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Effect of Fucoidan on Potato Virus X Accumulation and Ultrastructure of Mesophyll Cells of *Datura stramonium* L.

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Abstract—The accumulation of potato virus X (PVX) in mesophyll cells of detached *Datura stramonium* L. leaves treated with fucoidan from brown algae *Fucus evanescens* C. Ag. have been evaluated by electron microscopy during the early infection period (three days after infection). It was found that cells of the leaves treated with fucoidan 24 h before infection accumulated virus less than untreated control. Ultrastructure-morphometric assay showed that fucoidan treatment causes an increase in the protein-synthesizing capability of cells (nucleolus dimention and amount of both mitochondria and rough endoplasmic reticulum membranes increased). At the same time, fucoidan treatment slightly activates the lytic compartment, which leads to the destruction of virus particles. Therefore, it may be considered that fucoidan induces a cellular defense mechanism that limits virus accumulation. Stimulation by the fucoidan of the formation of PVX-specific laminar structures able to bind virus particles may be another antiviral cell defense mechanism induced by the virus that prevents PVX reproduction and its intra- and intercellular transport.

Key words: *Datura stramonium, Fucus evanescens.* fucoidan, potato virus X, mesophyll cells, infection.

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Abbreviations: TMV, tobacco mosaic virus; PVX, potato virus X; LS, laminar structures; ER, endoplasmic reticulum.

Much efforts has bee put into the search for ecologically safe, biologically active compounds capable of stimulating plant resistance to pathogens, including viruses (Lyon et al., 1995; Reunov, 1999). These compounds include various polysaccharides, including 1.3;1.6-β-D-glucans from fungi (Kopp et al., 1989; Rouhier et al., 1995) and brown algae (Elakova et al., 1994; 2007; Reunov et al., 1996, 200); 1.3;1.4-D-glucan from lichen *Cetraria islandica* (Stübler and Buchenauer, 1996); chitosan (Pospieszny et al., 1991; Chirkov, 2002); oligosaccharides derived from xyloglucan (Šubíková et al., 1994) and galactoglucomannan (Šlováková et al., 2000); κ/β-carragenan from red algae *Tichocarpus crinitus* (Reunov et al., 2004; Barabanova et al., 2006).

Recently, it was reported that sulfated polysaccharide fucoidan inhibits infection caused in host plants by the tobacco mosaic virus (TMV) (Lapshina et al., 2006, 2007). Fucoidan derived from brown algae *Fucus evanescens* suppresses the accumulation of TMV in cells of tobacco leaves. Here, we presented the results of an ultrastructural morphometric examination of the fucoidan effect on the development of infection induced by potato virus X (PVX) in leaves of *Datura stramonium* L.

MATERIALS AND METHODS

Experiments were conducted on leaves of 4-week-old plants of *Datura stramonium* L. grown in a greenhouse. Fucoidan was isolated from brown algae *Fucus evanescens* C. Ag. at the Laboratory of Enzyme Chemistry, Pacific Institute of Bioorganic Chemistry, Far-East Branch of the Russian Academy of Sciences. The compound is 1.3;1.4-α-L fucan sulfated mostly at the C-2 position of fucose residues (Kusaikin et al., 2004). A severe strain of PVX (Reifman and Kolesnikova, 1973) purified by the method described (Otsuki et al., 1974) was used for infection.

Detached young *D. stramonium* leaves (4–5 cm in length) were cut along the middle vein and dusted with Carborundum. The left halves of leaves (experimental) were rubbed with 1 mg/ml fucoidan, while the right halves (control) were treated with water. After 10 min, the leaves were washed with water and placed into a humid chamber. After 24 h, the left and right halves of the leaves were dusted again with Carborundum. Then, half of the leaves were rubbed with PVX suspension (2 mg/ml), while the other half was treated with water, after which they were washed and placed into the humid chamber. After 3 days, small pieces of healthy and infected tissue from leaf halves were fixed for 3 h in 6.5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.4), then for 2 h in 1% osmium tetraxide. The samples were dehydrated in a graded alcohol and acetone series and embedded in Araldite. The sections

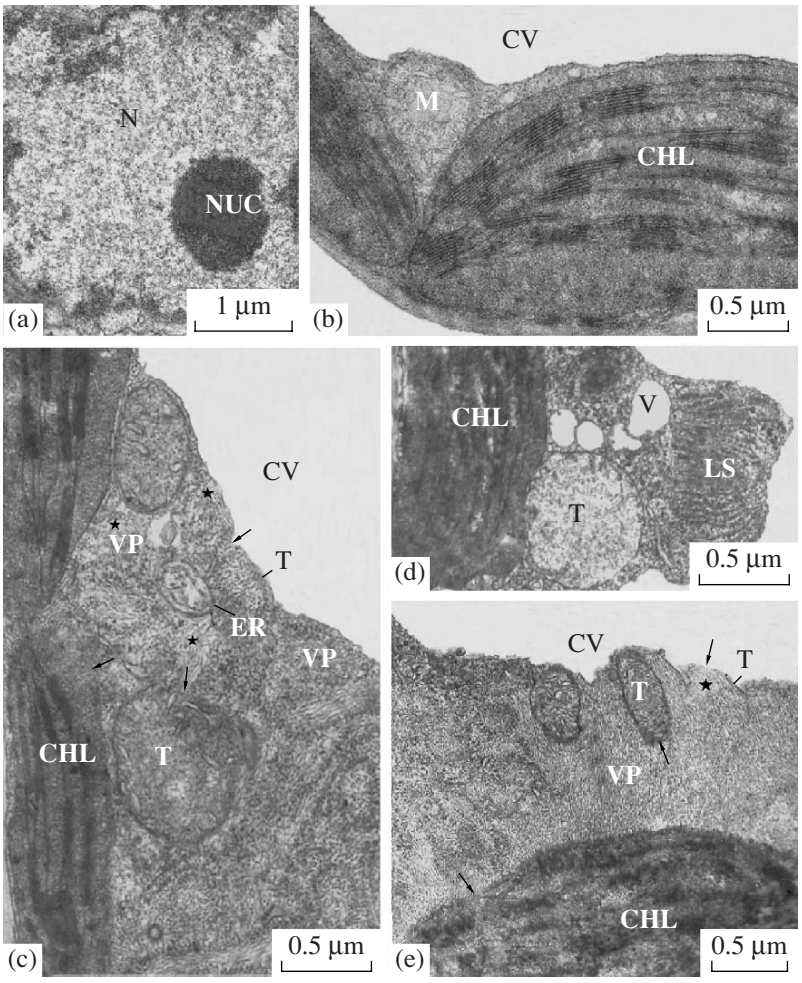

Fig. 1. Healthy (a, b) and PVX-infected (c–e) cells of palisade parenchyma in *Datura stramonium* leaves untreated (a, b, e) and treated (c, d) with fucoidan. Arrows points to disruptions in chloroplast and mitochondrion envelopes; asterisks points tonoplast damages. (c, e) electron light areas in cytoplasm. V, vacuole; VP, virus particles; LS, laminar structures; M, mitochondria; CHL, chloroplast; CV, central vacuole; ER, endopalsmic reticulum; N, nucleus; NUC, nucleolus.

obtained with ultratome LKB-III (LKB, Sweden) were contrasted with uranyl acetate and lead citrate and examined with an JEM-7A electron microscopy (JEOL, Japan). Morphometric analysis was performed on parenchyma palisade cells as described by Kiseleva et al. (1974). Three blocks were used for each variant. The sections were mounted on slot grids coated with formvar film stabilized with carbon. Twenty cells were assayed on each blend, i.e., 60 cells were examined in each variant. Intracellular PVX accumulation was assayed by counting the number of profiles of virus particles per unit of the area of the cell section (Reunov and Lapshina, 1983). Statistical treatment of the results obtained was performed according to the routine technique using Student's *t*-test (Lakin, 1973). Confidence probability was 0.95.

RESULTS

An ultrastructural study of palisade parenchyma of uninfected leaves untreated with fucoidan and incubated for 4 h in a humid chamber showed that main organelles usually retain intact morphology. Cell nuclei have light nucleoplasms with fibrillar-granular material and condensed chromatin mostly located at the cell periphery; nucleoli are of moderate density and size (Fig. 1a). Chloroplasts have fairly well-developed photosynthetic membranes (Fig. 1b). Mitochondria contain a relatively small number of cristae submerged in the light matrix (Fig. 1b). The endoplasmic reticulum (ER) and Golgi apparatus are weakly developed.

Leaf treatments with fucoidan did not notably affect the structure of cellular organelles. However, some morphometric cellular parameters increased, including the nucleolar volume density; the number and volume density of mitochondria, microbodies with crystalloids, and vacuoles; and the surface area of rough and smooth ER membranes and dictyosomes (table).

Three days after infection, abnormal modifications of chloroplasts and mitochondria were observed in both fucoidan-treated and untreated leaves. The most obvi-

Effect of fucoidan on morphometric parameters of healthy and PVX-infected cells of palisade parenchyma in *Datura stramonium* leaves

Parameter[a]	Halves of healthy leaves[b]		Halves of infected leaves[c]	
	untreated	treated with fucoidan	untreated	treated with fucoidan
Volume nucleolar density, % to nucleus volume	0.12 ± 0.01	0.17 ± 0.02	0.19 ± 0.02	0.26 ± 0.03
Volume density of mitochondria, % to cell volume	2.2 ± 0.20	3.1 ± 0.30	3.4 ± 0.40	4.6 ± 0.50
Number of mitochondria in 100 μm^3	2.8 ± 0.30	4.3 ± 0.40	4.6 ± 0.50	7.1 ± 0.70
Surface area (μm^2) of rough ER membranes in 1 μm^3	0.29 ± 0.03	0.40 ± 0.04	0.7 ± 0.07	1.2 ± 0.20
Surface area (μm^2) of smooth ER membranes in 1 μm^3	0.23 ± 0.03	0.31 ± 0.03	0.44 ± 0.04	0.66 ± 0.06
Volume density of microbodies with crystalloids, % to cell volume	0.21 ± 0.03	0.28 ± 0.03	0.32 ± 0.03	0.51 ± 0.06
Number of microbodies with crystalloids in 100 μm^3	0.3 ± 0.04	0.51 ± 0.05	0.56 ± 0.06	0.73 ± 0.08
Surface area (μm^2) of dictyosomes in 1 μm^3	0.12 ± 0.01	0.18 ± 0.02	0.31 ± 0.03	0.42 ± 0.05
Volume density of vacuoles, % to cell volume	1.6 ± 0.20	2.8 ± 0.30	4.1 ± 0.50	5.3 ± 0.50
Number of vacuoles in 100 μm^3	4.5 ± 0.50	6.0 ± 0.60	6.4 ± 0.70	7.9 ± 0.80
Number of virus particle profiles in 1 μm^2 of cell section	–	–	67.5 ± 7.0	43.3 ± 5.0
Volume density of LS, % to cell volume	–	–	4.6 ± 0.50	7.4 ± 0.80

Note: [a] Each value is the average of 60 cells. The standard errors were calculated by using the Student's *t*-test, $P = 95\%$. Experimental leaf halves treated with fucoidan and control ones treated with water were placed in a humid camera. After 24 h, the leaf halves were divided into two groups. One group was rubbed with water [b] and the other was rubbed with PVX[c]. Then, all leaf halves were placed in a humid camera.

ous disorders in chloroplasts caused by the virus were altered thylakoid and grana structure (Figs. 1c–1e). Local damage tot he chloroplast envelope was frequently apparent (Figs. 1c, 1e). Mitochondria lost cristae and matrix were visible as vacuole-like structures (Fig. 1d). Occasionally, mitochondria also had damaged envelopes (Figs.1c, 1e, 2a). Usually, mitochondria remain intact in infected cells and frequently had more cristae than healthy cells, which is an indication (Zavarzin and Charazova, 1982) of their high functional activity. The largest amounts of extended cristae were observed in mitochondria in leaf cells treated with fucoidan (Fig. 2a).

Unlike chloroplasts and mitochondria, other cellular organelles did not undergo notable degenerative changes; their morphological features show that their activity is higher than in uninfected cells. The most active organelles were found in infected cells of fucoidan-treated leaves. In the nuclei of these cells, nucleoli were the largest and had a significant granular component (Fig. 2b). The amounts of dictyosomes (Fig. 2c), as well as membranes of rough and smooth ER, were notably increased (Figs. 2c, 2d). The formation of smooth ER elements usually appeared to be swollen (Figs. 2c, 2d). Ringlike reticulum profiles were frequently observed (Fig. 1c, 2e). A characteristic of the infected cells, particularly for those treated with fucoidan, was the formation of cytoplasmic vacuoles (Fig. 1d) and microbodies with crystalloids (Fig. 2f), as well as round, osmiophilic bodies (spherosomes) (Fig. 2g). It should be noted that, in palisade cells of healthy leaves not treated with fucoidan, no spherosomes were observed; these structures were only visualized (though not frequently) in cells of healthy leaves treated with the polysaccharide.

Morphometric analysis showed that the nucleolar volume density; amount and volume density of mitochondria, microbodies with crystalloids, and vacuoles; and the surface area of rough and smooth ER membranes and dictyosomes in infected cells treated with fucoidan were larger than in other cells (table).

Large accumulations of PVX in the cytoplasm were frequently seen in leaf cells not treated with fucoidan (Fig. 1e). In cells treated with fucoidan 24 h before infection, the accumulation of viral particles was encountered less frequently. In the cytoplasm of many infected cells both treated (Fig. 2h) and untreated (Fig. 2i) with fucoidan, we observed relatively small virus aggregates associated with so-called laminar structures (LSs); PVX-specific inclusions were described by many workers (see review Reunov, 1999). The examination of serial sections (Shalla and Shepard, 1972) showed that LSs are membrane-like structures (3–7 nm thick) frequently associated with granular particles that have diameters of less than 80S-ribosomes. These structures contain protein that has no antigen similarity with PVX and, therefore, is not a viral protein (Shalla and Shepard, 1972). Morphometric analysis revealed that infected cells treated with fucoidan had a 1.56-times-smaller amount of PVX particle profiles per unit of cellular section area (1 μm^2) and a 1.6-times-

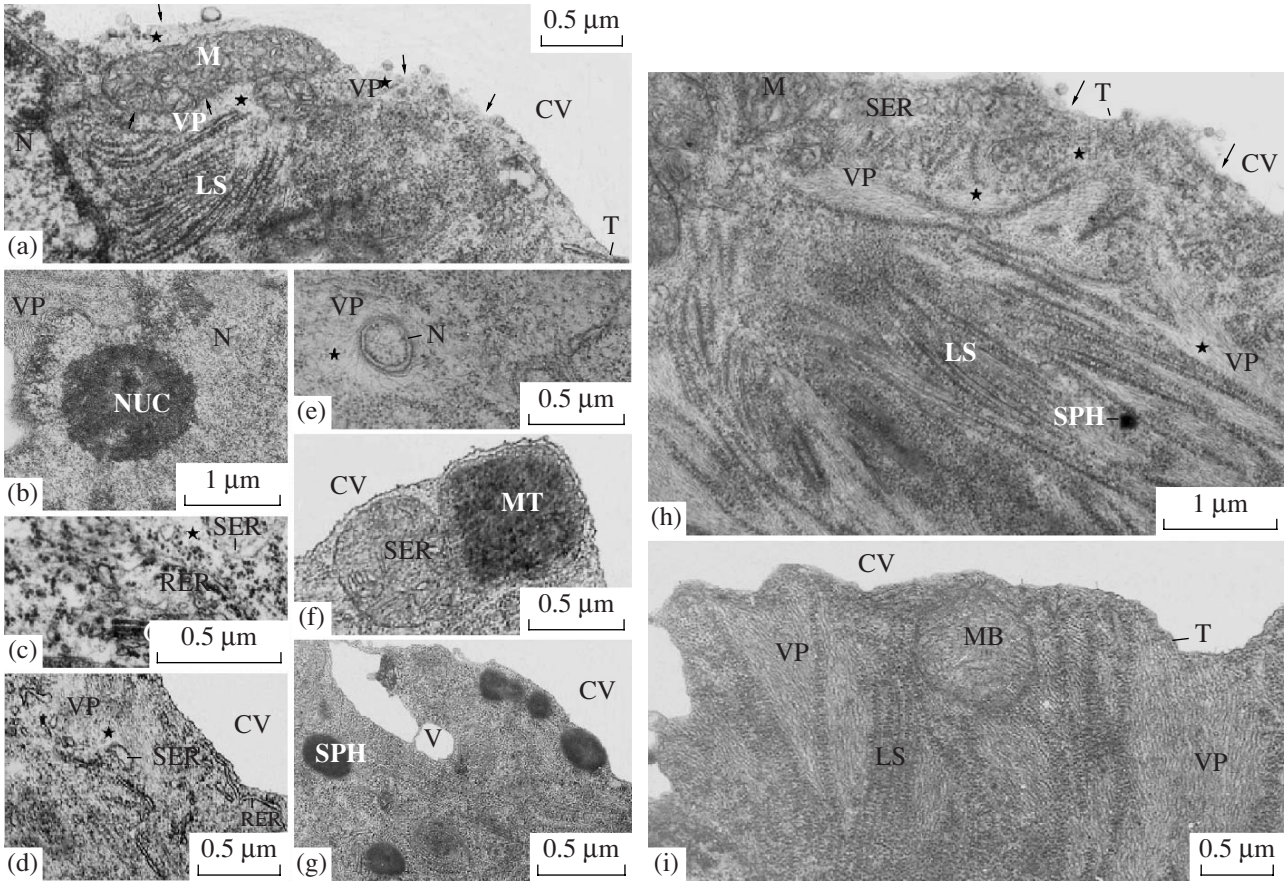

Fig. 2. Ultrastructure of PVX-infected cells of palisade parenchyma in *Datura stramonium* leaves treated (a–h) and untreated (i) with fucoidan. Arrows point to damaged mitochondrion envelope (a) and to cytoplasm areas close to the central vacuole without tonoplast (a, h); asterisks show (a, c–e, h) electron light cytoplasm areas; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; MB, microbody; SPH, spherosome; T, tonoplast. Other designations are the same as in Fig. 1.

greater volume density of LS in infected cells of untreated leaves (table).

It should be noted that the cytoplasm of infected cells, especially after treatment with fucoidan, often had electron light zones (Figs. 1c, 1e, 2a, 2c–2e, 2h). In similar zones close to the border with the central vacuole, the tonoplast may be damaged (Figs. 1c, 1e, 2a, 2h) and even not seen (Figs. 2a, 2h). Virus particles localized in electron light areas of the cytoplasm were usually weakly contrasted and thin in appearance (Figs. 1c, 2a, 2d, 2e, 2h).

DISCUSSION

The results showed that fucoidan isolated from algae *F. evanescens* inhibits PVX accumulation in mesophyll cells of detached leaves of *D. stramonium* L. during the early infection period.

Ultrastructure-morphometric assay showed that fucoidan treatment causes the activation of nucleoli (increased their size and the amount of granular component), mitochondria (increased their number and the amount of widened cristae), and the proliferation of rough ER. According to the current concepts (Andreeva, 1985; Kulaeva, 1985; Mashanski and Rabinovich, 1987), these structural features of cells treated with fucoidan attest to the stimulation of a protein-synthesizing cell apparatus that leads to a considerable increase in the cell's ability to generate defensive responses to pathogen invasion (Reunov, 1999).

However, fucoidan also activates the lytic compartment, which is expressed, in particular, in the stimulation of the formation of dictyosomes, smooth ER elements, and cytoplasmic vacuoles, which are components of this compartment (Matile, 1975; Belitser, 1978). Ringlike ER profiles are presumably sections of forming cytosegresomes (Belitser, 1978). According to some evidence (Matile, 1975; Reunov, 1999; Rinne et al., 2001), lytic compartments of plants may also include sherosomes, the number of which increases in infected cells, especially after fucoidan treatment.

The swelling of smooth ER cisternae probably mirrors abnormal alterations of reticulum membranes and, therefore, the disturbance of their barrier properties. It

may be accompanied by the leakage of hydrolases from reticulum cavities into the cytoplasm (Belitser, 1978). The detection of acid phosphatase in widened ER lumens, membranes, and adjoined cytoplasm areas in leaf cells of *D. stramonium* infected with PVX (Reunov and Lapshina, 1985) confirms this representation.

Hydrolases released from ER cisternae seem to induce the development of lytic processes, resulting in the degradation of the tonoplast and organelles and the appearance of electron light zones in the cytoplasm. Virus particles observed in these zones (especially in infected cells of leaves treated with fucoidan 24 h before infection) were faintly contrasted and thin in appearance. As was found previously (Reunov, 1989; Reunov et al., 2006), this is due to virion destructive changes produced by hydrolases.

It is interesting to note that the data on the fucoidan effect in plant cells show its resemblance to phytohormones. It is known that cytokinins activate protein synthesis in leaves by increasing the size of the protein-synthesizing apparatus (Kulaeva, 1973). Kinetin increased the nucleolar size and amount of granular component in nucleoli, as well as the number of mitochondria in TMV-infected cells of tobacco leaves (Reunov et al., 1989). The number of mitochondria increased in cells of potato leaves treated with gibberellin prior to PVX infection (Zherebchuk, 1984). However, there is evidence (Kulaeva, 1973) of the activation of various hydrolytic enzymes in plant cells treated with phytohormones (kinetin, indolyl acetic acid, gibberellin). It was noted that ER proliferation preceded the activation of hydrolase by gibberellin (Kulaeva, 1973). Potato treatment with gibberellin before PVX infection stimulates RNAase, which inhibits virus replication through the destruction of viral RNA (Zherebchuk and Olevinskaya, 1972).

The fucoidan-mediated activation of the lytic compartment, which causes destruction of PVX particles, can probably be considered to be a compound-induced cellular defense mechanism that limits virus accumulation. Another antiviral cellular mechanism realized through fucoidan action is, possibly, stimulation of PVX-specific LS. It was demonstrated that there is a reverse dependence between intensity of PVX replication and LS formation (Reunov, 1989). In addition, it was found that LS can bind PVX particles and, thus, prevent their transposition and reproduction (Lapshina and Reunov, 1997).

Microbodies with crystalloids formed in cells may play an adaptive role, in particular in the inhibition of the accumulation of hydrogen peroxide (Belitser, 1978). Therefore, the stimulation of their formation by fucoidan may prevent membrane structures from lipid peroxidation. This results in the inhibition of the development of intracellular destructive processes.

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