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Identification of Aster Yellows Phytoplasma in *Dendranthema grandiflorum*

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Phytoplasmas were identified from two chrysanthemum (*Dendranthema grandiflorum*) plants showing different symptoms; one with stunting, rosette, and excessive branching (Ph-ch1), and the other with stunting and chlorosis (Ph-ch2). Electron microscopy of midrib of the plants with the symptoms revealed that numerous phytoplasmas were localized in the phloem cells. The disease was transmitted from infected plants to healthy ones by grafting. Phytoplasma-specific DNA was detected in polymerase chain reaction (PCR) analysis with template DNA extracted from the leaves of Ph-ch1 and Ph-ch2, both of which yielded a same DNA band corresponding to 1.5 kb. Using a specific primer pair (R16F1/R1) synthesized based on aster yellows (AY) phytoplasma, a DNA fragment of 1.1 kb was amplified by PCR. Endonuclease restriction patterns of the 1.1 kb PCR products from Ph-ch1 and Ph-ch2, which were digested with each of the restriction endonucleases *Sau*3A, *Hha*, *Alu* and *Rsa*, were same as those of AY phytoplasma from periwinkle. This suggests that the chrysanthemum plants (Ph-ch1 and Ph-ch2) be infected with a phytoplasma belonging to AY phytoplasma.

Keywords : chrysanthemum, endonuclease restriction patterns, phytoplasmas, polymerase chain reaction.

Phytoplasmas (previously called mycoplasma-like organisms) are phloem-limited plant-pathogenic prokaryotes (Hopkins, 1977). They are known as the causal agents of yellowing, stunting and scorch diseases in various plants. Since the discovery of phytoplasmas in 1967, several hundreds of yellows-type diseases have been identified on a variety of economic crops worldwide (McCoy et al., 1989). In the past, identification and classification of phytoplasmas depended entirely on biological properties such as symptoms in diseased plants, plant host range (Chiykowski, 1967; Westdal and Richardson, 1969), and specificity of pathogen transmission by insect vectors (Freitag, 1967).

Clark et al. (1983) and Sinha and Benhamou (1983) reported the development of polyclonal antisera for the detection of phytoplasmas. Bertaccini et al. (1990) detected chrysanthemum yellows (CY) mycoplasma-like organism in *Catharanthus roseus*, *Chrysanthemum frutescens*, and *Chrysanthemum carinatum* by dot hybridization. Recently, polymerase chain reaction (PCR) was used to detect phytoplasma DNA in *Brassica*, *Chrysanthemum*, and *Hydrangea* (Bertaccini et al., 1992). Phytoplasma 16S rDNA sequences were amplified by PCR using a primer pair designed on the basis of a phytoplasma 16S rRNA gene, and the partial 16S rDNA sequences were compared through restriction fragment length polymorphism (RFLP) analysis (Schneider et al., 1993). Lee et al. (1993) classified 40 phytoplasmas into nine distinct 16S ribosomal RNA groups and 14 subgroups based on restriction band patterns derived from RFLP analysis. And also phytoplasmas infecting parsley and *Lilium martagon* were classified to the aster yellows (AY) (Khadhair et al., 1998) and aster yellows cluster (16Sr) subgroup-C (Vorácková et al., 1998) through RFLP analysis, respectively.

Chrysanthemum yellows have been reported in several species of chrysanthemum in Italy (Appiano et al., 1983) and the Netherlands (Bertaccini et al., 1990). In this study, we identified phytoplasmas in commercially cultivated chrysanthemums based on symptomatology, electron microscopy, and RFLP analysis. This is the first report on the observation of phytoplasmas in *Dendranthema grandiflorum*.

Materials and Methods

Phytoplasma sources. In 1998, chrysanthemum plants showing stunt, leaf proliferation, and shortening of internode (Fig. 1) were collected from a commercial farm in Ilsan, Kyunggi province, and other chrysanthemum plants showing yellows and stunt symptoms (Fig. 2) were collected from another commercial farm in Masan, Kyungnam province, Korea. The diseases were maintained on chrysanthemum seedlings inoculated by grafting in a greenhouse, and the plants were used for electron microscopic examination and molecular studies. Aster yellows (AY)-infected periwinkle DNA were provided by Abdul Hameed Khadhair (Alberta Research Council, Vegreville, Canada).

Electron microscopy. Small pieces from the leaf midribs of phy-

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toplasma-infected chrysanthemums were prefixed in 1% Karnovsky's fixative solution, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, and dehydrated in an ethanol series. Embedding was conducted in Spurr resin. Ultrathin sections were prepared with an ultramicrotome, stained with uranyl acetate, and examined with a Carl Zeiss LEO 906 transmission electron microscope.

Grafting assay. Healthy chrysanthemums were insertion-grafted on Ph-ch1 and Ph-ch2 chrysanthemums. Six weeks after grafting, inoculated chrysanthemums were cut and planted in soil. After rooting, the plants were pinched to induce branches.

Extraction of DNA. Two months after grafting, expanded young leaves were collected from Ph-ch1 and Ph-ch2 chrysanthemums showing disease symptoms. Approximately 0.3 g of leaf midrib tissues including petioles were cut, surface-sterilized with 1% sodium hypochlorite for 5 min, and rinsed three times in sterile distilled water. The sections were transferred into 2 ml solution of 0.8% cellulase (Sigma Chemical Co. St. Louis, MO, USA); 1 mM calcium chloride; 0.6 M mannitol and were incubated overnight at 4 (Lee and Davis, 1983). The tissues were ground with mortar and pestle in suspending medium (0.5 M mannitol, 30 mM HEPES, 0.1% polyvinylpyrrolidone 40, pH 7.0) and DNA was extracted following a method described by Lee and Davis (1983). DNA concentration was measured using a Beckman DU 530 spectrophotometer.

Primer. Two pairs of primer (P1/P6, R16F1/R1) were synthesized. A 16S rDNA universal primer pair P1/P6 was synthesized based on the common 16S rDNA sequences of phytoplasma. The sequence of the forward primer P1 was 5'-AAGAGTTTGATCCTGGCTCAGGATT-3' and that of the reverse primer P6 was 5'-TGGTAGGGATACCTTGTTACGACTTA-3' (Deng and Hiruki, 1991; Gundersen and Lee, 1996; Khadhair et al., 1998). The second primer pair, R16F1/R1 designed to specifically amplify 16S rDNA sequences from the AY group of phytoplasmas, was used in nested PCR to amplify DNA fragment of 1.1 kb. The sequence of the forward primer R16F1 was 5'-TAAAAGACCTAGCAATAGG-3' and that of the reverse primer R1 was 5'-CAATCCGAACTGAGACTGT-3'.

DNA amplification. The PCR reaction mixture contained 20 ng/μl of nucleic acid, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 100 μM of each dNTP, 0.4 μM of each primer, 2.5 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase (Perkin Elmer, Roche, Branchbug, NJ, USA). Amplification was performed in a DNA Thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The thermal conditions for the primer set P1/P6 included 35 cycles of denaturation at 94°C for 30 sec (except 2 min for the first cycle),

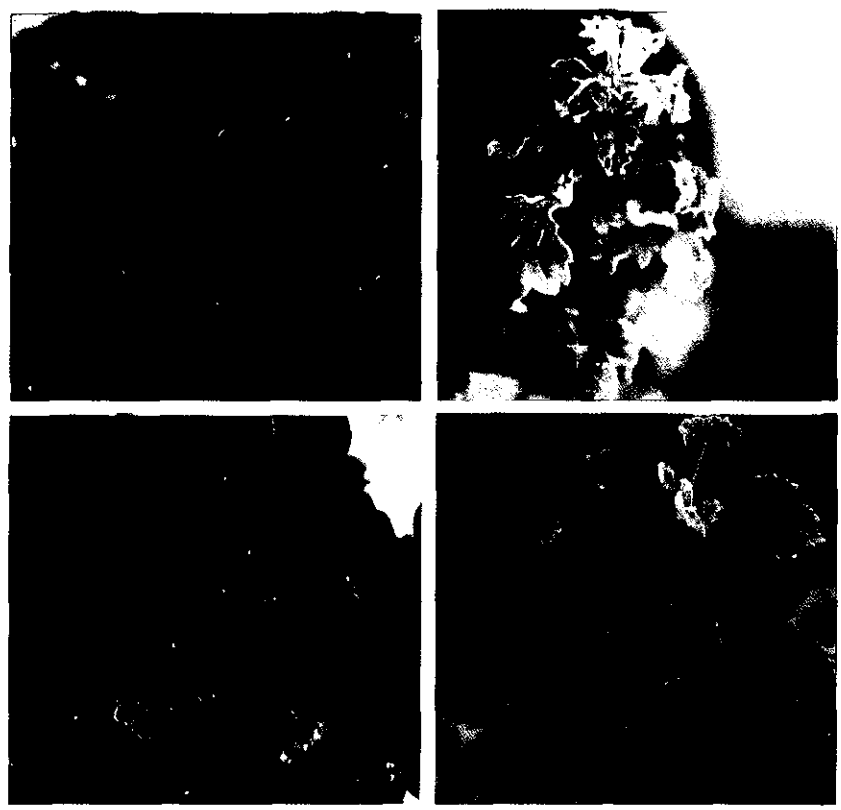

Fig. 1-4. Naturally infected (1 and 2) and graft-transmitted (3 and 4) chrysanthemum (*Dendranthema grandiflorum*) plants with phytoplasma-like symptoms. 1. One chrysanthemum plant (Ph-ch1) showing stunted growth, rosette and excessive branching. 2. Another and chrysanthemum plant (Ph-ch2) showing yellows and stunting. 3. Phytoplasma-infected chrysanthemum showing symptoms of leaf narrowing and shortening of internode 10 weeks after grafting with Ph-ch1. 4. Phytoplasma-infected chrysanthemum showing leaf chlorosis and narrowing by grafting with Ph-ch2.

annealing at 65°C for 50 sec and extension at 72°C for 1.5 min. The last cycle was extended for an additional 3 min at 72°C. The amplified nucleic acid was precipitated and used as templates in nested PCR by adding sodium acetate (3.0 M, pH 5.2) to 0.3 M followed by 2.5 volume of cold ethanol and standing at -70°C for 1 h. The precipitated nucleic acid was collected by centrifugation at 19,000 g for 25 min at 4°C, and the pellets dried in vacuum desiccator, and then dissolved in 30 µl of distilled water.

Nested PCR. Approximately 10 ng/µl of the precipitated nucleic acid from DNA amplification with primer pair P1/P6 was used as templates in nested PCR. The nested PCR reaction mixture was prepared as described above for DNA amplification. A total of 35 thermal cycles were carried out consisting of denaturation at 94°C for 1 min (4 min for the first cycle), annealing at 55°C for 1 min and extended for 1.5 min at 72°C and extended again for 7.5 min in the last cycle. The PCR product was separated by electrophoresis on 1.5% agarose gel in 0.5 × TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) (Sambrook et al., *1989*) at 100 V for 20 min and then visualized by staining in ethidium bromide solution under a UV transilluminator.

RFLP. The amplified nucleic acid was precipitated from PCR product of 1.1 kb as described above for the precipitation of PCR products of 1.5 kb. Approximately 1/4 volume of the precipitated nucleic acid (1.1 kb) was digested with each of the restriction endonuclease *Sau*3A, *Hha*I, *Alu*I, and *Rsa*I according to the manufacturer's instructions (Takara, Shiga, Japan). Endonuclease-treated PCR product (1.1 kb) was separated by electrophoresis on 1.8% agarose gel in 0.5 × TBE buffer.

Results and Discussion

Symptoms. Chrysanthemum plants inoculated by grafting with naturally phytoplasma-infected chrysanthemums did not show any disease symptoms early after inoculation, but exhibited somewhat narrowing and yellowing of the inoculated leaves in advance (data not shown). Symptoms were more advanced in laterals when the inoculated chrysanthemum plants were pinched. New leaves from laterals of cysanthemum inoculated with Ph-ch1 showed symptoms of narrowing and shortened laterals (Fig. 3). Chrysanthemum plants inoculated with Ph-ch2 showed leaf chlorosis and narrowing 6 weeks after grafting (Fig 4). These symptoms were similar to those of parsley infected with aster yellows (Khadhair et al., 1998).

Electron microscopy. Electron microscopy of ultrathin sections of the leaf midribs showed numerous membrane-

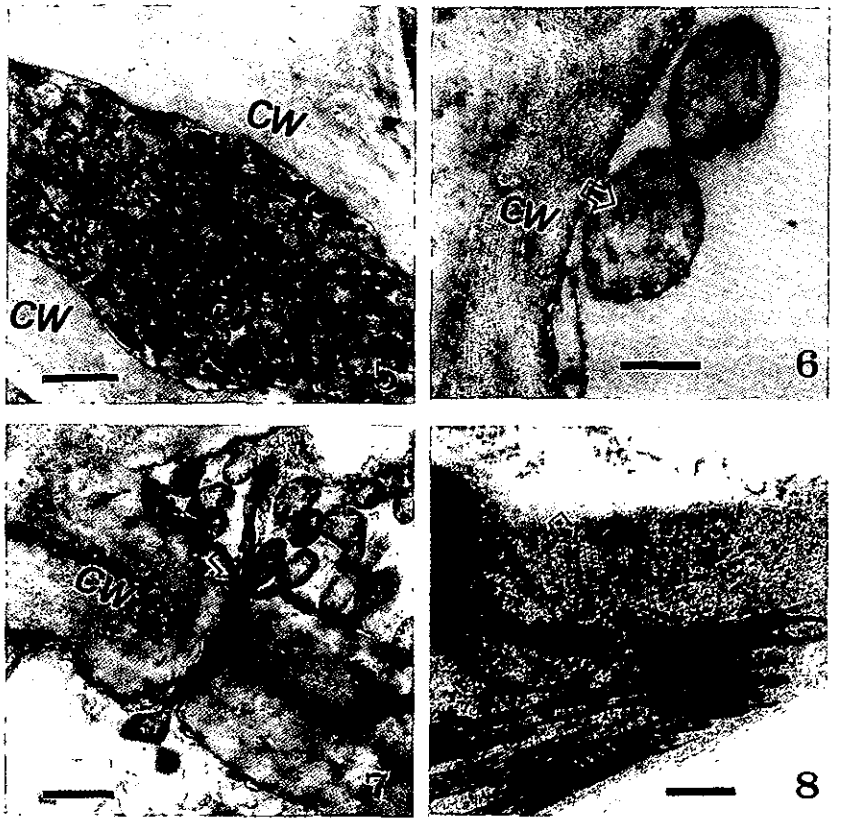

Fig. 5-8. Electron microscopy of midribs of phytoplasma-infected chrysanthemum (*Dendranthema grandiflorum*) plants. 5. Sieve element filled with phytoplasma bodies. CW, cell wall. Bar represents 500 nm. 6. Phytoplasmas containing fine fibrils (arrow). CW, cell wall. Bar represents 200 nm. 7. Some phytoplasmas passing through a sieve pore (arrow). CW, cell wall. Bar represents 400 nm. 8. Chloroplast containing phytoferritin crystals (arrow). Bar represents 300 nm.

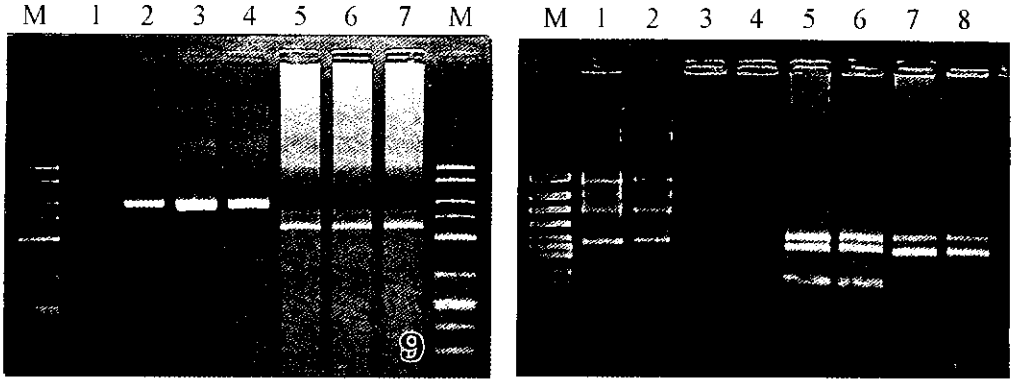

Fig. 9. Amplification of 16S rDNA sequences from infected chrysanthemum plants using universal primer pair R1/R6 (lane 1-4) and specific primer pair R16F1/R1 (lane 5-7). PCR products were separated by electrophoresis in 1.5 % agarose gel. Lane M, 50-2500 bp DNA size marker; lane 1, healthy chrysanthemum; lane 2 and 5, Alberta AY phytoplasma-infected periwinkle DNA; lane 3 and 6, Ph-ch1; lane 4 and 7, Ph-ch2.

Fig. 10. Restriction endonuclease analysis of 16S rDNA after PCR amplification with primer pair R16F1/R1 using endonuclease *Sau*3A (lane 1, 2), *Hha*I (lane 3, 4), *Alu*I (lane 5, 6) and *Rsa*I (lane 7, 8). Endonuclease treated PCR products were separated by electrophoresis in 1.8 % agarose gel. Lane 1, 3, 5 and 7, Alberta AY phytoplasma; lane 2, 4, 6 and 8, Ph-ch1; M, DNA size marker (Φ174-*Hinc*II).

bound structures resembling phytoplasmas. They were round or pleomorphic in shape, with a diameter of 70-250 nm and were localized in the phloem cells, sometimes so numerous as to fill the whole cell (Fig. 5). Appiano et al. (1983) and Siddique et al. (1998) characterized the phytoplasma cells as containing ribosomes and fibrillar material in the central region, presumed to contain DNA. Also phytoplasmas observed in the chrysanthemum of our study contained fine fibrils (Fig. 6). Phytoplasmas were often observed passing through the sieve pores (Fig. 7). In chloroplasts of mesophyll cells of phytoplasma-infected chrysanthemums, phytoferritin crystals were easily observed (Fig. 8). The occurrence of crystalline phytoferritin has been also reported in the chloroplasts of soybean plants infected with soybean mosaic virus (Cho et al., 1998).

DNA amplification. Universal primer pair P1/P6 amplified the 1.5 kb DNA fragment of phytoplasma 16S rDNA from Ph-ch1 and Ph-ch2 chrysanthemums. There was no amplification of the DNA from healthy plants (Fig. 9). These results are in agreement with previous reports in which the same primer pair was used for identification of AY phytoplasma in parsley (Khadhair et al., 1998).

Nested PCR. PCR products 1.1 kb were amplified with specific primer pair R16F1/R1 from the 1.5 kb PCR products. (Fig. 7). These results are in agreement with previous studies which used the same primer pair for identification of AY phytoplasma in parsley plants (Khadhair et al., 1998).

RFLP. The restriction profiles of the PCR products from pytoplasma-infected chrysanthemums and from those of Alberta AY phytoplasmas showed identical DNA patterns in gel electrophoresis (Fig. 10), indicating the presence of AY phytoplasma in the symptomatic chrysanthemums similar to the Alberta AY phytoplasma isolate (Khadhair et al., 1998). Digestion with restriction endonuclease *Rsa*and *Hha* produced two major DNA fragments while three and four restriction products were formed with *Alu*and *Sau*3A, respectively. There were no differences in RFLP band patterns between Ph-ch1 and Ph-ch2 (data not shown). The identity of the symptoms of Ph-ch1 and Ph-ch2 chrysanthemums was confirmed as an infection symptom of an isolate of AY phytoplasma. But, futher studies are needed to find the reason for the different disease symptoms in chrysanthemums (Ph-ch1, Ph-ch2) infected with the same AY group phytoplasma.

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References

Appiano, A., D'Giovanni, G. and Conti, M. 1983. Ultrastructural observations on chrysanthemum plants infected by a mycoplasma-like organism. *Giornale Botanico Italiano* 117:136-137.

Bertaccini, A., Davis, R. E. and Lee, I. M. 1990. Detection of chrysanthemum yellows mycoplasma-like organism by dot hybridization and southern blot analysis. *Plant Dis.* 74:40-43.

Bertaccini, A., Davis, R. E., Hammond, R. W., Vibio, M., Bellardi, M. G. and Lee, I. M. 1992. Sensitive detection of mycoplasmalike organisms in field collected and *in vitro* propagated plants of *Brassica, Hydrangea* and *Chrysanthemum* by polymerase chain reaction. *Ann. Appl. Biol.* 121:593-599.

Chiykowski, L. N. 1967. Reaction of some wheat varieties to aster yellows. *Can. J. Plant Sci.* 47:149-151.

Cho, E. K., Martin, E. M., Goeke, S. C. and Kim, K. S. 1998. Ultrastructural comparison of soybean differentials infected with a virulent SMV strain. *Korean J. Plant Pathol.* 14:563-566.

Deng, S. and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *J. Microbiol. Methods* 14:53-61.

Freitag, J. H. 1967. Interaction between strains of aster yellows virus in the spotted leafhopper *Macrosteles fascifrons*. *Phytopathology* 57:1016-1024.

Gibb, K. S., Schneider, B. and Padovan, A. C. 1998. Differential detection and genetic relatedness of phytoplasmas in papaya. *Plant Pathol.* 47:325-332.

Gundersen, D. E. and Lee, I. E. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathol. Medit.* 35:144-151.

Hopkins, D. I. 1977. Diseases caused by leafhopper-borne rickettsia-like bacteria. *Annu. Rev. Phytopathol.* 17:277-294.

Jiang, Y. P. and Chen, T. A. 1987. Purification of mycoplasma-like organisms from lettuce with aster yellows disease. *Phytopathology* 77:949-953.

Khadhair, A. H., Kawchuk, L. M., Taillon, R. C. and Botar, G. 1998. Detection and molecular characterization of an aster yellows phytoplasma in parsley. *Can. J. Plant Pathol.*20:55-61.

Lee, I. M. and Davis, R. E. 1983. Phloem-limited prokaryotes in sieve elements isolated by enzyme treatment of diseased plant tissues. *Phytopathology* 73:1540-1543.

Lee, I. M. and Davis, R. E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organism by using cloned DNA and RNA probes. *Mol. Plant-Microbe Interact.* 1:303 -310.

Lee, I. M., Hammone, R. W., Davis, R. E. and Gundersen, D. E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* 83:834-842

McCoy, R. E., Caudwell, A., Chang, C. J. Chen, T. A., Chiykowski, L. N., Cousin, M. T., Dale, J. L., de Leeuw, G. T. N., Golino, D. A., Hackett, K. J., Kirkpatrick, B. C., Marwitz, R., Petzold, H., Sinha, R. C., Sugiura, M., Whitecomb, R. F., Yang, I. L., Zhu, B. M. and Seem ller, E. 1989. Plant diseases associated with mycoplasmalike organisms, In: *The Mycoplasmas. Vol. 5. Spiroplasmas, Acholeplasmas, and Mycoplasmas of Plant and Arethropods*. ed. by R. F. Whitcomb, and J. G. Tully, pp. 545-640. Academic Press, New York.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schneider, B., Ahrens, U., Kirkpatrick, B. C. and Seemüller, E. 1993. Classification of plant pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *J. Gen. Microbiol.* 139:519-727.

Siddique, A. B. M., Guthrie, J. N., Walsh, K. B., White, D. T. and Scott, P. T. 1998. Histopathology and within-plant distribution of the phytoplasma associated with Australian papaya dieback. *Plant Dis.* 82:1112-1120.

Sinha, R. C. 1979. Purification and serology of mycoplasmalike organisms from aster yellows-infected plants. *Can. J. Plant Pathol.* 1:65-70.

Voráčková, Z. P., Fránová, J., Válová, P., Mertelik, J., Návrátil, M. and Nebesárová, J. 1998. Identification of phytoplasma infecting *Lilium martagon* in the Czech Republic. *J. Phytopathol.* 146:609-612.

Westdal, P. H. and Richardson, H. P. 1969. The susceptibility of cereals and wild oats to an isolate of the aster yellows pathogen. *Can. J. Plant Pathol.* 47:755-760.