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Elimination of Aster Yellows Phytoplasma from *Dendranthema grandiflorum* by Application of Oxytetracycline as a Foliar Spray

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Aster yellows phytoplasma-infected chrysanthemums showing stunt, rosette, and excessive branching were treated with a foliar spray of 400 mg/l oxytetracycline at three-day interval for 1, 2, 3 and 4 months. Two months after the final treatment, new shoots from the recovered chrysanthemums showed the recurrence of the disease symptoms. However, cuttings from chrysanthemums treated with oxytetracycline did not express any phytoplasma infection symptoms for more than 10 months. Also, chrysanthemums dipped in 100 mg/l oxytetracycline solution combined with a foliar spray of 400 mg/l oxytetracycline for 4 weeks showed the same results. Using an electron microscope, ultrathin sections of leaf midribs of chrysanthemum cuttings treated with oxytetracycline for 4 months did not show phytoplasma bodies 10 months after treatment. Nucleic acids from chrysanthemums, which did not express phytoplasma infection symptoms for more than 10 months, did not amplify 16S rRNA gene of phytoplasma by polymerase chain reaction. These results may have implications in the propagation of phytoplasma-free healthy stocks for a wide range of plant species.

Keywords : aster yellow, chrysanthemum, oxytetracycline, phytoplasma, recovery.

Phytoplasmas are phloem-limited plant pathogenic prokaryotes known as the causal agents of yellowing, stunt, 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). Chrysanthemum yellows have been reported in several species of chrysanthemum in Italy (Appiano et al., 1983), the Netherlands (Bertaccini et al., 1990), and Korea (Chung et al., 2001). Davis and Clark (1994) reported that phytoplasmas (formerly called as mycoplasma-like organism) could be eliminated by incorporation of tetracycline in growth medium of *Pyrus* species. Cha and Tattar (1993) reported temporary recovery of ash trees from ash yellows phytoplasma infection symptom with two times injection of 4% oxytetracycline using trunk infection. Zaim and Samad (1995) also reported the temporary recovery of *Withania somnifera* (L.) infected with witches broom disease with oxytetracycline spray.

In this study, phytoplasma-infected chrysanthemums were sprayed with oxytetracycline or dipped in the solution to control phytoplasmas and its effect were discussed.

Materials and Methods

Phytoplasma sources. A chrysanthemum plant showing stunt, leaf proliferation, and shortening of internode was taken from a commercial farm in Ilsan, Kyunggi Province, Korea. This chrysanthemum was maintained by grafting on healthy chrysanthemum seedlings in a greenhouse. The disease-infected chrysanthemum seedlings were propagated by cutting for this experiment.

Oxytetracycline treatment. To examine the effect of oxytetracycline on phytoplasma infected chrysanthemums, potted chrysanthemum plants at six leaf stage infected with phytoplasma diseases were applied with 0, 100, 200, 400, 800 and 1,200 mg/l oxytetracycline (Sigma, chemical) as a foliar spray at three-day interval for 1 month. To select the optimum concentrations of oxytetracycline for root dip experiment, chrysanthemum stems with four attached leaves, which rooted in water by 1 cm length, were dipped in 100, 200, 300, 400 and 500 mg/l oxytetracycline for 6 weeks. The dipping solution was changed every week. To eliminate the phytoplasma bodies from the infected chrysanthemums, 400 mg/l oxytetracycline was singly applied as a foliar spray at 6.5 ml/plant at three-day interval for 1, 2, 3 and 4 months in a pot experiment; and in combination with root dip experiments, stems which rooted in water by 1 cm length were dipped in oxytetracycline solution of 100 mg/l for 2 and 4 weeks. Immediately after the final treatment, potted chrysanthemums were grown with or without separation from the treated chrysanthemums, and root-dipped chrysanthemums combined with foliar spray were transplanted in pots. Twenty samples were replicated in each treatment for both pot and root dip experiment.

Cultivation method. All potting mixtures used in this experiment consist of one part each of peat and perlite; no fertilizer was added during cultivation. Chrysanthemums grown separately after oxy-

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tetracycline were treated as follows: chrysanthemum stems with 5-6 leaves attached were cut from the top of the oxytetracycline-treated stems, and the cut stems were grown for 7 months and propagated by cutting; plant height and leaf length of the propagated chrysanthemums were measured 3 months after cutting; then, growth of chrysanthemums grown without separation was measured 10 months after the final treatment.

Electron microscopy. Small pieces from the leaf midribs of oxytetracycline-treated chrysanthemum plants 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 (Chung et al., 2001). 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.

PCR amplification of 16S rDNA. Polymerase chain reaction (PCR) was carried out to confirm the elimination of phytoplasma bodies from the previously disease-infected chrysanthemums, which were treated with oxytetracycline for 4 months followed by propagation using cuttings, 10 months after treatment. The PCR reaction mixture contained 20 ng/μl of total nucleic acids, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 100 μM of each dNTP, 0.4 μM of each P1 and P6 primer (Chung et al., 2001), 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 seconds (2 minutes for the first cycle), annealing at 65°C for 50 seconds, and extension at 72°C for 1.5 minutes. The last cycle was extended for an additional 3 minutes at 72°C. The amplified nucleic acids were analyzed on 1.5% agarose gel.

Results

Application of oxytetracycline concentrations of 400, 800, and 1,200 mg/l to phytoplasma-infected chrysanthemums increased the length of internodes and leaves 1 month after treatment (Table 1, Fig. 1). Though the plants recovered from the damages within 1 month after treatment, 800 mg/l and 1,200 mg/l of oxytetracycline caused phytotoxicity (data not shown) resulting to leaf darkening. Therefore, it was assumed that 400 mg/l was the optimum concentration for a foliar spray of oxytetracycline to chrysanthemums. In the root dip experiment, stems of chrysanthemums with higher concentrations of oxytetracycline turned into black (Fig. 2) earlier than the leaves on stems in 100 mg/l of oxytetracycline in which blackening of stems appeared after 4 weeks. From this result, the optimum concentration of oxytetracycline for root dipping was assumed to be 100 mg/l, while the optimum duration was as long as 4 weeks. Infected chrysanthemums foliar sprayed with 400 mg/l oxytetracycline for 1, 2, 3 and 4 months recovered from infection 1 month after each treatment (data not shown).

Table 1. Effect of oxytetracycline treatment for one month by a foliar spray at three day intervals on the growth of phytoplasma-infected chrysanthemums one month after treatment

Oxytetracycline conentration (mg/l)	Internode length (cm)	Leaf length (cm)
0	0.33 x[a]	2.5 x
100	0.40 x	2.9 x
200	0.50 y	4.8 y
400	0.83 z	6.5 z
800	0.83 z	6.2 z
1,200	0.80 z	5.5 z

[a]Mean separation within a column by Duncan's multiple range test at $P=0.05$.

Two months after each final treatment, newly sprouting plants from the treated chrysanthemums showed typical phytoplasma infection symptoms of stunt and shortening of internodes (Fig. 3A). Meanwhile, chrysanthemums separated from those treated for 4 months followed by the final treatment have not expressed any phytoplasma infection symptoms for more than 10 months (Fig. 3B, C) and did not amplify phytoplasma 16S rRNA genome but amplified in not separated plants (Fig. 4). Also, leaf length, internode length, and plant height increased, and the plants flowered (Table 2). In the root dip experiment, nucleic acid from chrysanthemums treated for 2 weeks amplified 16S rRNA gene of phytoplasma but not those treated for 4 weeks (Fig. 4). Chrysanthemums dipped in 100 mg/l oxytetracycline solution for 4 weeks combined with a foliar spray of 400 mg/l oxytetracycline have not expressed any phytoplasma infection symptoms for more than 10 months after the final treatment (data not shown) and the growth were similar with those of healthy plants (Table 3). Through electron microscope, ultrathin sections of leaf midribs of chrysanthemums, which were grown with separation using cuttings from the ones applied with oxytetracycline for 4 months, 10 months after final treatment did not reveal any phytoplasma bodies in the sieve tubes of phloem. However, numerous phytoplasma bodies were observed in chrysanthemums grown without separation or were not treated (Fig. 5).

Discussion

Phytoplasma infected chrysanthemums applied with 400 mg/l oxytetracycline for a period of 4 months and then grown with separation using cuttings immediately after final treatment recovered from infection. Recovery was assumed to result from the low titer of phytoplasma bodies by oxytetracycline application. So, the recurrence of disease symptoms from recovered chrysanthemums after stopping oxytetracycline application was probably due to

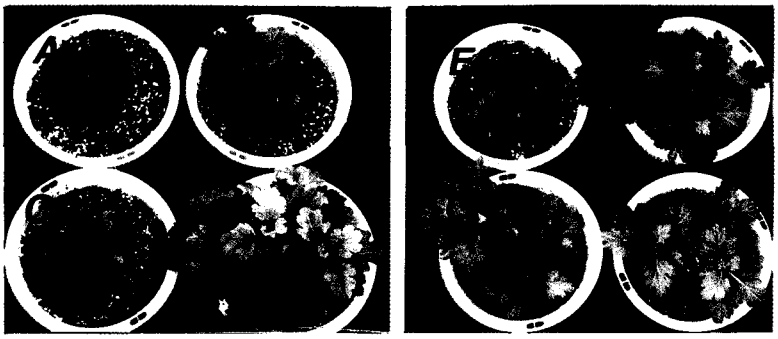

Fig. 1. Phthoplasma-infected chrysanthemums treated with oxytetracycline for 1 month using foliar spray. A and E, untreated chrysanthemum; B, 100 mg/l; C, 200 mg/l; D, 400 mg/l; F, 400 mg/l; G, 800 mg/l; H, 1,200 mg/l. Photo was taken 1 month after the final treatment.

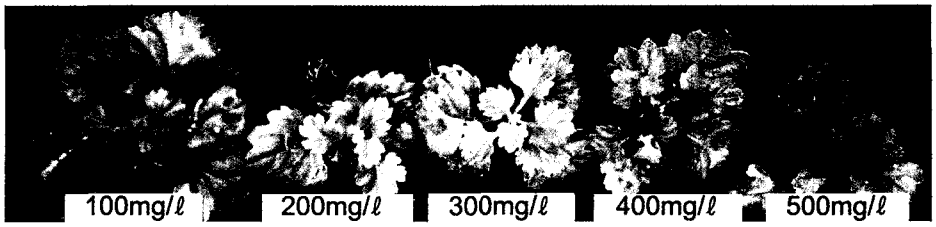

Fig. 2. Treatment of chrysanthemums root by dipping in oxytetracycline solutions for 6 weeks.

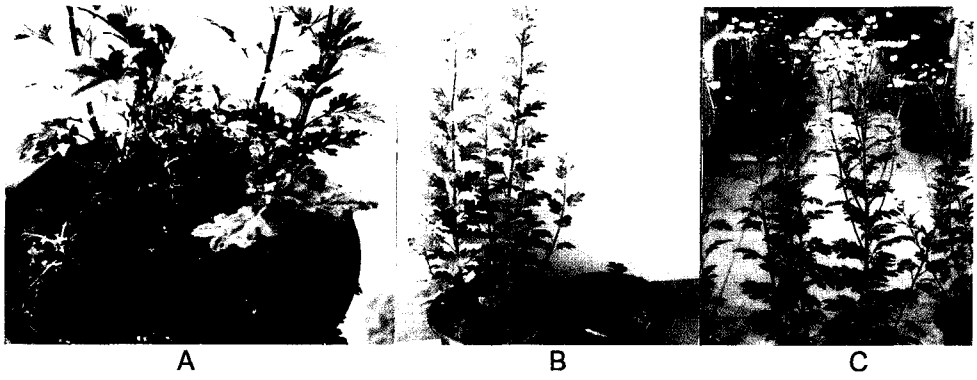

Fig. 3. Phytoplasma-infected chrysanthemums applied with 400 mg/l oxytetracycline for 4 months at three-day interval by foliar spray. A, reappearance of stunting and proliferation on new sprouts from the roots 2 months after the final treatment; B, separation effect in plants separated by cutting (left) and non-separated (right) after the final treatment; C, flowering of phytoplasma-infected and oxytetracycline-sprayed chrysanthemums.

translocation of phytoplasma bodies which remained in the roots to upper stems or leaves. Because the roots were not directly applied with oxytetracycline, it was assumed that high titer of phytoplasma bodies remained. Chrysanthemums grown with separation immediately after treatment from the ones which were foliar sprayed with oxytetracycline for 4 months did not show disease symptoms again.

In the root dip experiment, chrysanthemum roots absorbed the oxytetracycline solution. This was translocated to leaves such that phytoplasma bodies present in the roots could be eliminated during treatment. From the PCR analysis of chrysanthemums which did not show infection symptoms for more than 10 months, it could be concluded that phytoplasma bodies were completely eliminated from the previously phytoplasma-infected chrysanthemums. Soni and Thind (1989) reported the translocation of agrimycin-100 (1.5% oxytetracycline+15% streptomycin), which was used to control maize bacterial stalk rot, from root to leaf tips 24 and 48 hrs after root dip treatment. In the experiments of Cha and Tattar (1993) and Zaim and Samad (1995), the temporary recovery of the plants from infection was assumed to be caused by the phytoplasma bodies

Table 2. Effect of oxytetracycline application duration in foliar spray of 400 mg/l on the growth of phytoplasma-infected chrysanthemums 10 months after final treatment according to growing method after final treatment

Duration (months)	Growing methods	Leaf length (cm)	Plant height (cm)	Flowering
1	Separated	2.9±0.18[a]	8.1±0.65	Not flowered
	Non-separated	2.6±0.35	4.5±0.47	Not flowered
2	Separated	2.6±0.20	8.9±0.21	Not flowered
	Non-separated	3.0±0.21	4.2±0.22	Not flowered
3	Separated	2.0±0.15	7.9±0.31	Not flowered
	Non-separated	2.2±0.14	4.6±0.31	Not flowered
4	Separated	7.2±0.31	55.0±1.27	Not flowered
	Non-separated	2.4±0.10	4.5±0.22	Flowered

[a] Mean±standard deviation.

Table 3. Effect of oxytetracycline application by root dipping in a solution of 100 mg/l combined with 400 mg/l foliar spray on the growth of phytoplasma-infected chrysanthemums

Treatment		Duration (weeks)	Plant height (cm)	Leaf length (cm)	Flower width (cm)
Infected (control 1)		0	3.7	2.2	–[a]
Healthy (control 2)		0	42.5	9.7	8.9
Infected (treated)		2	3.5	2.5	–
		4	41.5	9.7	8.8
T test	Control 1	2	NS[b]	NS	·
		4	**	**	·
	Control 2	2	**	**	·
		4	NS	NS	NS

[a] Not flowered.
[b] NS : nonsignificant, **: significant at $P=0.01$.

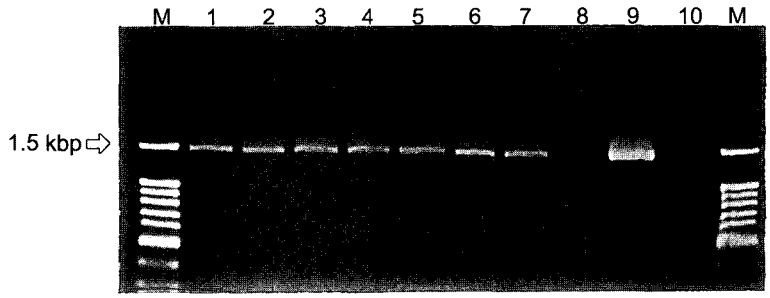

Fig. 4. Agarose gel electrophoresis of PCR products from phytoplasma-infected chrysanthemums grown without (lanes 1, 2, 3 and 4) or with (lanes 5, 6, 7, and 8) separation from the ones treated with 400 mg/l oxytetracycline for 1, 2, 3 and 4 months; and from chrysanthemums dipped in 100 mg/l oxytetracycline solution combined with a foliar spray of 400 mg/l oxytetracycline for 2 and 4 weeks (lanes 9 and 10). Lanes 1 and 5, 1 month; lanes 2 and 6, 2 months; lanes 3 and 7, 3 months; lanes 4 and 8, 4 months; lanes 9, 2 weeks; lanes 10, 4 weeks; M, 100 bp DNA size marker.

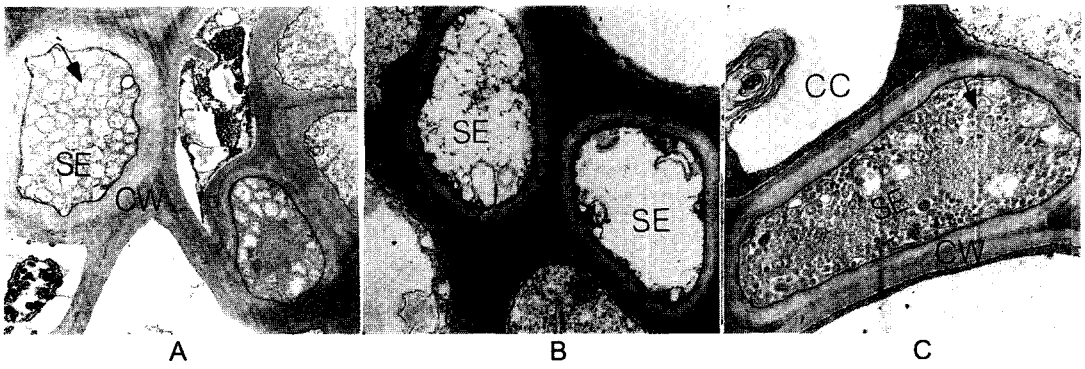

Fig. 5. Electron micrograph of phloem sieve element of phytoplasma-infected chrysanthemums foliar sprayed with oxytetracycline for 4 months (B and C) and untreated (A). B, Chrysanthemums grown for 10 months with separation after the final treatment. C, Numerous phytoplasma bodies in sieve element of chrysanthemums grown for 10 months without separation after the final treatment. CW: cell wall, CC: companion cell, SE: sieve element, arrow indicates phytoplasma bodies.

which remained in the roots.

These results could have implications in the propagation of phytoplasma-free stocks for a wide range of plant species. Phytoplasma-infected plants are known as having characteristics of premature drying and death of infected twigs (Siddique et al., 1998; Zaim and Samad, 1995),

which are usually maintained by grafting on healthy plants. It is believed that foliar spray with oxytetracycline on phytoplasma-infected plants can be useful for maintaining diseased plants for further use.

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