Detection of a Latent Infectious Agent That Protects Against Infection by Chrysanthemum Chlorotic Mottle Viroid

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ABSTRACT

A latent infectious agent (ChCMV-NS) that protects against chrysanthemum chlorotic mottle viroid (ChCMV) was found in cultivars of *Chrysanthemum morifolium*; it produced no recognizable symptoms, but is detectible by its complete protection against the severe strain of chrysanthemum chlorotic mottle viroid. At least 8 days of incubation are required after inoculation of ChCMV-NS for complete protection against ChCMV. Optimum detection of ChCMV-NS occurred at 21 C and the optimum for detection of ChCMV from crude extracts of source plants was 21 to 24 C. Detectible infectivity of ChCMV was increased with illumination intensities up to 30,128 lx. The existence of a latent infectious agent is important in indexing programs, since current indexing procedures will not detect the latent agent.

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Chrysanthemum chlorotic mottle (ChCMV) was first described in 1971 by Dimock et al. (4) as a disease induced by a virus-like agent infecting *Chrysanthemum morifolium* (Ramat.) Hemsl. Since that report, we have determined (13) that the causal agent is a viroid such as those which cause potato spindle tuber (1, 3), citrus exocortis (14), and chrysanthemum stunt (2).

The florists' chrysanthemum is the only reported host plant for ChCMV. All cultivars of florists' chrysanthemum tested are susceptible to ChCMV, but not all cultivars exhibit recognizable symptoms. Cultivar Deep Ridge gave the most distinct and reliable foliar symptoms; however, symptom development is dependent on appropriate temperature and light conditions. Symptoms are best expressed with minimum light intensities of 10,760 lx, photoperiods of 12 hours (9), and temperatures of 24-27 C (11). No symptoms are expressed at constant temperatures less than 21 C and light intensities less than 5,380 lx. Symptoms were expressed within 14 days when plants were placed in optimal temperature and light conditions, even if they previously had been maintained in suboptimal conditions. Even when all these environmental conditions are controlled, symptom development on inoculated plants is erratic, and problems are experienced during summer months. Thus, attempts were made in the present work to determine what factors influence the titer of ChCMV in chrysanthemums used to supply inoculum.

In some cases, clones of cultivar Deep Ridge failed to express symptoms, even under optimal environmental conditions when inoculated mechanically with ChCMV or with tissue implants from ChCMV-infected chrysanthemums. This phenomenon was first observed in tests to detect ChCMV in commercial cultivars. Deep Ridge plants were inoculated with tissue from known ChCMV-infected chrysanthemums. It was first thought that environmental conditions were not optimal for symptom expression, but no symptoms were induced even under optimal temperature and light conditions. Nonsymptomatic Deep Ridge (NS-Deep Ridge) was checked along with symptomatic Deep Ridge (S-Deep Ridge) to authenticate cultivar trueness and the cultivars were found to be identical.

It was hypothesized that a latent strain of ChCMV may be the cause of the failure of NS-Deep Ridge to exhibit symptoms and of erratic symptom development from ChCMV inoculations made during the summer months. There was reason to suspect the existence of more than one strain of ChCMV since, occasionally, inoculated plants exhibited sparse and indistinct symptoms under optimal conditions. Single plants have also been observed that exhibit severe, sparse, or no symptoms on different shoots of the same plant. Thus, efforts were made to determine the presence of diverse strains of ChCMV and their possible effect on induction of symptoms by the common strain.

MATERIALS AND METHODS.—*Chrysanthemum morifolium* 'Deep Ridge' was used as a test plant, and ChCMV-infected Deep Ridge was used as a source of inoculum in all experiments. Inoculum for mechanical inoculations was prepared by removing young newly expanding leaves from plants exhibiting chronic symptoms (severe mottling to complete chlorosis) and grinding the leaves in a precooled mortar with one part (w/v) of bentonite-borate buffer (0.1 M H_3BO_3 and 0.1 M $Na_2B_4O_7$ adjusted to pH 8.0 and mixed 1:1 with a 1% bentonite solution shaken on a Burrell Wrist-Action shaker for 30 minutes and chilled at 4C). All mechanical inoculations were made as described by Horst et al. (10). Tissue implantation inoculations were performed as previously described by Dimock et al. (5) using 14- and 16-gauge Aspiration Trocars® (Popper & Sons, Inc.; 300 Benton Ave., New Hyde Park, N.Y. 11040).

The total number of plants that exhibited symptoms was recorded on alternate days for 21 days after inoculation. Inoculated plants were then trimmed to approximately 15 cm above the soil surface to induce new growth from lateral buds. Final observations were made 9 days later, and data were expressed as an infectivity index (1, 3); i.e., a summation of the number of plants reacting after 30 days, but no dilution factor was used in the data reported here. The infectivity index reflects the total number of plants that had reacted at the completion of the experiment, and also the time at which symptoms appeared.

Environmental growth chambers were used to maintain constant temperatures and light conditions (23,672 lx). Inoculum source plants and test plants for greenhouse experiments were maintained at approximately 24C. Supplemental lighting was used during the winter months to maintain a minimum of 10,760 lx for 14 hours of every day. Plants were fertilized

weekly with 230 g of 20-20-20 fertilizer per 18.9 liters of water applied at a 1:10 dilution rate.

RESULTS AND DISCUSSION.—*Chrysanthemum chlorotic mottle viroid infectivity.*—Preliminary experiments were made to determine when maximum detectible levels of ChCMV are attained. Three leaves were inoculated on each of 30 plants maintained at 27C. Newly formed leaves on inoculated plants were sampled 7, 10, 13, 16, 19, 22, 30, 60, and 90 days after inoculation. Inoculated leaves were sampled 10, 16, 22, and 30 days after inoculation. Detectible infectivity appears to reach maximum levels within 90 days at 27 C and highest levels of infectivity were demonstrated from newly formed apical leaves (Fig. 1).

Although source plants for all further experiments were maintained under optimum environmental conditions, symptom development was erratic during the summer months. Temperature was first investigated with illumination maintained at 23,672 lx with a 14-hour photoperiod. A high level of infectivity was obtained within 14 days when inoculated plants were maintained at 32 C; whereas at lower temperatures, the rate of increase in infectivity was slowed. Highest levels of infectivity were not achieved until after 24 days at 21 C in mechanically inoculated plants (Fig. 2). Inoculated plants were also maintained at diurnal temperatures of 18-37 C and 21-32 C using a 14-hour photoperiod. Fourteen-hour illumination coincided with 14 hours at the higher temperature of a given regimen. After 18 days, highest levels of infectivity were obtained from plants maintained under 21-32 C; however, after 28 days the highest levels were obtained from plants maintained at 18-37 C (Fig. 2). Maximum degree of infectivity was obtained within 10 days from high-intensity illumination (30,125 lx) as compared to low-intensity illumination (5,380 lx) (Fig. 3). Other experiments showed that radiant energy under controlled environmental conditions did not affect infectivity. In addition, Temik, which was used during summer months as a systemic insecticide, had no effect on the detectible infectivity of ChCMV.

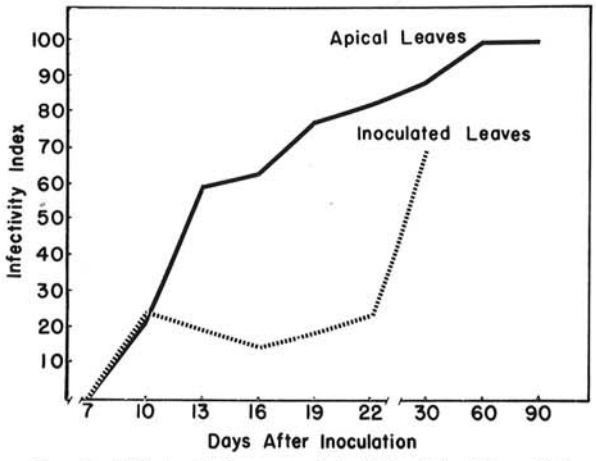

Fig. 1. Effect of time on detectible infectivity of the chrysanthemum chlorotic mottle viroid (ChCMV) from ChCMV-infected chrysanthemum cultivar Deep Ridge. Data are the average of three experiments. Infectivity index is the summation of the number of plants that had reacted after 30 days.

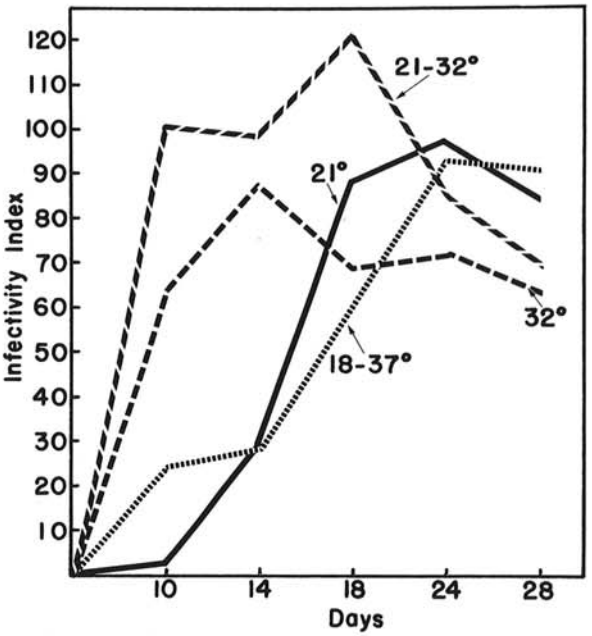

Fig. 2. The effect of temperature on detectible infectivity of the chrysanthemum chlorotic mottle viroid (ChCMV) from chrysanthemum cultivar Deep Ridge. Infectivity assayed 10, 14, 18, 24, and 28 days after inoculation. These data are the average of two experiments. Infectivity index is the summation of the number of plants that had reacted after 30 days.

Infectivity of a latent infectious agent.—Even under optimum conditions for induction of symptoms and detectible infectivity of ChCMV, instances were found in which clones of Deep Ridge failed to express symptoms. The existence of latent strains of ChCMV was therefore investigated. Cuttings removed from S-Deep Ridge (potentially symptomatic plants) were grafted as scions to NS-Deep Ridge (clone which exhibits no symptoms) used as the understock. After 4 weeks, cuttings were removed from the scion and rooted. When implant inoculations from ChCMV-infected chrysanthemums were then made to these rooted cuttings, no symptoms developed. Cuttings which were removed and rooted from scions of a graft of S-Deep Ridge to S-Deep Ridge and inoculated with ChCMV exhibited symptoms. These results suggested cross-protection [a phenomenon well known with "conventional" plant viruses (12)] by a latent strain of ChCMV (ChCMV-NS) present in NS-Deep Ridge.

Therefore, an experiment to establish the existence of a latent infectious agent that protects against ChCMV was made with a group of 15 plants of Deep Ridge which were first inoculated with implants from NS-Deep Ridge, and 1 week later challenge-inoculated with S-Deep Ridge (infected and exhibiting typical ChCMV symptoms). As a control, 15 plants of Deep Ridge were inoculated with implants from healthy Deep Ridge, and then challenge-inoculated 1 week later with implants from symptomatic S-Deep Ridge. No plants inoculated first with NS-Deep Ridge followed by symptomatic S-Deep Ridge exhibited symptoms after 30 days, whereas all plants implanted first with healthy Deep Ridge and then inoculated with symptomatic S-Deep Ridge expressed symptoms typical of severe ChCMV.

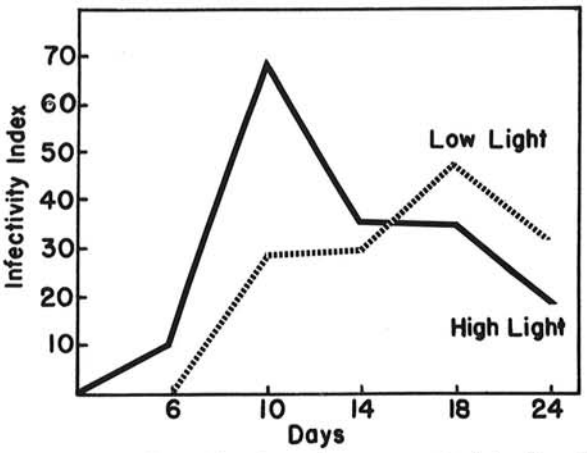

Fig. 3. The effect of light intensity on detectable infectivity of the chrysanthemum chlorotic mottle viroid (ChCMV) from chrysanthemum cultivar Deep Ridge. Infectivity assayed 6, 10, 14, 18, and 24 days after inoculation. High-intensity illumination was approximately 30,125 lx and low-intensity illumination was approximately 5,380 lx. These data are the average of two experiments. Infectivity index is the summation of the number of plants that had reacted after 30 days.

Another experiment was run to test the incubation time required for ChCMV-NS to give complete protection against ChCMV. Deep Ridge was implanted with ChCMV-NS and, at intervals, challenge implants were made with ChCMV. Inoculated plants were grown at 21, 24, and 27 C to determine the effect of temperature on ChCMV-NS. No protection was obtained at 2 days, some protection was apparent after 5 days (Table 1), and complete protection was obtained against ChCMV after 10 days. There appeared to be more protection at 21 C than at 27 C. The experiment was repeated at the same temperature regimes to determine more precisely the incubation time required for complete protection against ChCMV. In this experiment, 12 Deep Ridge plants were implanted with ChCMV-NS tissue at four 2-day intervals and challenge implants were made with ChCMV 10 days later so that challenge inoculations were made 2, 4, 6, and 8 days after inoculation with ChCMV-NS. The results of the second experiment were similar to those of the first, and complete protection was achieved after 8 days (Table 1). There again appeared to be more protection at cooler temperatures. This difference may be due to slower growth of chrysanthemums at 21 C than at 27 C. The effects of plant growth on changes in viroid titer are very difficult to separate, since rate of increase or decrease in infectivity may be intimately associated with plant growth. However, based on symptom severity (brightness of symptoms), more protection was obtained under cooler temperatures, since even after 2 days of ChCMV-NS incubation, symptom severity was reduced in comparison with control plants inoculated first with healthy Deep Ridge tissue and then challenged with ChCMV.

A comparison was made with challenge inoculations made to Deep Ridge plants grown in the greenhouse which had previously been inoculated with ChCMV-NS for 2, 4, 6, and 8 days. No protection against ChCMV was achieved after 8 days' incubation of ChCMV-NS; however, these plants were then trimmed to allow new growth from lateral buds and no symptoms developed in this new growth on those plants inoculated with ChCMV-NS for 8 days. Thus, complete protection was achieved (according to results in the final read-out of symptoms) after 8 days' incubation of ChCMV-NS in the greenhouse. It is not known what caused the delay in protection in the greenhouse, but this does indicate that the interactions between the proposed latent and severe strains of ChCMV can differ greatly under varying conditions. It is quite possible that spontaneous mutant strains of ChCMV may arise and be favored by plant growth under specific environmental conditions as described by Hariharasubramanian et al. (7) for defective tobacco mosaic virus mutants. It is also possible that a mixed population of the proposed latent-to-severe strains of ChCMV, rather than a pure severe strain of ChCMV, was in the inoculum source and that environmental factors such as temperature and light may play an important role in the prevalence of one strain over another at different times of the year.

The latent infectious agent was also detected in commercial chrysanthemum cultivars Albatross, Mefo, and Good News. These cultivars were suspected of carrying ChCMV-NS, since attempts to inoculate and then recover ChCMV infectivity from these cultivars were unsuccessful. Recovery inoculations to Deep Ridge were later challenged with ChCMV and no symptoms were expressed after 30 days. Efforts were also made to induce the prevalence of latent strains in ChCMV-infected Deep Ridge by maintaining plants at constant 23 and 32 C. Inoculations were made from green and chlorotic portions of leaves with mottle symptoms. Plants exhibiting sparse and/or no symptoms were more readily obtained at 32 C. This may also reflect the suscept's ability to support viroid multiplication without symptom development.

TABLE 1. Protection by a latent infectious agent against the chrysanthemum chlorotic mottle viroid

Days between NS-Deep Ridge inoculation and challenge inoculation[a]	Temperature (C)		
	21	24	27
Expt. 1			
2	26[b]	31	41
5	6	14	10
10	0	0	0
14	0	0	0
Expt. 2			
2	32	36	44
4	14	22	27
6	7	16	20
8	0	0	0

[a]Symptomatic chrysanthemum cultivar Deep Ridge (S-Deep Ridge) plants were inoculated with implants from nonsymptomatic (NS-Deep Ridge) plants and were then challenge-inoculated with implants from S-Deep Ridge infected with a severe strain of ChCMV which causes symptoms in that cultivar.

[b]Infectivity index = the summation of the number of plants which had reacted after 30 days.

The occurrence of diverse strains in "conventional" viruses is well established (8). Mild strains of potato spindle tuber viroid have been reported by Fernow (6) but, to my knowledge, latent viroid strains have not heretofore been reported. The results of experiments reported here establish that some infectious agent, which I termed ChCMV-NS, does occur in some sources of the chrysanthemum cultivar Deep Ridge, and that at least 8 days of incubation are required after inoculation with ChCMV-NS for complete protection against ChCMV. ChCMV-NS has also been detected in other chrysanthemum cultivars which likewise exhibited no symptoms in response to its presence. This, of course, does not preclude the possibility that some chrysanthemum cultivars may exhibit symptoms when inoculated with ChCMV-NS. No symptoms are exhibited on cultivars used to test for other viruses which affect chrysanthemums, nor on test plants commonly used to bioassay conventional plant viruses.

The existence of strain(s) of ChCMV is quite important because their presence in assay plants could obviate detection of symptom-inducing strains. Their presence in source plants would normally go undetected. The only method of detection now known is to challenge-inoculate test plants with ChCMV 8 days after inoculation with the test material. Mixed populations of strains (varying from latent to severe) of ChCMV may exist in commercial chrysanthemum cultivars and the relationships and/or interactions of these strains are dependent on the existing environmental conditions.

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