33	16	22	16	5
1 + 2	165	54	44	32	26	9

TABLE 3
CULTURING AND ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY) TESTS FOR *SPIROPLASMA CITRI* IN PERIWINKLES EXPOSED TO NATURAL INFECTION IN THE TADLA REGION OF MOROCCO

Periwinkles	Severity of wilting*	Stolbur*	ELISA† (eye readings)	Culture of *S. citri*‡
1	5/5		+++	
2	5/5		+++	+
3	5/5		++	
4	5/5		++	+
5	3/5		++	
6	3/5		++	
7	5/5		++	
8	2/5		++	
9	2/5		+	+
10	2/5		+	
11	2/5		++	+
12		5/5	-	+
13		5/5	-	-
14		5/5	-	-
15		5/5	-	-

* 0/5 = no symptoms, 5/5 = very severe symptoms.
† - = negative, + = positive, +++ = highly positive assay.
‡ + = culture of *S. citri* was obtained, - = no culture was obtained.

Fig. 1. Symptoms induced on healthy periwinkles graft inoculated with shoots from diseased periwinkles collected in various regions of Morocco. A) Healthy periwinkle. B and C) Leaf dwarfing and internode shortening (B) and virescent flower (C) on plants inoculated respectively with phyllody-affected periwinkle M2 from Rabat and periwinkle 2 from Kenitra. D) Stolbur C (Giannotti strain) affected plant. E) Stolbur-like symptoms on periwinkle inoculated with shoots from Beni Mellal plant PML 4. F and G) Symptoms of *S. citri* (Israeli strain) infection on periwinkle grown at 20-25° C (G) and identical symptoms on plant (F) inoculated with shoots from Rabat periwinkle M4.


B
A
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1

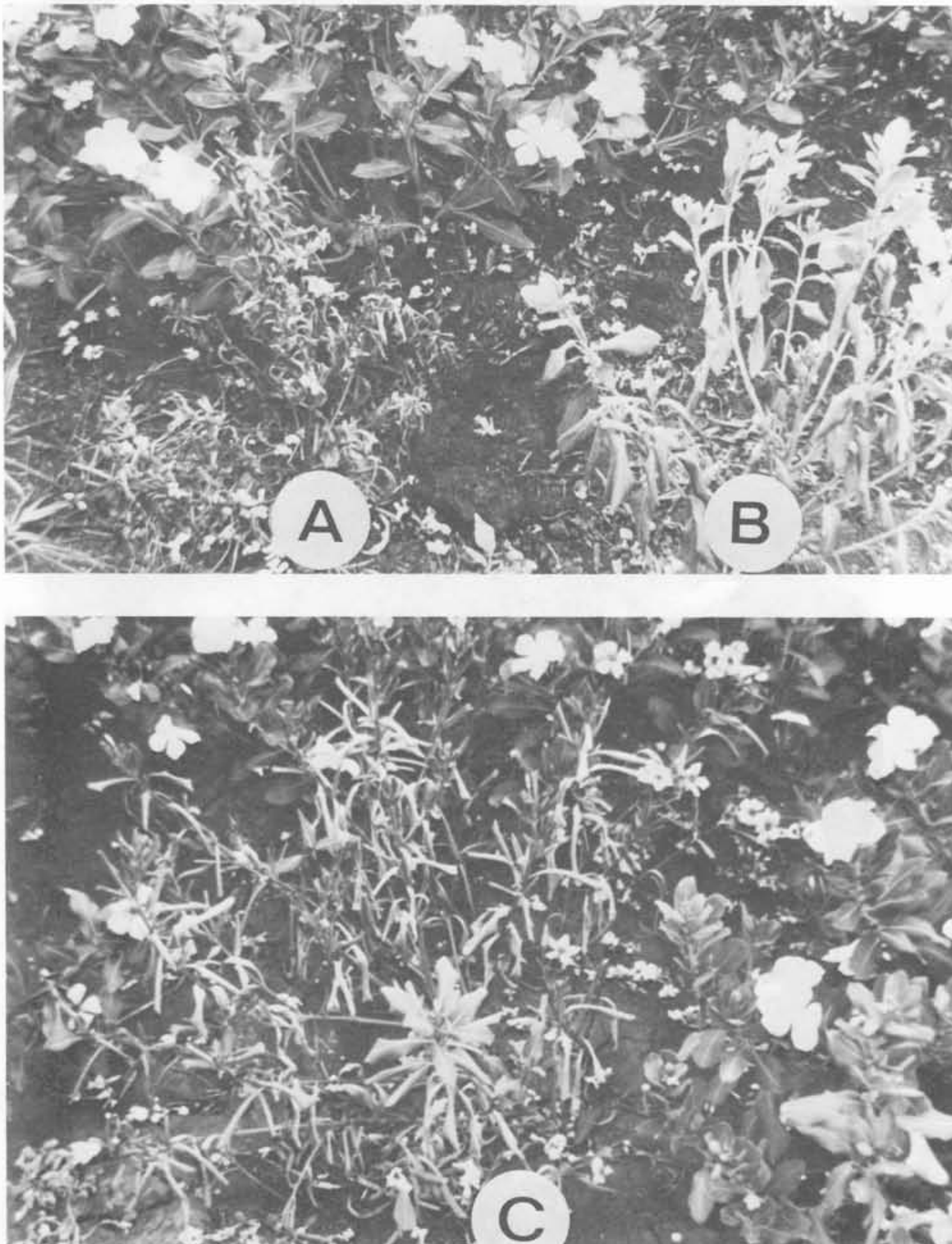

Fig. 2. Various stages of wilting of periwinkles infected by *S. citri* under natural conditions in the Tadla region of Morocco. A) Late stage of wilting; many leaves are dry. B) Early stage; some leaves are still turgid, while many are rolled. C) More advanced stage than B, except for one shoot. Notice stolbur-like periwinkles with dwarfed flowers.

were tested by ELISA and culturing of *S. citri* from these plants was also tried. Table 3 shows that all wilted periwinkles gave a positive ELISA reaction, and that a spiroplasma characterized as *S. citri* by ELISA and polyacrylamide gel electrophoresis, could be cultured from such periwinkles. Stolbur-affected plants were negative by ELISA; however, a *S. citri* culture was obtained from one of the periwinkles showing stolbur (plant 12). This plant was probably doubly infected like a periwinkle reported by Oldfield *et al.* (1977*b*).

Shoots of 8 wilted periwinkles and 2 stolbur-affected plants were carried to Bordeaux. Electron microscopy showed the presence of helical mycoplasmas in the phloem of the wilted material and MLO's in the plants with stolbur symptoms. Graft inoculation of shoots from the wilted plants to healthy periwinkles grown at 25°C induced symptoms typical of *S. citri* infection (fig. 1 E), and *S. citri* could be cultured from these inoculated plants. Periwinkles inoculated with stolbur tissue developed symptoms similar to those shown in fig. 1D, and *S. citri* was not recovered by culturing.

Three species of planthoppers or leafhoppers were collected in the two periwinkle beds on October 8, 1978: *Laodelphax striatellus* (Fallen) (28 individuals), *Psammotettix striatus* (Linnaeus) (43 individuals) and *Neoaliturus (Circulifer) haematoceps* (Mulsant and Rey) (4 individuals). All 3 species tested positive for *S. citri* by ELISA, and a spiroplasma, characterized as *S. citri*, was cultured from *N. haematoceps*. The *S. citri* strains isolated from the leafhopper and the infected periwinkles on which the insects were collected produced identical protein patterns on polyacrylamide gels. Hoppers of the above 3 species and of 2 additional species: *Toya propinqua* (Fieber) and *Euscelis alsius* (Ribaut) collected in the stubborn-affected Valencia late sweet orange orchards close to the periwinkle beds also tested positive for *S. citri* by ELISA.

DISCUSSION

Twenty-six of 165 experimental periwinkles grown outdoors from June to October 1978 in the Tadla region of Morocco were infected by *S. citri*. Under the hot summer climate of Tadla, *S. citri* infection causes lethal wilting similar to that observed at 27-32°C in the glasshouse. This lethal collapse might explain the discrepancy between the number of periwinkles that were planted in June (200) and the number of plants that were still alive in October (165). If so, more than 26 plants became infected by *S. citri*. The significant incidence of natural *S. citri* infection is probably due to the following factors:

1) the periwinkles were planted near a sweet orange orchard severely affected by stubborn disease, which provided a reservoir of *S. citri*;

2) no insecticide treatments were applied in the orchard or on the periwinkle beds, thus allowing high insect vector populations;

3) the planthopper and leafhopper populations in the periwinkle beds were probably increased by the yellow trap in each bed;

4) the land was kept clear of vegetation on the eastern side of the orchard, except for the 2 periwinkle beds and there were no other plants on which insects could feed between the orchard and the periwinkles;

5) in California, Calavan *et al.* (1976) have shown that most natural spread of *S. citri* occurred during the months of June to October, the period of the experiment reported here.

It is highly probable that the vectors responsible for *S. citri* transmission in Morocco are plant or leafhoppers for the following reasons:

1) three leafhoppers are known to transmit *S. citri* in California;

2) one planthopper, *L. striatellus*, and 2 leafhoppers, *P. striatus* and *N. (C.) haematoceps*, collected on the periwinkle beds or in the sweet orange orchard and closely related to *Circulifer*

tenellus, a vector of stubborn in California, tested positive for *S. citri* by ELISA; also, the stubborn pathogen could be isolated and cultured from the leafhopper, *N. haematoceps*, collected on the periwinkles. *C. tenellus* exists in Morocco but was rare in our surveys (Moutous *et al.*, 1980). Four additional leafhopper species collected in Morocco have tested positive for *S. citri* by ELISA: *Exitianus capicola* (Stål), *E. alsius* and *Recilia angustisectus (Linnavuari)* (Bové *et al.*, 1979*a*; Moutous *et al.*, 1980).

3) The strain of *S. citri* isolated from the leafhopper *N. haematoceps* collected in the periwinkle beds and the strains of*S. citri* cultured from 4 periwinkles in these beds produced identical protein patterns on polyacrylamide gels.

4) *S. citri* infection of the periwinkles is not seed transmitted. We have grown many periwinkles from seed collected on *S. citri*-infected plants and no symptoms of *S. citri* infection were observed on these seedlings.

There is also a high incidence of phyllody-virescence and stolbur infection of periwinkles in Morocco. The 1977 survey has revealed the presence of these 2 diseases on periwinkles (table 1) and the 1978 experiment confirmed their natural transmission (table 2). In addition to the leafhopper species mentioned above, we have found 2 additional species with potential vector capacity: *Cicadulina bipunctella bipunctella (Matsumara)* and *Euscelis lineolatus* (Brulle). Hence it is not surprising that natural transmission of MLO's occurs in Morocco. It is interesting to note that at Beni Mellal the survey revealed the presence of both phyllody-virescence and stolbur (table 1), and the experimental periwinkles exposed to natural infection there also became infected by these two diseases.

The infection of periwinkles by CMV is probably due to natural transmission by some of its aphid vectors present in Morocco.

In conclusion, these studies have revealed that a significant natural spread of *S. citri* and of MLO's occurs during the summer months in the Tadla region of Morocco, and that probably leafhopper vectors are involved. In that area, hundreds of acres of citrus have been pulled out because of unproductiveness due to stubborn. The high incidence of stubborn in Tadla is probably due to the use of *S. citri*-infected budwood and also to natural spread of the disease by insect vectors. The natural spread of *S. citri* in Morocco is a hazard to the production of disease-free nursery trees.

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