Association of Asparagus Virus II with Pollen from Infected Asparagus (*Asparagus officinalis*)

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ABSTRACT

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Asparagus virus II (AV-II), a member of the ilarvirus group, was determined to be readily mechanically transmissible to asparagus using diluted sap prepared from spear tips of AV-II-infected asparagus plants. Infectious virions of AV-II were detected on pollen and in wash solutions of pollen collected from AV-II-infected asparagus plants. Asparagus virus II antigens were localized on the exine of asparagus pollen using enzyme-linked immunosorbent assay and protein A-linked latex antiserum.

Asparagus virus II (AV-II) is a member of the ilarvirus (isometric labile ringspot virus) group and is widespread in asparagus (*Asparagus officinalis* L.) plantings in Washington (13) and California (6) and has been detected at low levels in asparagus in New Jersey (4). The virus has been detected widely within most commercial asparagus plantings in Michigan, and 50–70% of asparagus plants are infected with AV-II in most plantings 5 yr or older (11).

The route of transmission of AV-II within asparagus plantings has not been well studied. Some ilarviruses are known to be seed-transmitted, one is pollen-transmitted, and most are readily mechanically transmissible to a relatively wide range of herbaceous hosts (15). AV-II has been mechanically transmitted to asparagus by some researchers (7) but not by others (15), and the transmission of AV-II in asparagus seed has been well documented (7,13,15). Most researchers agree that asparagus is a symptomless host of AV-II (4–6,11–13,15). One researcher, however, indicated that some asparagus seedlings showed a faint mosaic pattern after 20 days on shoots that had been inoculated with partially purified preparations of AV-II (7). The research reported here was undertaken to verify the mechanical transmissibility of AV-II in asparagus and to attempt to localize AV-II on or within asparagus pollen.

MATERIALS AND METHODS

Mechanical transmission of AV-II to asparagus. A Michigan isolate of AV-II was purified from systemically infected leaves of *Chenopodium quinoa* Willd. using the method of Brunt and Stace-Smith (1) and further purified using three cycles of linear-log sucrose density gradient centrifugation (11). An extinction coefficient $E(mg/ml)/1cm$ (260 nm) of 5.3 was used to estimate the concentration of the purified AV-II (15). Partially purified AV-II (1 mg/ml) in 0.01 M sodium phosphate buffer, pH 7.0, was used to inoculate asparagus seedlings determined to be free from AV-II by rub-inoculation to *C. quinoa* (13). Two-month-old virus-free asparagus seedlings were maintained in the dark for 24 hr, then dusted with Carborundum (320 mesh); stems were rub-inoculated with purified virus or buffer using sterile cotton swabs. Plants were evaluated for the development of faint mosaic symptoms over an 8-wk period, after which young spears were harvested and tested for latent infection with AV-II by rub-inoculation to *C. quinoa*.

Mechanical transmission from asparagus to asparagus was assessed in a similar manner. Asparagus seedlings were first screened for AV-II by rub-inoculation to *C. quinoa*. Young spears from AV-II-infected and virus-free asparagus plants were triturated at 4 C and diluted 1:10 (w/v) in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol. These diluted sap preparations were used to rub-inoculate virus-free asparagus seedlings either directly on the stem, as previously described, or on the wounded spear tips immediately after hand-snapping.

Collection of asparagus pollen. During the winter of 1985, flowers were collected from greenhouse-grown male asparagus seedlings previously indexed for the presence of AV-II by indirect enzyme-linked immunosorbent assay (ELISA) and rub-inoculation to *C. quinoa*. Anthers were first examined under a dissection microscope, and mature anthers were manually removed with fine forceps before dehiscence. The anthers were dried at room temperature for 48 hr and stored at 4 C. Pollen samples for individual experiments were prepared using the method of Cole et al (3). Dried anthers were swirled in a dry glass tube using a vortex tube agitator, and pollen grains that adhered to the tube walls were collected. No evidence of damage was noted when suspensions of pollen were examined by light microscopy. Pollen samples were usually processed within 1 wk of their collection.

Test for the presence of infective virus in pollen. Pollen samples (0.05 ml dry-packed volume) were tested for the presence of infective virus by suspending samples in 5 ml of extraction buffer, 0.01 M sodium phosphate, pH 7.0, or in the same buffer containing 1% polyvinyl-pyrrolidone (PVP) (w/v) mol wt = 40,000 (Sigma Chemical Co., St. Louis, MO 63178), then shaking the tubes with a vortex tube agitator at maximum speed for 1 min (10). No evidence of pollen damage was observed when suspensions were viewed under the light microscope. Pollen grains were sedimented at 6,000 g for 5 min, and a 2-ml sample of the supernatant was withdrawn with a sterile pipette, examined by light microscopy, and found to be pollen-free. Samples were rub-inoculated on single leaves of two *C. quinoa* seedlings, which were observed for 5–14 days for symptom development.

Identification of AV-II. The identification of AV-II in pollen samples, in wash solutions, or in ruptured pollen that had been extensively washed was carried out by indirect ELISA using the methods of Voller and Bidwell (16) as modified by

Hamilton et al (10). Pollen samples were divided into equal test samples with a dry-packed volume of 0.05 ml and suspended in 0.05 ml of extraction buffer, 0.01 M sodium phosphate, pH 7.0, as previously described. One-half of the pollen sample suspended in extraction buffer was added to duplicate wells of flat-bottomed polystyrene Microtiter plates (Dynatech Laboratories, Chantilly, VA 20201) and incubated overnight at 4 C. Plates were washed three times with phosphate-buffered saline with 0.05% Tween 20 (v/v) (PBS-Tween).

To test for the presence of AV-II antigen on or in asparagus pollen, pollen was suspended in extraction buffer as previously described and incubated for 60 min at room temperature, then centrifuged as previously described; a 200-μl sample of pollen wash solution was withdrawn and used as antigen in similar indirect ELISA tests. Pollen samples were washed two more times in this manner and resuspended in extraction buffer. No pollen damage or tapetal fragments were evident when suspensions were examined under the light microscope. Washed pollen samples were divided in half, and intact pollen, with whatever AV-II virions might be attached to the their surfaces, were used as antigen in indirect ELISA. The remaining half of the sample was ground manually in a glass tissue homogenizer until all pollen grains were ruptured, and the homogenate was tested for internal and surface virions, as previously described (3).

Immunoglobulin specific for the Michigan isolate of AV-II was purified using the procedure of Clark and Adams (2) and diluted 1:50 (v/v) with PBS-Tween; 200 μl was then added to each well and incubated for 2 hr at room temperature. Plates were washed as before, and 200 μl of alkaline phosphatase conjugated with goat γ-globulin prepared against whole-molecule rabbit γ-globulin (Sigma Chemical Co.), diluted 1:1,000 (v/v) in PBS-Tween, was added. After a 3-hr incubation at 37 C, plates were again washed and 200 μl of enzyme substrate solution (p-nitrophenylphosphate [1 mg/ml] dissolved in 10% diethanolamine [v/v], pH 9.8, in distilled water with 0.02% sodium azide [w/v]) was added. Color change after 30–60 min was determined spectrophotometrically at A_{405nm} with a microELISA Minireader II (Dynatech Laboratories). The threshold value used for a positive reaction for each plate was three times the mean A_{405nm} value of the healthy control (two replicate wells per plate).

Detection of AV-II antigen on the surface of asparagus pollen. Protein A-linked latex antisera (PALLAS) was used to determine if AV-II antigen was present on the surface of asparagus pollen, using the methods of Querfurth and Paul (14) and Hamilton et al (10). A standard suspension of Bacto latex beads

(0.81 μm, Difco Laboratories, Detroit, MI 48232) was first diluted with 14 volumes of 0.9% NaCl (w/v) solution, then mixed 1:1 (v/v) with a diluted protein A solution (Sigma Chemical Co.) composed of 0.1 mg of protein A dissolved in 2.0 ml of 0.1 M glycine buffer, pH 8.2. This solution was incubated for 2–4 hr at room temperature with occasional stirring, then allowed to stand overnight at 4 C. The protein A-latex bead complexes were washed by centrifuging at 6,000 g for 30 min, then resuspended in 0.1 M glycine buffer, pH 8.2, with 0.02% PVP (w/v) added. The washing step was repeated two more times, and the final pellet was resuspended in 2 ml of 0.1 M glycine buffer, pH 8.2, containing 0.05% sodium azide (w/v).

Purified immunoglobulin specific for the Michigan isolate of AV-II was conjugated with the protein A-latex bead complex. The globulin suspension (100 μg/ml in 0.1 M glycine buffer, pH 8.2) was mixed 1:1 with the protein A-latex bead complex, allowed to incubate for 2–4 hr at room temperature, and washed as previously described.

Pollen samples were dissolved in 0.1 M glycine buffer, pH 8.2, mixed 1:1 (v/v) with the protein A-latex bead complex suspension, and allowed to incubate for 1

hr at room temperature with occasional shaking. The pollen and protein A-latex antibody complex was centrifuged at 6,000 g for 5 min, and the supernatant was drawn off with a pipette. Pellets were resuspended in 0.1 M glycine buffer, pH 8.2, and the wash step was repeated four times. The final pellet of the pollen and protein A-latex antibody complex was resuspended in a small volume of glycine buffer. The solution was pipetted onto the surface of aluminium specimen supports (15 mm diameter) previously coated with adhesive and allowed to settle for 10 min. Excess fluid was drained by touching the edge of the drop with a piece of filter paper, and the pollen was allowed to air-dry overnight at room temperature. The pollen samples were gold-coated and examined with a Super-Mini scanning electron microscope (International Scientific Instruments, Tokyo, Japan) at a magnification of ×2,500. To further verify that protein A-latex bead antibody complexes were binding specifically to AV-II antigen, a blocking experiment was carried out. Pollen samples were pretreated with a purified anti-AV-II immunoglobulin suspension (0.5 mg/ml in 0.1 M glycine buffer, pH 8.2) or buffer alone for 1 hr at room temperature with gentle agitation.

Table 1. Mechanical transmission of asparagus virus II (AV-II) in *Asparagus officinalis* L. using sap or concentrated, purified virus preparations

Inoculum	Site of rub-inoculation	No. of plants infected[a]/ no. of plants tested	Percent infected
Purified[b] virus from *Chenopodium quinoa*	Stem	1/30	3.3
Diluted sap[c]			
AV-II-infected asparagus	Stem	16/20	80
	Wounded stem tip	15/20	75
Virus-free asparagus	Stem	0/20	0
	Wounded stem tip	0/20	0

[a] Plants were tested for AV-II before and 2 mo after virus inoculation by rub-inoculation to *C. quinoa* leaves.
[b] Concentrated, purified AV-II (1 mg/ml) in 0.01 M sodium phosphate buffer, pH 7.0.
[c] Spear tips from AV-II-infected plants were triturated in 0.01 M sodium phosphate buffer, pH 7.0.

Table 2. Detection of asparagus virus II (AV-II) antigens on asparagus pollen, in pollen washes, and within pollen by enzyme-linked immunosorbent assay

Sample	Virus content[a]	A_{405nm} values[b]			
		Pollen	Wash[c]	Washed pollen	Ruptured pollen[d]
1	AV-II	0.08[e]	0.08	0.12	0.08
2	AV-II	0.20	1.60	0.09	0.05
3	AV-II	0.14	1.88	0.09	0.07
4	AV-II	0.09	0.09	0.09	0.08
5	None	0.01	0.00	0.03	0.04
6	None	0.00	0.02	0.03	0.03
7	None	0.01	0.01	0.01	0.03
8	None	0.00	0.02	0.04	0.02

[a] Plants were previously indexed for presence of AV-II by rub-inoculation to *Chenopodium quinoa*.
[b] Values are means of duplicate wells.
[c] Pollen was washed in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% polyvinylpyrrolidone for 60 min, then centrifuged at 6,000 g for 5 min.
[d] Washed pollen was ruptured by grinding in a glass tissue homogenizer.
[e] The threshold value for virus detection was three times the mean of the healthy.

Samples were then washed three times as previously described and tested for the presence of surface antigen using PALLAS.

RESULTS

Mechanical transmission of AV-II to asparagus. Only one of 30 asparagus seedlings inoculated with concentrated (1 mg/ml) purified AV-II, prepared from systemically infected *C. quinoa*, became infected (Table 1). Most virus particles from these preparations appeared partially disrupted when observed in the electron microscope and were only mildly infectious when rub-inoculated onto leaves of *C. quinoa* plants. Manual inoculation of healthy asparagus seedlings with sap prepared from spears of AV-II-infected asparagus plants was an effective means of transmitting the virus, with 75–80% of the test plants becoming infected (Table 1). Rub-inoculation of sap to stems or to wounded stem tips was equally effective. All AV-II-infected asparagus plants were without apparent symptoms.

Association of AV-II with asparagus pollen. Pollen samples collected from asparagus plants known to be infected with AV-II were determined to contain infective virus. In a preliminary study, pollen and pollen wash solutions from two samples of AV-II-infected asparagus induced chlorotic local lesions in *C. quinoa* followed by systemic mottling, typical of infection with AV-II. Single lesions from these plants were transferred, sequentially, three times on *C. quinoa*, and the symptoms induced were identical to those of AV-II. AV-II antigen was determined by indirect ELISA to be present on pollen and in pollen wash solutions of pollen samples from two AV-II-infected plants. No virus was detected using indirect ELISA on pollen collected from virus-free asparagus seedlings.

In a second study, AV-II antigen was determined by indirect ELISA to be present on pollen and in pollen wash solutions from AV-II-infected asparagus (Table 2). Pollen samples 2 and 3, collected from AV-II-infected asparagus, had mean A_{405nm} values of 0.20 and 0.14, respectively, and pollen wash solutions from both samples had mean A_{405nm} values of 1.60 and 1.88, respectively. Stringent washing of pollen samples collected from AV-II-infected asparagus plants did not remove all AV-II antigen from these pollen samples. Finally, washed pollen that had been ruptured with a glass tissue homogenizer was determined to contain no more AV-II antigen than washed pollen.

Detection of AV-II antigen in the exine of asparagus pollen. AV-II antigen was localized in the exine of hand-collected asparagus pollen by PALLAS. Scanning electron microscopy revealed many more latex particles bound to the exine of

pollen collected from AV-II-infected asparagus treated with anti-AV-II-protein A-latex conjugate than to the same pollen blocked by pretreatment with anti-AV-II γ-globulin or pollen collected from virus-free asparagus (Fig. 1). An average of 11.6 latex particles per pollen face were bound to pollen collected from AV-II-infected asparagus, whereas only 4.2 and 1.6 particles per face were observed on pollen collected from virus-free asparagus or blocked pollen, respectively (Table 3).

DISCUSSION

The experimental data presented here demonstrate that AV-II is readily transmissible from asparagus plant to asparagus plant by mechanical means via diluted sap (Table 1). Concentrated, purified AV-II prepared from *C. quinoa* was largely ineffective as an inoculum source. Purified virus particles were badly disrupted when observed in the electron microscope, and the inability of these preparations to infect asparagus plants is probably due to the partial degradation or inactivation of virus particles during sucrose density gradient centrifugation.

AV-II was determined to be an external contaminant of some pollen grains collected from virus-infected asparagus plants. Our data suggest that large numbers of virus particles may be sequestered within the exine of asparagus pollen grains where they cannot be readily detected. Some virus particles may be more tightly bound to the pollen wall than others, and stringent washing may release most but not all particles to the surrounding environment. At least one member of the ilarvirus group has been shown to be pollen-transmitted (8). Prunus necrotic ringspot virus (PNRSV) is pollen-borne in cherry and infects trees when they are pollinated by virus-contaminated pollen (8). Virus particles have been localized on the outer walls of some pollen grains. Hamilton et al (10), using ELISA, demonstrated the presence of PNRSV antigens on the exine of bee- and hand-collected sweet cherry pollen. When virus-containing pollen was washed, antigen was easily released and induced chlorotic local lesions when rub-inoculated onto *C. amaranticolor* Coste & Reyn. and *Cucumis sativus* L. 'Straight Eight.' Scanning electron microscopic observations of pollen treated with latex conjugated antibody specific for PNRSV revealed more latex beads bound to the surface of virus-infested than of virus-free pollen (10).

It has been suggested that pollen surfaces contaminated with infectious plant viruses may serve as vehicles by which certain mechanically transmissible viruses may spread within some plant species (9). This route of virus spread would, by necessity, be more common and efficient in open-pollinated than in

self-pollinated plants and could play a role in the seed transmission of some plant viruses. Asparagus is an open-pollinated plant, and it is possible that plants could become infected with AV-II by mechanical means via windblown or bee-carried pollen infested with the virus.
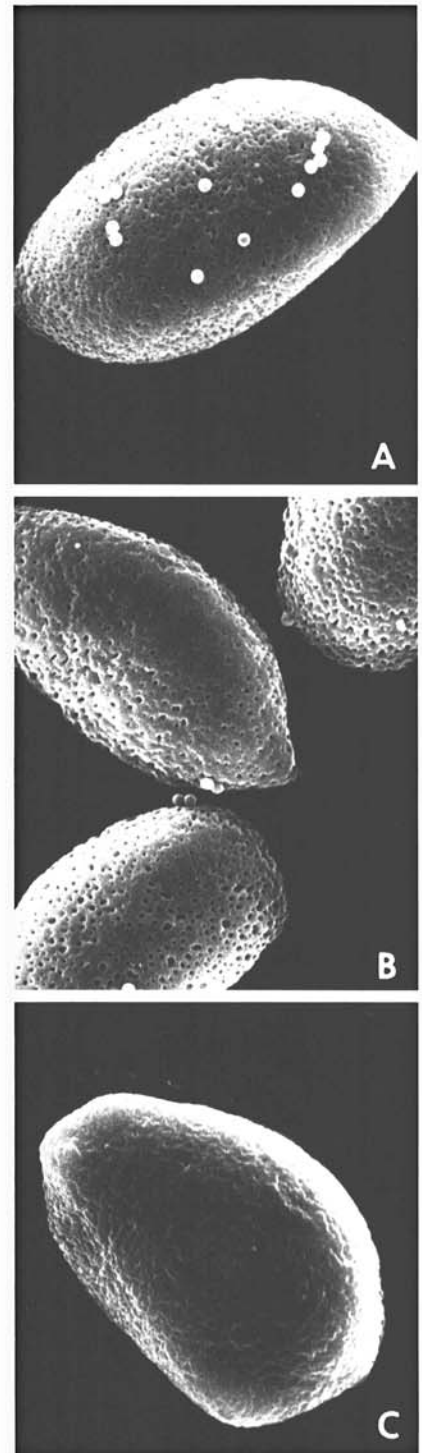

Fig. 1. Scanning electron micrographs of hand-collected asparagus pollen after exposure to latex spheres (0.81 μm diameter) conjugated with asparagus virus II (AV-II) antibodies ($\times$ 2,500). Pollen from **(A)** AV-II-infected asparagus, **(B)** virus-free asparagus, and **(C)** AV-II-infected asparagus blocked with antiserum specific for AV-II.

Table 3. Localization of asparagus virus II (AV-II) antigen in the exine of hand-collected asparagus pollen using protein A-linked latex antiserum

Virus content of pollen[a]	No. of pollen grains examined[b]	No. of latex beads per pollen face
AV-II	77	11.6 ± 3.3[c]
None	82	4.2 ± 1.4
AV-II, blocked[d]	50	1.6 ± 1.4

[a] Determined by enzyme-linked immunosorbent assay (ELISA) and rub-inoculation to *Chenopodium quinoa.*

[b] Samples were gold-coated, then observed on a scanning electron microscope at ×2,500.

[c] Values indicate the mean for a minimum of 50 pollen faces plus or minus the standard deviation.

[d] Pollen grains determined by indirect ELISA to contain AV-II antigen on or in the exine wall were pretreated with purified AV-II-specific γ-globulin, then treated with protein A-γ-globulin-conjugated latex beads.

Honeybees have been observed routinely visiting asparagus flowers, especially in seed production blocks (T. Evans, *unpublished*). Conceivably, bees could mechanically inoculate plant surfaces or floral parts during their visitations. This could result in the transmission of the virus to male plants or female plants and possibly to developing seed (9). Experimental infection of asparagus by AV-II-contaminated pollen has not yet been demonstrated. The increasing use of new all-male asparagus hybrids, however, could provide a larger reservoir of AV-II-contaminated pollen within asparagus-growing areas, resulting in an increase in the spread of AV-II. Therefore, the role of pollen in the field transmission of AV-II needs to be assessed.

In Washington and California, knives are usually used to sever asparagus spears at harvest, and it is possible that AV-II could also be spread by the knives. The hand-snapping of asparagus spears in Michigan probably provides a less efficient means of mechanical transmission of AV-II. In many asparagus-growing regions of the world, the foliage or fern is cut in late fall. This cultural practice could also be a means by which AV-II is spread within and between asparagus plantings. Contaminated blades could mechanically transmit AV-II if asparagus ferns are not allowed to completely senesce before cutting. Falloon et al (6) suggested that the spread of AV-II within one California seed production block was the result of cutting green fern stalks with a machete or a tractor-mounted slasher, both methods providing a means of mechanical transmission of AV-II. Direct evidence for mechanical transmission of AV-II in the field in asparagus is still lacking.

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