Viruslike Particles Associated with a Rhododendron Necrotic Ringspot Disease

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ABSTRACT

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A disease characterized by concentric necrotic rings on cultivated rhododendron (*Rhododendron* sp.) leaves is described. A survey in Oregon and British Columbia revealed similar symptoms on numerous plants of at least 13 cultivars and seedlings of rhododendron and a *Kalmia latifolia* plant. The causal agent was graft-transmitted to a healthy *K. latifolia* plant, but attempts to transmit it mechanically to 21 herbaceous hosts failed to induce symptoms. Electron microscopy of ultrathin sections and leaf-dip preparations showed that flexuous viruslike rods 460-540 nm long, and about 13 nm wide were associated with infected rhododendron plants. The virus is presumably a member of the potato virus X group. In thin sections of leaves viruslike particles occurred in bundles of four. Although symptoms appeared only on 2-yr-old leaves, the viruslike particles were abundant in current season's growth. We believe this to be the best substantiation to date of the viral etiology of a disease in rhododendron.

A seedling rhododendron (*Rhododendron* sp.) with concentric necrotic ringspot symptoms on the older leaves (Fig. 1-A) was found in Hood River, Oregon in 1968, and was propagated by cuttings in 1973. The original rhododendron and all plants propagated from it have shown similar symptoms each year since. Rings of necrotic tissue appear on 2-yr-old leaves shortly after growth begins in the spring. No symptoms occur on the current season's growth or on the flowers. As the season progresses, the rings typically become more numerous, and the affected leaves often redden and drop prematurely.

There have been few reports of virus diseases of rhododendron. Pape (4) reported a blistering and mottling symptom of rhododendron leaves in Germany which is typical in some virus-caused diseases. White (6) noted that the disease described by Pape was frequently observed in the United States, but that attempts to transmit a causal agent to healthy plants by inarching had failed. The true nature of this condition was unknown.

A concentric necrotic ring pattern in *Kalmia latifolia* L. (Fig. 1-D) was reported from England by Pearse (5) who suggested that it might be the result of virus infection, but transmission tests were not attempted. Rhododendron leaves showing a similar ring pattern were collected in Oregon in 1966 by L. Loring, plant pathologist from the Oregon State Department of Agriculture. Similarly affected plants have been observed by the senior author since that time. This paper reports the results of a limited survey for the disease on rhododendron and the results of transmission studies. It also describes the morphology of disease-associated flexuous rod-like particles found in symptomatic plants.

MATERIALS AND METHODS

Field survey.—To assess the distribution of the disease, we inspected rhododendron cultivars grown in nurseries, home gardens, and in municipal parks in the vicinities of Portland, Oregon, southern Vancouver Island, and the metropolitan area of Vancouver, British Columbia.

Inoculations.—Plants used for mechanical inoculation were grown from seed in a 15-24 C greenhouse with supplemental lighting to give a 16-hr day. Crude sap was used to inoculate the following host plants: *Antirrhinum majus* L., *Chenopodium amaranticolor* Coste et Reyn.,

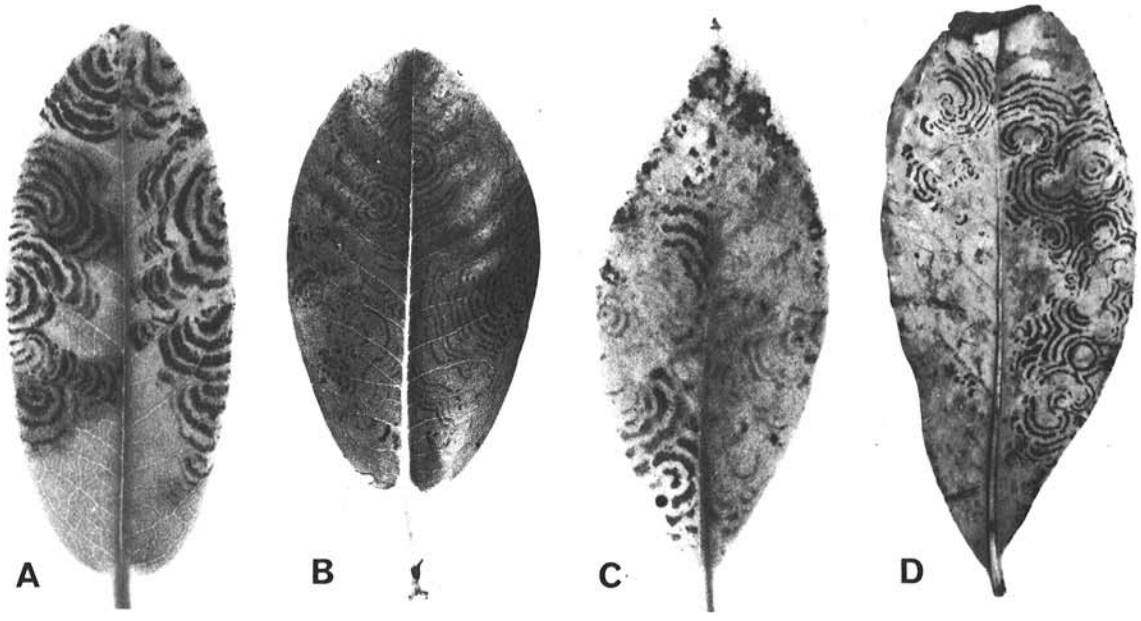

Fig. 1-(A to D). Rhododendron necrotic ringspot symptoms on rhododendron and *Kalmia latifolia* leaves. **A)** Lower leaf surface of rhododendron seedling from Hood River, Oregon. **B)** Upper leaf surface of rhododendron cultivar Unique. **C)** *K. latifolia* collected from a nursery in Portland, Oregon. **D)** *K. latifolia* collected by A. G. E. Pearse in England (photo courtesy A. G. E. Pearse).

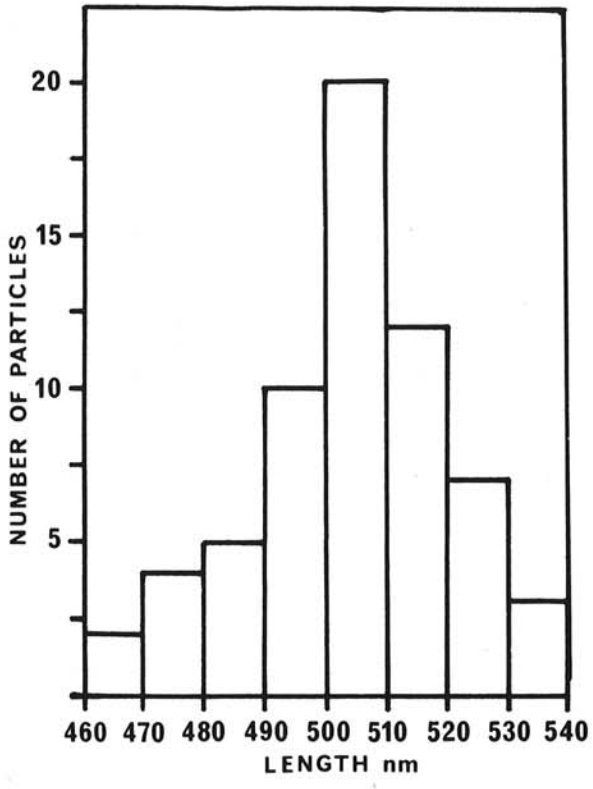

Fig. 2. Size distribution of viruslike particles found in leaf material collected from rhododendron plants which displayed necrotic ringspot symptoms.

C. quinoa Willd., *Cucumis sativus* L., *Datura stramonium* L., *Glycine max* L., *Gomphrena globosa* L., *Lycopersicon esculentum* Mill., *Medicago sativa* L., *Nicotiana clevelandii* Gray, *N. glutinosa* L., *N. tabacum* L., *Petunia hybrida* Vilm., *Phaseolus vulgaris* L., *Phlox drummondii* Hook., *Pisum sativum* L., *Spinacea oleracea* L., *Trifolium pratense* L., *T. repens* L., *Vicia faba* L., and *Vigna unquiculata* (L.) Walp.

Young symptomless leaves, and old leaves with and without symptoms, were homogenized in 2% nicotine buffer to which a small quantity of Celite® had been added. Inoculum was applied to test plants with pipe cleaners; then the inoculated leaves were rinsed with tap water. In a few tests the inoculum leaves were frozen in dry ice, macerated, and immediately applied to the cotyledons of young cucumber plants.

Graft transmission tests included approach grafting, whip grafting, and budding from infected rhododendron plants (cultivar Unique) to apparently healthy rhododendron plants of the following cultivars: Purple Splendor, Jean Marie de Montague, Kluis Sensation, Sappho, Roseum Elegans, and Dora Amateis, and to azalea [*Rhododendron obtusum* (Lindl.) Planch.], kinnikinnick [*Arctostaphylos uva-ursi* (L.) Spreng. 'Oregon hybrid'], and mountain laurel (*K. latifolia* L.).

Electron microscopy.—Most leaf-dip preparations were made by the standard technique of dipping a freshly cut edge of an infected leaf in a solution of 2% phosphotungstic acid (PTA) on a collodion-carbon coated grid. In a modification of this technique, a 2×3 mm piece of leaf was placed in a drop of distilled water, cut several times with a razor blade, and the droplet then transferred to the grid, treated with 2% PTA, allowed to stand 10 minutes, then dried. Photographs were taken on

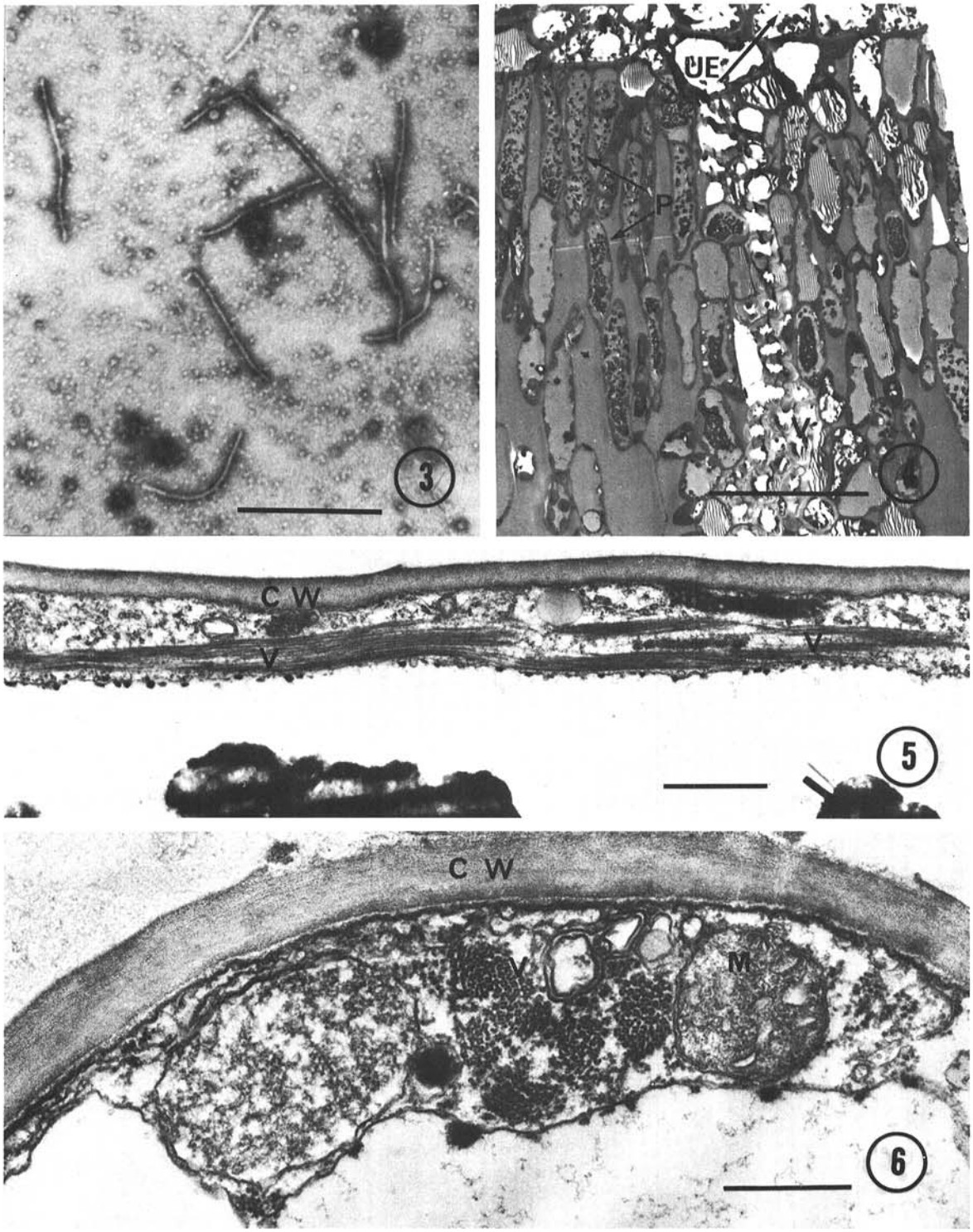

Fig. 3-6. Viruslike particles in leaf dip preparations and ultrathin sections of rhododendron leaf tissue. **3)** Particles in a preparation stained by washing in a solution of 2% phosphotungstic acid. Bar = 500 nm. **4)** Cross section of rhododendron leaf showing upper epidermis (UE), palisade cells (P), and vein (V). Bar = 50,000 nm. **5)** Longitudinal section of palisade cell showing the cell wall (CW) and aggregates of viruslike particles (V) in the cytoplasm. Bar = 1,000 nm. **6)** Transverse section of a palisade cell showing the cell wall (CW), mitochondrion (M) and aggregates of viruslike particles (V) within the cytoplasm. Bar = 500 nm.

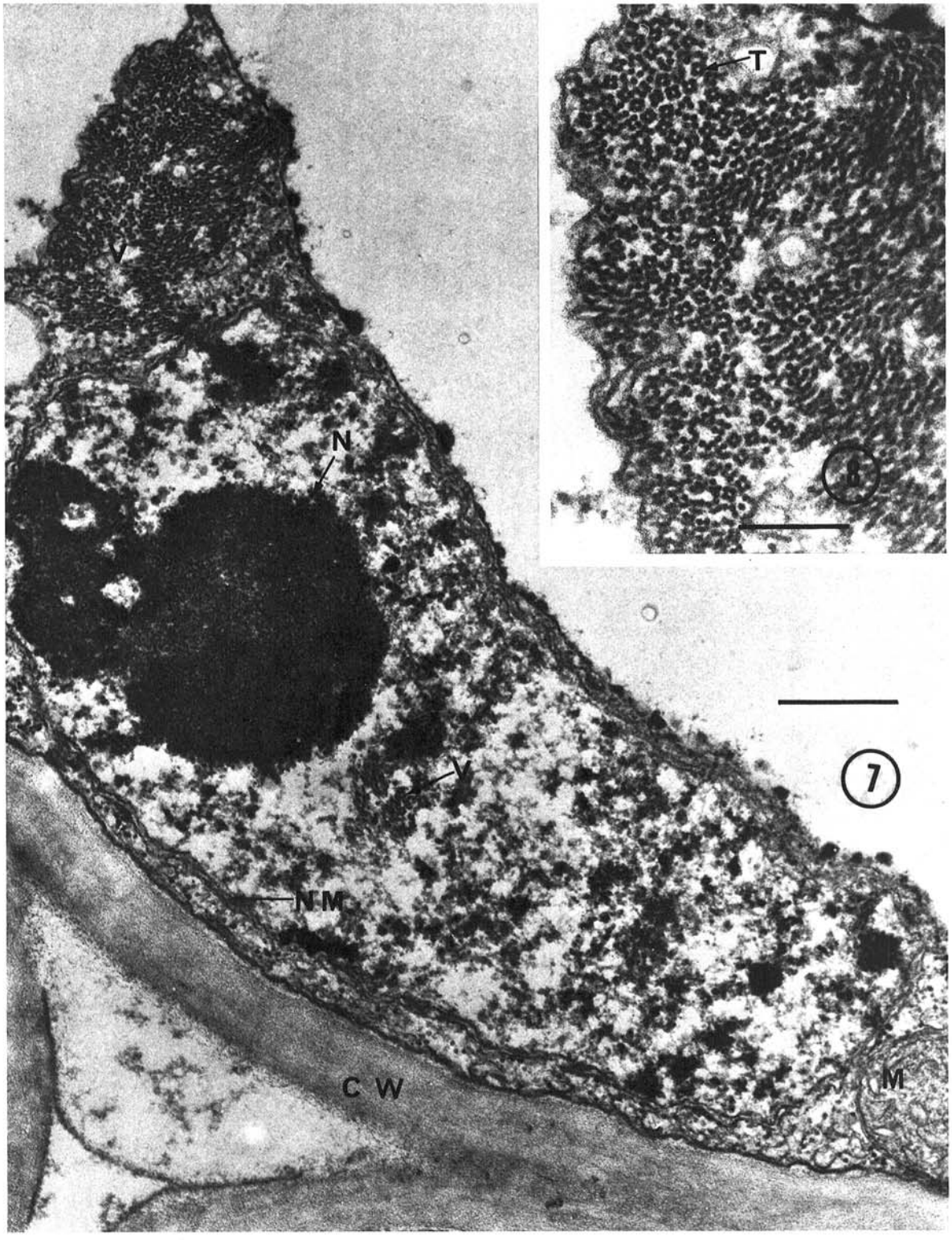

Fig. 7-8. Occurrence and arrangement of viruslike particles within the cytoplasm and nucleus. **7)** Transverse section of a palisade cell showing cell wall (CW), nuclear membrane (NM), mitochondrion (M), a large aggregate of viruslike particles (V) in the cytoplasm, and a small aggregate of particles in the nucleus. Bar = 500 nm. **8)** Enlarged portion of Fig. 7 showing individual particles aggregated side by side in groups of four to form tetrads (T). Bar = 200 nm.

35-mm film in a Phillips EM 300 electron microscope. Viruslike particles were measured from ×7 enlargements of the negatives and were grouped into 10-nm size categories.

Tissue thin-sections for electron microscopy were prepared from leaves with conspicuous symptoms and from bud scales of young symptomless shoot tips. Tissue pieces 1 × 2 mm were fixed for 1 hr at 18 C with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), then rinsed in phosphate buffer and postfixed for 1 hr at 18 C in 1% osmium tetroxide in Palades veronal buffer (pH 7.2). The doubly-fixed tissues were dehydrated by passage through a graded series of ethanol, treated with propylene oxide, infiltrated overnight in a mixture of Epon and propylene oxide, and then embedded in undiluted Epon 812. Ultrathin sections were cut with a diamond knife on a Reichert Ultratome, stained in uranyl acetate and lead citrate and then examined in a Phillips EM 300 electron microscope.

RESULTS

Field distribution.—Typical necrotic ring pattern symptoms were found on rhododendron cultivars Cosmopolitan, Goldfort, Harvest Moon, J. G. Milais, Loderi King George, Loderi Venus, Mrs. Betty Robertson, Mrs. W. C. Slocock, Souvenir of W. C. Slocock, and Unique. We also found these symptoms on two sister seedling selections (Karin and Lynda) in a nursery near Victoria, B. C., and on an unnamed rhododendron seedling in a home garden at Hood River, Oregon. Nearly all plantings of the cultivar Unique that were examined had some plants whose leaves showed the typical ring pattern (Fig. 1-B), but not all were equally severe. Symptom development among plants of this cultivar appeared to be inversely related to the light intensity to which plants were exposed; i.e., the most severe symptoms usually occurred on leaves of plants grown in partial shade. In the Loderi hybrids, however, leaves in full sun were the most severely affected, developing extensive necrosis. In one instance, two plants of the cultivar Unique grown in full sun were nearly symptomless, whereas 48 plants propagated from them and grown in 50% shade all developed severe symptoms the 2nd yr after rooting. In a nursery on Vancouver Island and in one near Portland, Oregon, we found cultivar Unique plants without symptoms.

In an Oregon nursery, in addition to the infected rhododendrons, we found a plant of *K. latifolia* with symptoms identical to those described in England (5). According to the nurseryman, this plant had shown symptoms for several years and had lost much vigor. Plants of *K. latifolia* seed in other Oregon nurseries were symptomless.

Transmission.—All attempts to transmit the causal agent to herbaceous hosts by sap inoculation failed, and only one graft transmission was successful. Establishing bud and graft unions from infected to healthy rhododendron plants was difficult. Infected scions usually died within 2 mo after grafting and necrosis extended to the understock. Even when the stock-scion union occurred, few of the buds grew, and from those few that grew, there was no apparent transmission. The only successful transmission was from rhododendron cultivar Unique approach-grafted to a *K. latifolia* plant. The plant was grafted on 26 June 1975 and developed a necrotic ring pattern on two leaves immediately above the graft about 2 mo later. Viruslike particles were seen in thin sections of young tip leaves of the inoculated *K. latifolia* shoot. Noninoculated *K. latifolia* plants showed no leaf symptoms and contained no viruslike particles.

Electron microscopy.—In an initial examination of leaf-dip preparations, involving more than 20 samples collected from rhododendron plants with typical symptoms, no viruslike particles were detected. Later, when thin sections revealed what appeared to be intracellular virus aggregates, a renewed effort was made to detect particles in leaf-dip preparations. Limited success was achieved by a modification of the standard technique (i.e., cutting a piece of infected tissue several times in a drop of distilled water, then transferring the droplet to a grid and staining). Two grids prepared in this manner from rhododendron leaves showed a scattering of viruslike particles. Areas containing such particles were photographed at a magnification of ×7,605. There were 63 intact, clearly visible particles and all were measured. Most particles were 460-540 nm long; 20 were 500-510 nm (Fig. 2). The modal length was 504 nm, the width was about 13 nm, and the particles were flexuous (Fig. 3), characteristics typical of a virus belonging to the potato virus X (PVX) group. A few long particles indicated limited end-to-end aggregation. No central core was visible in any of the particles.

In subsequent tests, tissue was taken from older leaves with symptoms, from young symptomless leaves, and from leaf bud scales. No more grids with viruslike particles were observed.

Leaves from two symptomless and four rhododendron plants which displayed necrotic ring patterns were examined with the electron microscope. No obvious ultrastructural or cytopathic changes were observed, but aggregates of filamentous particles were detected in the palisade cells of some of the diseased material. Transverse sections of leaf tissue showed that the aggregates were confined to the palisade cells (Fig. 4), occurring in loose bundles parallel to the long wall of the cell. In longitudinal sections through palisade cells, the bundles appeared as long strands of varying thickness in the cytoplasm. The strands frequently overlapped to extend the full length of the cell (Fig. 5). In longitudinal sections of palisade cells the aggregates were not always evident, but they could be detected in virtually every palisade cell that was cut in cross section (Fig. 6). Each cell contained three or four bundles of varying size scattered throughout the cytoplasm. Smaller bundles were occasionally found within the nucleus (Fig. 7). The particles usually aggregated side by side in groups of four. In bundles cut at right angles, these tetrads were clearly visible as a square with a hole in the center (Fig. 8), but when the bundles were cut at a slightly oblique angle, the tetrad structure was more difficult to discern. No aggregates were found in thin sections of leaves from the two healthy plants.

DISCUSSION

Rhododendron necrotic ringspot disease appears to be widely distributed in the Pacific Northwest and may also

be present in England, although we have not confirmed the presence of viruslike particles in the English material. Commercial losses due to the disease are not serious. The symptoms are usually mild on infected rhododendron, consisting of the necrotic ring pattern and a slightly premature leaf drop. In the Loderi rhododendron hybrids, however, associated leaf necrosis often disfigures the plants. In *K. latifolia*, the disease is usually more severe and may cause loss of vigor, but infections in this host are more readily recognized and diseased plants can be rogued out.

Finding typical symptoms in several rhododendron seedlings suggests transmission of the disease by seed or pollen. Low levels of seed transmission have been reported among several viruses of the PVX group (1) and the viruslike particles we found may be seed-transmitted. Aphids frequently colonize the new growth on rhododendron, where the viruslike particles were concentrated, but viruses of the PVX group usually are not aphid-transmitted, and aphid transmission of rhododendron ringspot was not attempted.

An examination of the parentage of the affected cultivars suggests that susceptibility to rhododendron necrotic ringspot disease may be an inherited character. Most of the infected cultivars we found were progeny of Campylocarpon or Griffithianum rhododendrons, although many cultivars of this genetic background were symptomless.

Although we experienced difficulty in finding many particles in leaf-dip preparations, the fact that most intact particles were within the 460- to 540-nm size range is reasonable evidence that the modal length of the viruslike particles is approximately 500 nm. Because the characteristics of the rhododendron necrotic ringspot-associated virus particles most closely parallel those of the potex virus group (2), we believe that it should be tentatively classified accordingly. We are unable to explain why particles are rarely seen in leaf-dip preparations of infected leaf tissue, whereas electron microscopy of comparable tissue reveals numerous bundles of flexuous particles. Possibly the unusual forces that bind the particles together in regular tetrads tend to restrict dispersal of particles from the cytoplasm of cut cells and may explain the failure of sap-transmission. To our knowledge, this in situ grouping of virus particles in tetrads is a phenomenon that has not been observed previously.

Another unusual aspect of the virus is the highly restricted distribution of particle aggregates. Most ultrastructural studies involving viruses with flexuous rods reveal particles in a variety of cells, whereas particles of the rhododendron virus appeared to be restricted to palisade cells. A similar, but less restricted, occurrence of PVX particles was reported by Kozar and Sheludko (3), who found that the virus was most frequently found in the palisade cells of *Datura stramonium* during the early phase of infection.

Rhododendron is an intractable host for the study of virus diseases. Although we have not shown that the viruslike particles associated with rhododendron necrotic ringspot are the cause of this disease, the evidence suggests such a relationship. Further, we believe that these results are the best substantiation to date of the viral etiology of a disease in this genus.

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