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Morphology and Intracellular Appearance of *Euonymus* Vein Clear Virus.

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사철나무 葉脈 바이러스의 形狀과 細胞內出現

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ABSTRACT

Negatively stained dip preparations from *Euonymus* showing vein clear symptoms revealed bacilliform particles. The particles tentatively referred to as the *Euonymus* vein clear virus(EVCV) have a relatively complex structure, measuring 230-280nm in length and 70-80nm in diameter. They have an envelope, 8-10nm thick, provided with evenly spaced beadlike projection about 5-6nm long. The inner tubular core which had no envelope showed helical structures, 200-220nm long, and 50-55nm in diameter. This inner tubular core is interpreted as the virus nucleocapsid. A striking association of virus particles with the nuclei of infected cells was apparent from sections which showed numberous virus particles at the nuclear periphery and in what appeared to be intranuclear virus particle inclusions. Careful examination of these apparent inclusions revealed the presence of the nuclear envelope surrounding them, in addition to cytoplasmic organelles within them. Such profiles were interpreted as having arisen when the sections passed through invaginations of the cytoplasm into the nucleus. In all the sections showing virus particles associated with the nucleus, large number of virus particles were found to be present in expanded areas between the two lamellae of the nuclear envelope. This location is suggested as a possible site of virus assembly. Serveal micrographs of particles found in this location suggested incorporation of the inner lamella of the nuclear envelope into the viral envelope. Various micrographs indicated a possible helical arrangement of certain components present in the virus core.

Key words: Euonymus vein clear virus, morphology, election microscope.

要 約

Dip-method에 依한 電子顯微鏡的 觀察의 結果, 사철나무 罹病葉에서 길이 230－280nm, 폭 70-80nm의 短桿形(一端이 둥근 彈丸形 : bullef-shaped, 兩端이 둥근 桿菌形 : bacilliform)의 바이러스 粒子가 多數 檢出되었다. 이 바이러스 粒子를 사철나무 葉脈黃化 바이러스(*Euonymus* vein clear virus : EVCV)로 命名하였다.

EVCV는 被膜(두께 8-10nm)을 갖고 있고, 이 被膜의 表面에는 5-6nm의 염주모양의 돌기가 있다. 핵단백(nucleocapsid)은 길이 200-220nm, 폭 50～55nm의 나선구조를 갖고 있다. 바이러스 粒子는 切片試料의 各種細胞의 細胞質內 또는 核內와 核膜間隙內에 散在 혹은 集團으로 관찰되었다. 이는 바이러스 粒子의 封入體는 核膜으로 둘러싸여 있었다. 대부분의 切片像에서는 바이러스 粒子의 集團이 核膜의 內膜과 外膜 사이의 間隙에서 관찰되었는 데, 이것은 바이러스 粒子의 成熟場을 示唆하고 있는 것으로 생각된다. 어떤 切片像에서는 바이러스 粒子의 被膜이 核膜의 內膜과 연결되어 있는 것도 관찰되어, 이 被膜이 核膜의 內膜으로부터 由來된 것으로 示唆되었다.

INTRODUCTION

A virus, designated *Euonymus* mosaic virus, was first described from *Euonymus* by Doi(4) in Japan. Doi detected a bacilliform virus, 70 x 230nm, in thin sections of *Euonymus* sp..

A virus capable of inducing vein clear on *Euonymus* leaves was recently isolated in our laboratory during one of the routine tests of recovery of plant viruses. The Korean virus has been tentatively referred to as the *Euonymus* vein clear virus(EVCV) until definite evidence of its relationship to the Japan virus is obtained.

The present paper describe the morphology of these particles in negatively stained preparations, and the fine structure and intracellular localization of EVCV inside host cells.

Cytopathic effects of the virus and a possible mechanism for its synthesis are also described.

MATERIALS AND METHODS

Host plants. Healthy plants and diseased plants which showed leaf symptoms of vein clearing and vein mosaic were collected from 5 different places in the southern part of Korea.

Dip-negative staining method: Dip-negative stained preparations were obtained by the direct negative staining method (DN-method, 5) or a modification of DN-method. The procedure was as follows; a small piece of an *Euonymus* leaf was cut several times with a razor blade in a drop of 2% osmium tetroxide on a clean slide glass.

A carbon-coated grid was touched to the surface of the drop and was air-dried for a few seconds. The grid was then dipped in a drop of 2% PTA, air-dried, and examined in an electron microscope.

Thin-sectioning. Small pieces of leaves from healthy or vein clearing plants were fixed in 5% glutaraldehyde in 0.1M phosphate buffer containing 0.25M sucrose at pH 7.0 for 3 hr, and post fixed in 2% osmium tetroxide in the same buffer at pH 7.0 for 1 hr before being dehydrated through a graded series of ethanol and embedded in Epon-812 (13). Sections were cut on a Porter-Blum model MT-2 ultramicrotome with glass knives, and stained with uranyl acetate and lead citrate. The specimens were examined under a Hitachi H-600 electron microscope.

RESULTS

Negatively stained preparations. Bacilliform particles, although the majority of particles were bullet-shaped, were easily detected in dip-preparations from diseased plants, whereas the same particles were not detected in those healthy plants (Fig. 1). The particles were relatively uniform in size, measuring 230-280nm long and 70-80nm in diameter. They will be referred to here-after as either EVCV particles or virus particles.

When the staining materials did not penetrate into the virus particles they appeared uniformly dense, were uniform in size, and showed no internal structures (Fig. 1-F). However, very often the particles were penetrated by the staining materials, probably because of disruption of their envelope, and then internal structure or inner tubular core was revealed (Fig. 1-A and D). EVCV particles showed an envelope (8-10nm thickness) with bead-like projections of 5-6nm long (Fig. 1-D, E and F). The inner tubular core which had no envelope showed helical structures with a pitch of about 5nm (Fig. 1-B and C). It measured 200-220nm in length and 50-55nm in diameter.

Its axial channel was about 30-35nm wide, and it showed about 90-100 alternate light and dark cross bands. The extremities of the inner core were rounded

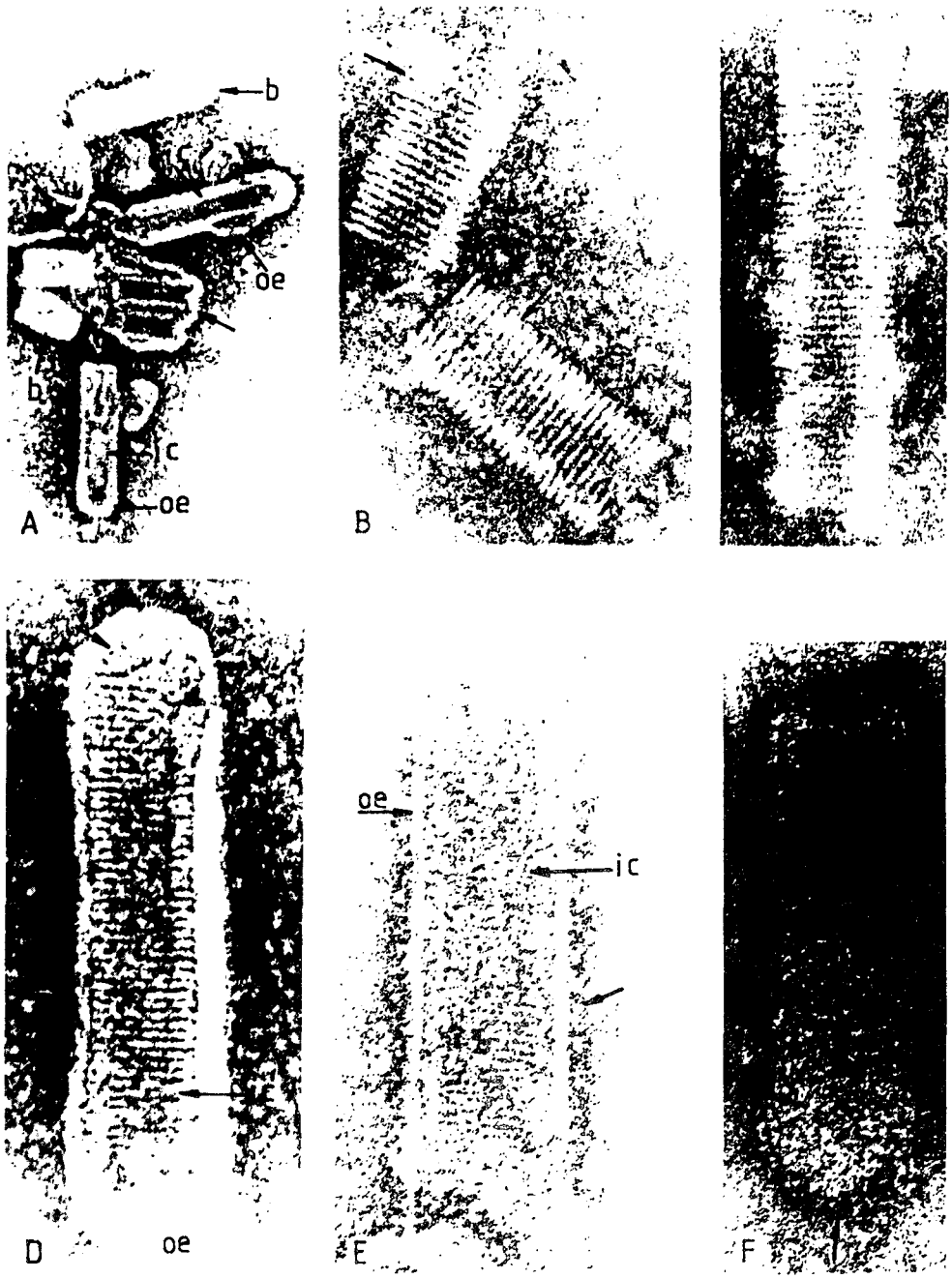


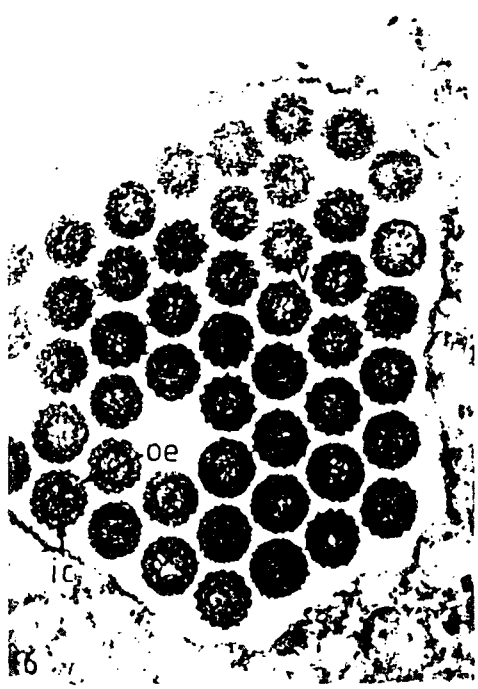
Fig. 1. Virus particles in negatively stained preparations from infected *Euonymus* leaf. A. A group of EVCV particles showing various shapes and size. (X 100,000) Arrow shows two inner cores enveloped by a common outer coat. B. and C. Three inner tubuler core particles, of variable length, lacking the viral envelope. Note cross striations(arrows). (X 355,000) D. A complete bacilliform particle of EVCV showing strong penetration by negative stain. Note the rounded extremity (arrow). (X 355,000) E and F. Bullet-shaped particles which have lost one of their ends. Note the beadlike projections clearly delineated at the periphery(arrow). (X 360,000) KEY FOR LABELING: ac, axial channel; b, bullet-shaped particle; c, cytoplasm; ch, chloroplast; cw, cell wall; er, endoplasmic reticulum; g, golgi complex; ic, inner core; il, inner lamella; m, mitochondrion; n, nucleus; ne, nuclear envelope; np, nuclear pore; nu, nucleolus; oe, outer envelope; ol, outer lamella; r, ribosome; se, sieve tube; t, tonoplast; v, virus particle; vac, vacuole.

or flattened (Fig. 1-D and F). On rounded ends the bands were disposed almost radially (Fig. 1-D arrow). Some particles, especially those of bullet-shape, were occasionally penetrated only partially by the staining materials, from the flat end, so that axial cavity about 20nm wide and about a half of the particle length were

Fig. 2. Spongy parenchyma cell of *Euonymus* infected with EVCV. Note the virus particles within the perinuclear spaces and also the virus containing cytoplasmic vesicles (X 18,000).

Fig. 3. A high magnification of the inset in Fig. 2. Note the formation of the virus-containing vesicles from the outer lamella of the nuclear envelope (X 50,000).

Fig. 4. A highly magnified transverse and longitudinal section of virus particles in cytoplasmic vesicle. Note slightly thicker inner core and the axial channel(arrows) (X 120,000). For abbreviation See Fig. 1.



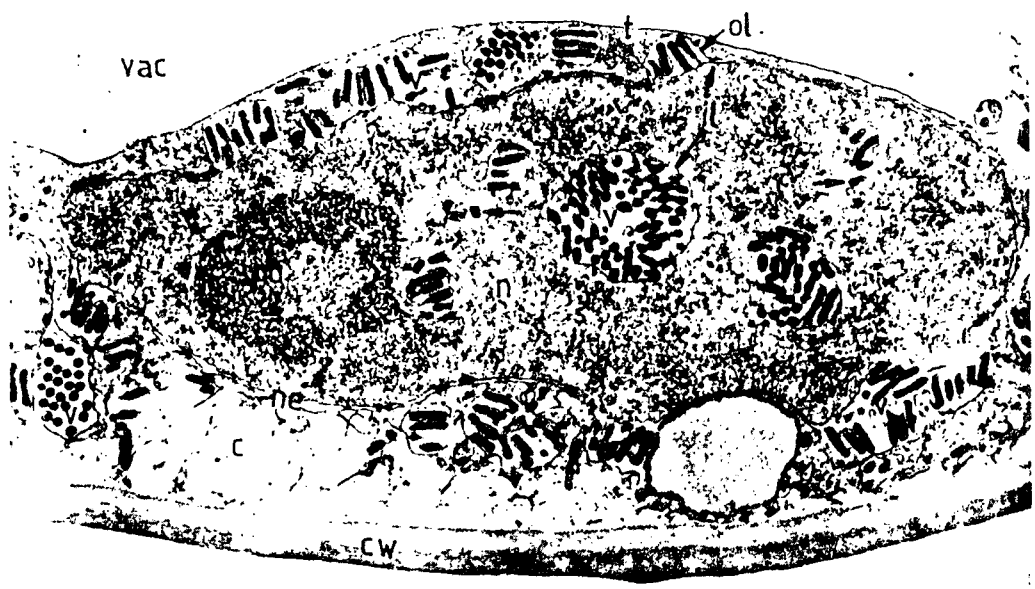


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Fig. 5. Transverse and longitudinal section through an ordered array of inner tubular core particles(v). Note the presence of the axial core(arrows) (X 58,000).

Fig. 6. A highly magnified transverse section of the virus particles in cytoplasmic vesicle. Note side-by-side arrangements of the virus particles (X 150,000).

Fig. 7. Palisade parenchyma cell *Euonymus* in fected with EVCV. Note the virus particles within a nucleus, the perinuclear spaces, and also the containing cytoplasmic vesicles. Some particles are apparently free in the nucleoplasm(arrows) (X 24,000). For abbreviations see Fig. 1.

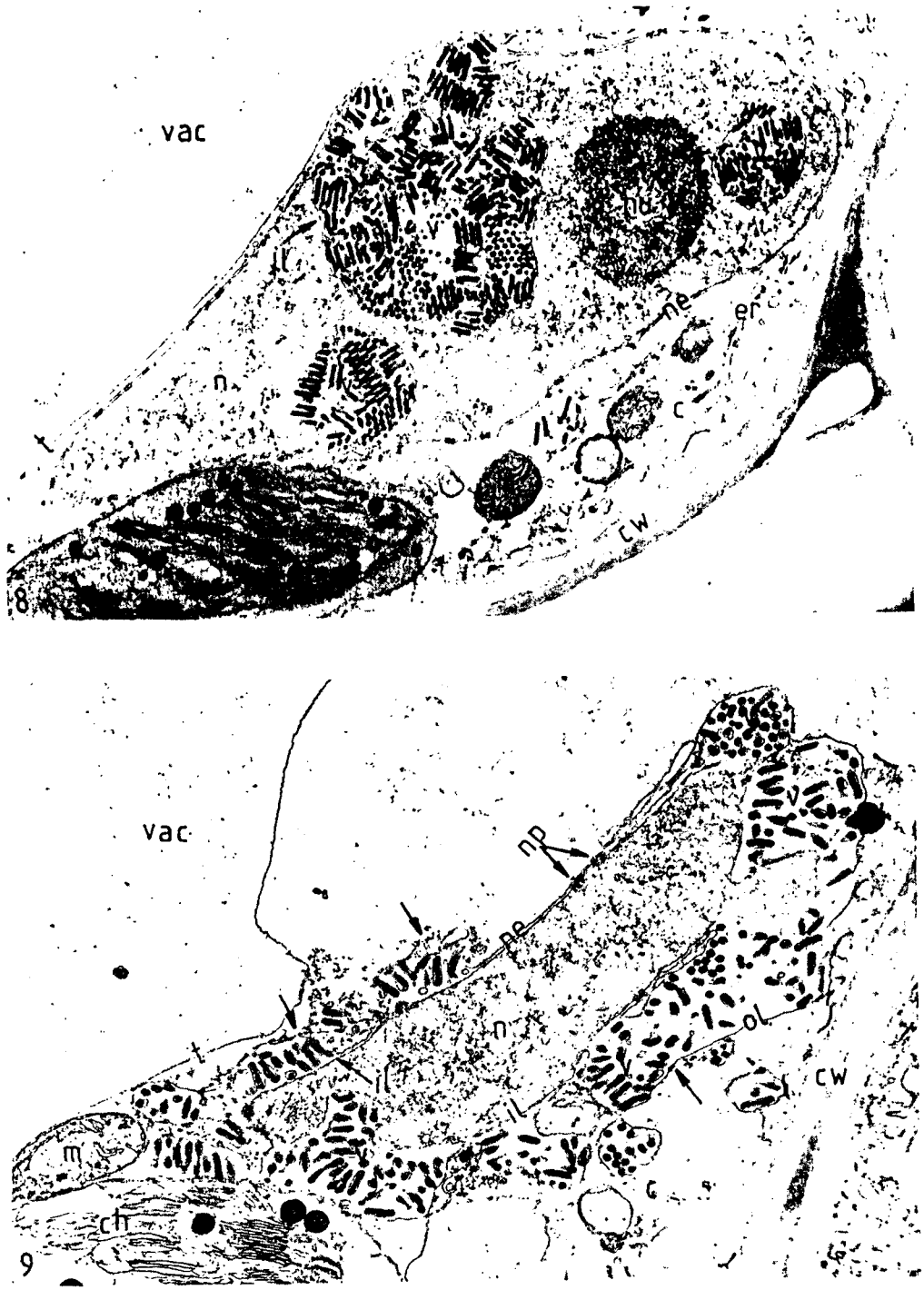


Fig. 8. A large number of bacilliform virus particles in the perinuclear spaces of a spongy parenchyma cell of *Euonymus* infected with EVCV (X 18,000).

Fig. 9. Palisade parenchtma cell of *Euonymus* infected with EVCV. Groups of bacilliform particles are found within the perinuclear spaces and cytoplasmic vesicles. This section showed possible origin of the virus-containing cytoplasmic vesicles. Arrows indicate ribosomes attached to the outer lamella (X 60,000). For abbreviations See Fig. 1.

revealed (Fig. 1-A). Occasionally, two or more inner tubular cores were found inside an envelope (Fig. 1-A arrow). Apparently free inner tubular cores, of variable length, were observed (Fig. 1-B).

Electron microscopy of thin sections from infected tissues. In thin sections of EVCV-infected leaf tissues a large number of virus particles were seen both in the nuclei and in the cytoplasm of infected cells (Fig. 2, 7, 8, 9 and 16). Fig. 3, 4, 5, 6, 10, 11 and 12 show the particles in longitudinal and transverse sections respectively.

The virus particles were bacilliform, being uniform in size and shape. The particles were morphologically similar to those detected in negatively stained preparations, although their dimensions were slightly smaller. In longitudinal views, the bacilliform of bullet-shaped particles were 200-250nm long, 60-70nm in diameter (Fig. 4, 6, 10 and 11). Most of the variations seen in the diameter and length of the particles were attributed to different planes of sectioning through bacilliform particles (Fig. 3 and 4). When the particles were regularly arranged in a crystalline array, center-to-center distance was about 70nm (Fig. 4 and 11). The envelope was easily discernible, but internal structures, appearing uniformly dense, were less conspicuous (Fig. 3, 4, 10 and 11). Particles sectioned in certain planes, however showed patterns which suggested an internal structures (Fig. 12 arrow). In transverse sections, the virus particles had a circular profile (Fig. 4, 6 and 12), 60-65nm in diameter, with an outer dense layer of 8nm thickness.

All the thin sections of the diseased tissues examined showed aggregates of particles at the periphery of nuclei of infected cells and occasionally in what appeared to be inclusions deep within the nucleoplasm (Fig. 2, 7, 8 and 9). The presence of a mitochondrion and cytoplasmic ribosomes within these inclusions, however, made it apparent that the section had passed through invaginations of the cytoplasm into the nucleus. Furthermore, the nuclear membrane was seen to surround them (Fig. 2, 3 and 8). Thus, it was assumed that although the virus appeared to be deep within the nucleoplasm, it was, in fact, located on the nuclear periphery or free in the cytoplasm (Fig. 7, 8 and 9).

Fig. 10. A high magnification of the perinuclear space shown in Fig. 9. Note ribosomes attached to the outer lamella(arrows) (X 60,000).

Fig. 11. A high magnification of the cytoplasmic vesicle. Bacilliform virus particles are regularly arranged in a crystalline array and being uniform in size and shape (X 60,000).

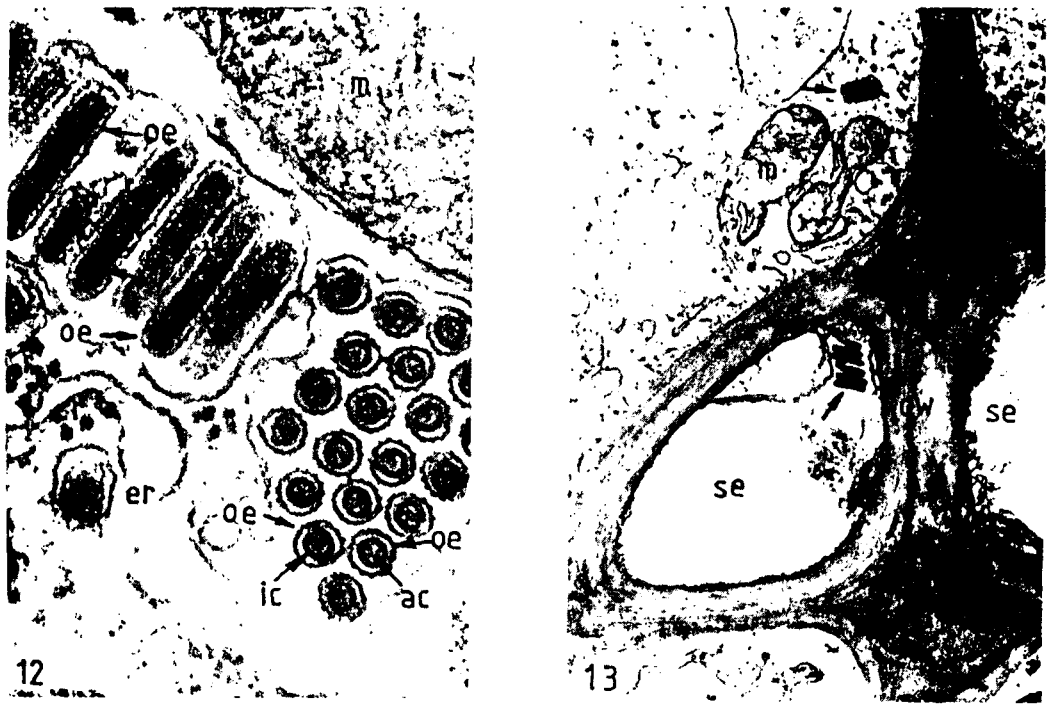


Fig. 12. A highly magnified transverse and longtudinal section of virus particles in cytoplasmic vesicles. Note virus particles in various planes with arrows indicating a possible helical arrangement of materials present in the virus core (X 120,000).

Fig. 13. Part of the vascular region of *Euonymus* infected with EVCV. Virus particles are found in the lumen of a sieve tube and in the cytoplasm of parenchyma (arrows) (X 22,500). For abbreviations See Fig. 1.

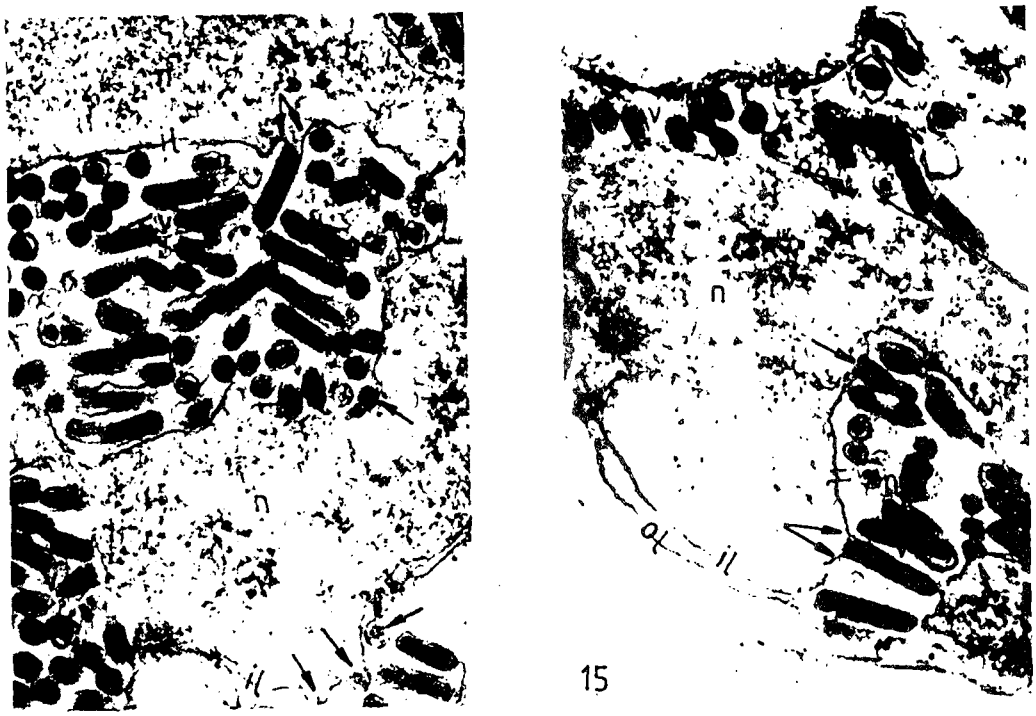
Virus particles were seen in large numbers in expanded spaces between the lamellae of the nuclear envelope. In Fig. 2, 3, 7, 8 and 9 small groups of virus particles are present under expanded areas of the outer lamella of the nuclear envelope, and a single large group in a deep invagination of the inner lamella. Groups of particles similarly arranged were frequently seen at the periphery of the nuclei of most of the infected cells examined.

Virus particles seen in the cytoplasm were invariably found in groups enclosed within a single membrane to which ribosomes were frequently attached (Fig. 3, 9, 10, 11 and 12). Moreover, many profiles were seen which suggested that these cytoplasmic vesicles were derived from the outer lamella of the nuclear envelope (Fig. 3, 7 and 9).

Intermediary stages of virus particle maturation through a budding process toward the perinuclear space, at the inner lamella of the nuclear envelope, were observed (Fig. 14 and 15).

During this process the tubular particles present in the nucleus acquired the envelope, which apparently is derived from the inner lamella of the nuclear envelope, and concomitantly they passed from the nuclear side to the perinuclear space. Thus the tubular particles found in the nucleus and the inner core of the virus particles might be considered as identical. The budding process seemed to occur with the tubular particle attached parallel to the inner lamella of the nuclear envelope, and being gradually surrounded by it. This produced two different images in sections, one showing the tubular particles sectioned longitudinally and the other transversally (Fig. 14 and 15 arrows).

Virus particles were also observed in sieve tubes of infected plants (Fig. 13). The infected cells commonly showed signs of degeneration such as a low concentration of ribosomes in the ground matrix of the cytoplasm; a small amount of endoplasmic reticulum, which when present was usually represented by small vesicles; plastids showing disorganized lamellae, amoe-



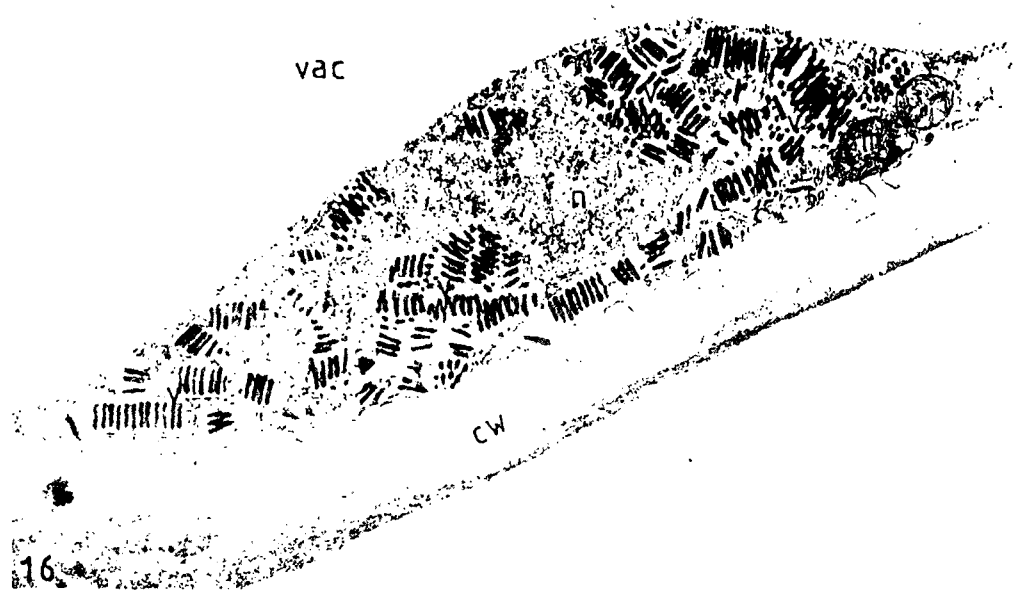


Fig. 14 and 15. Detailed aspects of the intermediary steps of the EVCV particles budding process(arrows) in EVCV-infected leaf cells. Double arrows indicate apparent continuity of the inner lamella of the nuclear envelope with the viral envelops (X 60,000).

Fig. 16. Spongy parenchyma cell of *Euonymus* infected with EVCV. Groups of bacilliform particles are found within the perinuclear space and cytoplasmic vesicles (X 16,000). For abbreviations See Fig. 1.

boid projections, vacuoles, and accumulation of starch. But, in the infected cells, the most striking effect, besides the accumulation of EVCV particles in the perinuclear space, was the abnormal growth of the nucleolus and the reduction or disappearance of the chromatic material from the nucleus (Fig. 2, 9 and 16).

DISCUSSION

The bacilliform and the bullet-shaped particles observed in negatively stained dip preparations and in the perinuclear space of the sectioned infected cells must represent EVCV. The relatively complex morphology of these particles is similar to that of particles of the plant rhabdoviruses (6, 12).

The most commonly used negative stain for the electron microscopic examination of virus particles is 2% PTA(pH 7.0) (8).

A commonly encountered problem is that many viruses are unstable in this stain and the particles disrupt (6). The majority of plant rhabdovirus particles observed in sections of infected cells are bacilliform whereas when negatively stained preparations are examined the variation in size and shape is quite bewildering. In preparations stained with PTA at pH between 6.5 and 7.0, most plant rhabdovirus particles appear bullet-shaped (3, 7, 10, 11, 17, 18). However, it has been demonstrated with both sowthistle yellow vein virus (XYVV) (14) and wheat striate mosaic virus (WSMV) (1) that when virus preparations are fixed with glutaraldehyde prior to negative staining the particles appear bacilliform and this is more evident when sections of infected cells are observed. This paper also demonstrated with EVCV that when virus preparations are fixed with osmium tetroxide prior to negative staining the particles appear bacilliform. There now seems to be little doubt that the apparent morphlogy of plant rhabdovirus particles of various shapes and size observed in negatively stained preparations are derived from bacilliform particles as a result of preparative artifacts (6).

Many of the plant rhabdoviruses have been studied in sections of infected cells and the information has been reviewed in some detail by Knudson (12) and Francki (6). It has been suggested by Hull (9) that plant rhabdoviruses can be divided into two subgroups depending on their cellular sites of synthesis and assembly.

Francki (6) also point out that some plant rhabdoviruses tend to be accumulated in the perinclear space of infected plant cells whereas others appear to form cytoplasmic, membrane-bound inclusions. It was concluded that EVCV belong to plant rhabdovirus accumulated in the perinuclear space of infected cells.

In electron micrographs of cells infected by EVCV, the envelopes of some particles are seen to be continuous with the inner lamellae of the nuclear membranes. It has also been demonstrated that in cells infected by EVCV, structures which appear to be viral nucleocapsids can be observed in the nucleoplasm. These observations suggest that the nucleocapsids of EVCV are synthesized in the nucleus of infected cells and are then extruded into the perinuclear space, at the same time acquiring their viral envelopes from the inner lamella of the nuclear membrane as indicated by the observations made by Kitajima and Costa (10) for Gomphrena virus, by Rubio-Huertos and Bos (16) clover enation mosaic virus (CEMV). Thus, the complete virion is formed by budding from the nuclear membrane. Electron micrographs such as those in Fig. 14 and 15, are highly suggestive of such a mechanism of virus particle assembly.

In cells where groups of virus particles are observed scattered in the cytoplasm as well as forming aggregates in the perinuclear space, the cytoplasmically located groups usually appear to be surrounded by membranes forming vesicles (Fig. 5, 6, 10, 11, 12 and 13). It seems that these vesicles are derived from the endoplasmic reticulum. Since it is now reasonably well documented that the endoplasmic reticulum is a continuation of the outer lamella of the nuclear membrane, it seems likely that the virus particles scattered throughout the cytoplasm may be those which have been transported from the perinuclear space in the enchylema of the endoplasmic reticulum.

Fig. 16 shows the reduction and disappearance of the chromatic material from the nucleus. A suggestive hypothesis to explain it is that the chromatic material is being broken down to provide raw material, mainly nucleotides, for virus synthesis. This might alter the cell metabolism, including the subsequent disorganization and even its death.

Virus particles have also been observed in sieve tubes of infected plants. Some plant rhabdoviruses have also been observed in phloem and xylem cells of clover, sowthistle, and gerbera plants, respectively (2, 11, 15). It is possible that vascular bundle could also be involved in the traslocation of plant rhabdoviruses.

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