Witches'-Broom Disease of Lime Affects Seed Germination and Seedling Growth But Is Not Seed Transmissible

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Abstract

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Witches'-broom disease of lime (WBDL), caused by '*Candidatus* Phytoplasma aurantifolia', has devastated many Mexican lime orchards and is currently a threat to lime production in neighboring provinces of southern Iran. Several reports have suggested transmission and spread of WBDL phytoplasma through the seed of infected plants. In the present study, claims of seed transmission of this phytoplasma were examined. Fruit were collected from infected trees in the infested areas of Minab (Hormozgan Province) and from symptomless trees in noninfested areas. Lime seed from symptomless and witches'-broom-affected trees were sown in separate beds in an insect-proof screenhouse and the resulting seedlings were examined for phytoplasma infection. Leaf, stem, and root samples were collected from both groups of seedlings every 3 months for 2 years and tested for WBDL phytoplasma using direct and nested polymerase chain reaction (PCR).

Repeated PCR tests on the seedlings did not reveal the presence of phytoplasma DNA. Likewise, symptoms of the disease were not observed on these seedlings after 2 years. PCR assays detected the phytoplasma in coats of some seed from infected trees; however, no excised embryos were positive for the phytoplasma. All positive PCR results were confirmed by restriction fragment length polymorphism assay. One-year-old seedlings derived from seed of noninfected plants appeared more vigorous in terms of height, number of leaves, and fresh weight of shoot compared with those from infected trees. The germination percentage, mean daily germination, peak value, and germination value were significantly higher for seed of fruit from noninfected trees and seed from fruit on asymptomatic branches of infected trees than those from fruit on symptomatic branches of infected trees.

Witches'-broom disease of lime (WBDL), caused by '*Candidatus* Phytoplasma aurantifolia' was found first in a small area in southern Iran in 1997 (17). Since then, WBDL has spread to many areas, devastating Mexican lime plantations in major lime-growing provinces of Hormozgan, Sistan-Baluchistan, and Kerman (16). In the field, the phytoplasma is naturally spread by the vector leafhopper *Hishimonus phycitis* (18). The pathogen has been experimentally transmitted to several species of citrus by graft inoculation, and to periwinkle and a number of solanaceous plants such as tomato, eggplant, and tobacco by dodder or graft inoculation (19–21). Several studies have focused on the detection of WBDL phytoplasma in various parts of the lime seed. El-Kharbotly et al. (5) were the first to suggest the possibility of seed transmission of WBDL phytoplasma. Later, Khan (9) used the polymerase chain reaction (PCR) assay to study seed transmission of WBDL phytoplasma in 6-month-old seedlings. Based on this report, the majority of seed from infected plants in Oman yielded infected seedlings. The author also reported the presence of phytoplasma-like structures in seed tissues by electron microscopy. Such a high rate of seed transmission is unusual for a phloem-limited organism. Our observation in affected citrus groves of southern Iran does not support seed transmissibility of this phytoplasma. On the other hand, there is no information on the effect of phytoplasma infection of lime trees on the viability of seed and growth of seedlings from fruit from infected trees. Lime trees in southern Iran are mainly propagated through seed. In addition, lime seedlings are used as rootstock for many citrus species and cultivars. Therefore, the issue

of seed transmissibility of '*Ca. Phytoplasma aurantifolia*' must be clarified because it relates directly to the implementation of control strategies. In the present study, we evaluated the effect of WBDL infection on certain horticultural parameters of lime seed, presence of the phytoplasma in seed tissues, and seed transmission of the WBDL phytoplasma.

Materials and Methods

Seed collection, sowing, germination, and sampling. Fruit were harvested at commercial maturity from severely infected trees (Fig. 1A and B) in the Minab region and from noninfected trees in noninfested areas of Hormozgan Province, Iran, and seed were manually extracted. Approximately 6,000 seeds from infected and healthy trees, respectively, were sown in field soil in separate 2.5-by-10-m beds in a leafhopper-proof screenhouse at the Minab Agricultural Research Station in September 2007. Seedlings were observed weekly for abnormalities and sampled for PCR assays every 3 months over a period of 2 years. Leaves, stems, and roots from 75 randomly selected seedlings were subjected to DNA extraction followed by direct and nested PCR to detect phytoplasma. In another experiment to facilitate germination of all embryos, 30 seeds from infected plants and 10 seeds from noninfected plants were surface sterilized with 1% sodium hypochlorite, rinsed three times with sterile distilled water, and cultured (2 seeds per tube) on half-strength Murashige-Skoog basal salts (12) for 2 to 4 weeks. All seedlings were then tested using direct and nested PCR.

DNA extraction. Total DNA was extracted from lime tissues using a modified cetyltrimethylammonium bromide (CTAB) procedure (4) as follows. Approximately 0.3 to 0.5 g of either leaf midrib, root, or crown tissue was powdered in liquid nitrogen, transferred to 1.5-ml centrifuge tubes, and mixed with 700 μ l of hot (60 to 65°C) CTAB buffer (20 mM EDTA, 100 mM Tris-HCl [pH 8.0], 2% CTAB, 1.4 M NaCl, 1% polyvinyl pyrrolidone, and 0.2% 2-mercaptoethanol in sterile distilled water). The homogenate was incubated at 60 to 65°C for 1 h with gentle inversion every 15

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min, then extracted with 700 μ l of chloroform/iso-amyl alcohol (24:1). The mixture was centrifuged (Eppendorf centrifuge 5415 R) at 12,000 rpm ($13,400 \times g$) for 10 min and the supernatant was transferred to a fresh microcentrifuge tube. DNA was precipitated by adding 0.7 volume of cold isopropanol and the mixture was incubated for 30 min at -20°C and centrifuged for 10 min at 13,000 rpm ($15,700 \times g$). The supernatant was removed and the pellet was washed three times with 200 μ l of 70% ethanol and centrifuged for 5 min at 5,000 rpm ($2,300 \times g$). The pellet was dried, dissolved in 100 μ l of Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0) or sterile double-distilled water and stored at

-20°C . DNA was separately extracted from whole seed (50 samples of 3 seeds/sample; 150 seeds total), seed coats from infected plants (10 samples of 3 seeds/sample; 30 seeds total), and 600 excised embryos (three lots of 200 embryos each) from the seed of infected trees. DNA was extracted from the powdered tissues following the above-mentioned procedure (4) after grinding seed or seed parts in liquid nitrogen.

PCR. Both direct and nested PCR were performed to detect the phytoplasma in various tissues. Each reaction mixture of 20 μ l contained 13 μ l of H_2O , 2 μ l of 10 $\times$ PCR buffer, 1.2 μ l of MgCl_2 (25 mM), 0.4 μ l of each dNTP (10 μM), 1.25 U of *Taq* DNA polymerase (Cinagen, Iran), 1 μ l of each oligonucleotide primer (100 μM), and 20 to 30 ng of genomic DNA template. Universal primer pair P1/P7 (22) was used to amplify a fragment of 1,800 bp of ribosomal operon that extended from the 5' end of 16S rRNA to the 5' region of the 23S rRNA gene. Amplification was carried out with an initial denaturing temperature of 94°C for 2 min; followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min; and a final extension cycle at 72°C for 10 min. To increase the sensitivity of assays, nested PCR was performed using primer pairs R16F2n/R16R2 (6) with 1:20 dilution of P1/P7 primed PCR products as template. The thermocycling program consisted of an initial denaturing step for 3 min at 95°C ; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and a final extension cycle at 72°C for 10 min. Each PCR product (10 μ l) was analyzed by electrophoresis in a 1% agarose gel in 1 $\times$ Tris-borate-EDTA buffer. PCR products were visualized on 1% agarose gels stained with ethidium bromide. In each experiment, the symptomatic leaves of infected lime were included as positive controls and leaves of noninfected plants and sterile distilled water were used as negative controls.

Restriction fragment length polymorphism. Samples yielding visible DNA bands after PCR were examined by restriction fragment length polymorphism (RFLP). The PCR product was digested by restriction enzymes *AluI*, *HhaI*, *HpaII*, *RsaI*, and *TaqI* (Roche, Germany) following the manufacturer's instructions. Usually, 8 μ l of PCR product was digested for 4 h at 37°C (65°C for *TaqI*) in a 20- μ l reaction volume using 1 unit of each enzyme. Digested products were separated on 2% agarose gels and stained with ethidium bromide.

Seed germination parameters and vegetative growth of seedlings. Four replicates of 100 seeds from each healthy and infected tree were placed on wet filter paper in petri dishes in an incubator at 25°C with 16 h of light and 8 h of darkness. Seed originating from symptomatic and symptomless branches of infected trees were tested separately. The data on seed germination was recorded daily as germinated or nongerminated seed up to 37 days and, at the completion of the test, germination percentage (GP), mean daily germination (MDG), peak value of germination (PV), and germination value (GV) were calculated (7). GP is the percentage of germinated seed at the end of the germination period, while GV is an index, combining both speed and completeness of germination. GP and GV eliminate the need of subjective value judgment, and can be expressed as $\text{GV} = \text{MDG} \times \text{PV}$, where MDG is the number of germinated seed divided by the number of days at the end of examination and PV is the daily cumulative percent germination divided by days since the start of the test (3). Weights of 1,000 seeds originating from noninfected trees, asymptomatic branches of infected trees, or symptomatic branches of infected trees were compared by weighing four samples of 300 seeds each, and extrapolating the means to 1,000 seeds. To compare the vegetative growth of seedlings derived from seed of severely infected plants to those derived from seed of asymptomatic plants, 100 1-year-old seedlings from each source were randomly removed and several parameters of seedling vigor, including plant height, stem diameter, internode length, number of leaves, length and width of leaves, shoot fresh weight, and root fresh weight were measured after 1 year and means were compared using Tukey's test.

Results

Phytoplasmal assay. Witches'-broom symptoms were not observed on the approximately 6,000 seedlings grown from seed of

Fig. 1. Effect of witches'-broom disease of lime (WBDL) phytoplasma infection on fruit and seed of Mexican lime. **A**, Fruit on a WBDL-infected Mexican lime tree; **B**, fruit aggregates on symptomatic branches of an infected tree; **C**, seed from symptomatic fruit of infected tree (right) compared with those from noninfected trees (left).

infected or healthy trees after 2 years. PCR analysis of leaf, stem, and root tissues of 600 samples did not detect any phytoplasma-positive samples. None of the in vitro-grown plantlets from seed of infected trees showed symptoms or were positive by PCR. Direct and nested PCR detected WBDL phytoplasma in 20 and 34%, respectively, of seed samples from infected trees. Neither direct nor nested PCR detected phytoplasma in the excised embryos, seedlings, and in vitro-grown seedlings from seed of infected trees (Table 1). The rates of phytoplasma detection in whole seed, seed without embryos, and seed coats were similar, indicating confinement of the phytoplasma to the seed coats. Although the WBDL phytoplasma was detected in seed harvested from infected trees, seed transmission of WBDL to seedlings was not observed. In all cases of positive PCR, the RFLP pattern was the same as that for '*Ca. Phytoplasma aurantifolia*' (11; Fig. 2).

Seed size, seed germination parameters, and vigor of resulting seedlings. Seed from symptomatic fruit on witches'-broom branches of infected trees showed some necrosis of the seed coat and were smaller than the seed from nonsymptomatic branches of infected trees and those from noninfected trees (Fig. 1C). In all, 1,000 seeds from symptomatic fruit on witches'-broom branches of infected trees weighed 16.2 ± 0.2 g compared with 29.3 ± 0.3 g for seed of fruit on normal branches and 33.1 ± 0.9 g for seed from noninfected trees. Compared with seed from symptomatic branches of infected trees, seed from asymptomatic branches of infected trees and normal plants showed significantly ($P < 0.01$) higher GPs, MDG, PV, and GV. No significant differences were observed at $P < 0.01$ in the germination parameters between seed from fruit on asymptomatic branches of infected trees and those from normal trees (Table 2). There were no significant differences in vegetative growth parameters such as crown diameter, internode length, leaf length, leaf width, and fresh weight of root in 1-year-old seedlings derived from seed of normal trees and those derived from seed of infected trees (Table 3). Significant differences at $P < 0.01$ in plant height and leaf number were observed between the seedlings derived from symptomatic and nonsymptomatic branches (Table 3). One-year-old seedlings from seed of infected plants had fewer shoots, lower fresh weight, and lower plant height.

Discussion

Seed transmission of phytoplasmas is a matter of controversy. Some researchers dispute seed transmission of these phloem-limited organisms because of the absence of direct connection between sieve elements and embryos (10). Phytoplasmal seed transmission has recently been reported in tomato, oilseed rape, and lime plantlets by Botti and Bertaccini (1). Others have shown the presence of phytoplasmas in seed coat or even in the embryo of affected plants with no concrete evidence of phytoplasma passage into seedlings (2,13–15). However, one cannot absolutely rule out seed transmission of phytoplasmas when these organisms infect seed embryos. Although lime trees are propagated by seed in southern Iran and seedlings of lime and several other citrus species are highly susceptible to WBDL infection by graft inoculation (20),

symptoms of the disease have seldom been observed in lime seedlings or young plants in affected areas. Furthermore, examination of many field-grown seedlings by PCR has shown no indication of their infection. Based on our results, we conclude that seed transmission of WBDL phytoplasma does not occur in lime.

Our results show that WBDL phytoplasma may be detected in seed of infected plants but is confined to the seed coats. These results are in agreement with the findings of Jiang et al. (8), who used direct and nested PCR to detect mulberry dwarf (MD) phytoplasma in seed coats but not in seed embryos of MD-affected mulberry. No symptoms of the disease were observed in the resulting seedlings from seed of infected mulberry and seed transmission of this disease did not occur (8).

Contrary to our findings with WBDL, the European stone fruit yellowing (ESFY) phytoplasma was detected in the seed embryos of stone fruit, and such infected seed showed very low viability

Fig. 2. Restriction fragment length polymorphism analysis of polymerase chain reaction products from a witches'-broom disease of lime (WBDL)-infected lime seed coat (left) and WBDL-positive control (right). M is DNA marker.

Table 2. Germination parameters of seed from infected (symptomatic and asymptomatic branches) and noninfected trees

Germination parameters ^z	Seed source ^y		
	Noninfected trees	Infected trees	
		Asymptomatic branches	Symptomatic branches
GP	92.0 a	92.0 a	80.0 b
MDG	3.1 a	3.0 a	2.2 b
PV	3.2 a	3.1 a	2.6 b
GV	9.9 a	9.3 a	5.7 b

^y Means with the same letter in each row are not significantly different at $P < 0.01$ using Tukey's test.

^z GP = Germination percentage, MDG = Mean daily germination, PV = peak value, and GV = germination value.

Table 1. Direct and nested polymerase chain reaction (PCR) detection of Witches'-broom disease of lime phytoplasma in whole seed, seed coats, embryos, and seedlings originated from infected and noninfected Mexican lime trees

Samples	Origin	No of samples	No of detections	
			Direct PCR	Nested PCR
Seed	Infected trees	50 (3 seeds each)	10 (20%)	17 (34%)
Excised embryos	Seed from infected trees	3 (200 embryos each)	0.0	0.0
Seed without embryos	Infected trees	10 (3 seeds each)	2 (20%)	4 (40%)
Seed endosperms	Infected trees	10 (5 endosperms each)	0.0	0.0
Seed coats	Infected trees	6 (10 seeds each)	1 (16.6%)	2 (33%)
Seedlings	Seed from infected trees	600	0.0	0.0
In vitro-grown seedlings	Seed from infected trees	15	0.0	0.0
Seed	Noninfected trees	10 (1–2 seeds each)	0.0	0.0
Seedlings	Seed from noninfected trees	60	0.0	0.0
Seed coats	Noninfected trees	2 (10 seeds each)	0.0	0.0
In vitro-grown seedlings	Seed from noninfected trees	3	0.0	0.0

Table 3. Vegetative growth parameters of seedlings derived from seed of infected and noninfected trees^z

Seed source	Plant height (cm)	Crown diameter (mm)	Internode length (cm)	No. of leaves	Leaf length (mm)	Leaf width (mm)	FW shoot (g)	FW root (g)
Noninfected trees	60.9 a	3.4 a	15.1 a	39.4 a	46.8 a	26.8 a	8.4 a	0.9 a
Infected trees	46.7 b	3.3 a	15.3 a	30.4 b	46.8 a	26.6 a	6.5 b	0.9 a

^z FW = fresh weight. Means with the same letter in each column are not significantly different at $P < 0.05$ or, for number of leaves and plant height, at $P < 0.01$ using Tukey's test.

(21.6%) and poor GP (9.4%). However, similar to WBDL phytoplasma, transmission of ESFY phytoplasma via flower structures and seed has not yet been proven (13). As opposed to WBDL phytoplasma, Nipah et al. (14) detected Cape Saint Paul wilt disease (CSPWD) phytoplasma in seed embryos of West African Tall palms infected with CSPWD. Moreover, Cordova et al. (2) reported the presence of lethal yellowing phytoplasma in seed embryos from infected coconut trees. Nevertheless, as in WBDL phytoplasma, seed transmission of the two mentioned disease agents was reported to be unlikely.

Fruit were rarely observed on symptomatic branches of WBDL-infected trees. However, unusual aggregates of small, deformed fruit were occasionally seen on symptomatic branches (Fig. 1B). Seed coats derived from such fruit showed necrotic spots. The average weight of seed from fruit of symptomatic branches was 50% less than those from fruit of asymptomatic branches of infected and normal trees. Our results showed that the germination value of lime seed obtained from healthy trees is nearly 1.7 times higher than the seed of symptomatic fruit on WBDL phytoplasma-positive trees, whereas GP of seed from normal plants was only 1.2 times higher than the seed of symptomatic fruit of infected trees. Thus, the majority (80%) of the seed obtained from fruit on symptomatic branches of infected plants were viable. Moreover, the seed of the fruit on symptomless branches of infected trees germinated as well as the seed of normal trees. There were also no significant differences in other germination parameters between seed from uninfected trees and those from asymptomatic branches of infected trees. This may be due to uneven distribution of the phytoplasma in the newly infected plants. The presence of WBDL phytoplasma in the embryos and seedlings derived from seed of infected lime has been reported (1,9), although production of witches'-broom trees from such seed has never been demonstrated.

In the present study, we were not able to detect phytoplasmal DNA in any seedlings or plantlets derived from seed of infected trees. We also did not observe any symptomatic plants among several thousand seedlings from seed of infected trees grown under controlled condition in the present study. These findings are in accordance with the scarcity of WBDL-infected seedlings or young trees in affected plantations of southern Iran. Our conclusion that WBDL is not seed transmissible in southern Iran could greatly reduce the concern associated with the routine practice of lime propagation by seed. Although, in our experiments, WBDL phytoplasma was not transmitted through the seed of infected lime, seedlings derived from such seed were less vigorous. Therefore, planting of seed derived from healthy trees may still be of benefit to growers.

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