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Reovirus-Like Particles Associated with Rice Ragged Stunt Diseased Rice and Insect Vector Cells

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日比野啓行*・Nasir SALEH**・Martoadmojo ROECHAN** : Rice Ragged Stunt 罹病イネおよび媒介昆虫細胞内のレオウイルス様粒子

Abstract

Rice ragged stunt causes symptoms distinct from rice grassy stunt : the symptoms are stunting of plants, twisted, ragged, and shortened leaves, branching of tillers, panicle emptiness, and galls along the veins. Virus-like particles of 55-60 nm in diameter occurred in dip preparations from the leaf and gall tissues of ragged stunt-diseased rice plants stained with phosphotungstate. Similar particles were found in extracts obtained from infectious vectors *Nilaparvata lugens* Stal. When the tissues were fixed with glutaraldehyde before dipping, particles had "spikes" around their periphery and 1-6 filaments were extended from the particles. Galls resulted from hyperplasia of phloem tissues and gall cells contained amorphous inclusions visible with the light microscope. Virus-like particles were embedded in viroplasm-like inclusions or were scattered in the cytoplasm of phloem and gall cells. Particles were about 65 nm in diameter and had electron dense cores of about 45 nm surrounded with less electron dense shells. Thirty to ninety percent of insect vecotrs in infectious colonies contained virus-like particles and about 1/2 to 1/3 of these insects were transmitters. Thin sections of insect transmitters showed that virus-like particles were embedded in viroplasm-like inclusions in the cells of the salivary glands, nerve tissues, muscles, fat bodies, and fore-gut. Particles were abundant and aggregated in crystalline arrangements in salivary glands and fat-body cells. Tubules with virus-like particles occurred in fat-body cells. (Received January 8, 1979)

Introduction

Ragged stunt of rice occurred in Indonesia in 1976[5] *and in The Philippines in* 1977[6]. The disease is called "kerdil hampa" in Indonesia and caused severe damage to rice production locally in Bali, Java, and Sumatra in 1977. The disease causes stunting of plants, twisted, ragged, and shortened leaves, branching of tillers, panicle emptiness, and galls along the abaxial surface of leaf veins. The disease agent is transmitted by the brown planthopper *Nilaparvata lugens* Stal in a persistent manner. The minimal incubation period of the pathogen in the insect is 5 days[5,6,12]. Transmission of the disease agent is similar as that of rice grassy stunt by the same vector ; however, ragged stunt causes symptoms distinct from grassy stunt and cross protection did not occur between the pathogens of these two diseases on rice plants[5]. Virus-like particles associated with rice ragged stunt (RRS) are about 60 nm in diameter in phosphotungstate stain[5,12], but no such particles are associated with

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grassy stunt[8,10].

Here, we report the occurrence and morphology of particles associated with RRS in negative stain, and in the infected rice and insect vector cells.

Materials and Methods

RRS was isolated from a rice plant collected in West Java in 1976 and was maintained on rice plants by successive transfers using the insect vector[5]. A virus-free colony of the brown planthopper *N. lugens* was reared on rice plants in a screened cage. Seedlings of the rice variety Taichung Native 1 at the first or second leaf stage were inoculated with RRS using infectious insects and inoculated seedlings were grown in a greenhouse.

Leaf samples were collected from young leaves showing twisted or ragged leaf symptoms 1 month after inoculation, and were also collected from healthy seedlings. Galls on flag leaves or from other upper leaves were collected 2 to 3 months after inoculation. Dip preparations were obtained by dipping cut faces of these samples in a drop of neutral 1% phosphotungstate or 1% uranyl acetate, and were examined with a Hitachi HS-9 electron microscope. In other experiments, samples were fixed with 3% glutaraldehyde or 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7) before dipping. For thin sectioning, samples were fixed with 3% glutaraldehyde in the same buffer for 1 hour in an ice bath. After washing with cold buffer, they were post fixed in buffered 1% osmium tetroxide for 2 hours in a refrigerator, dehydrated in an acetone series, and embedded in Epoxy resin. Thin sections were cut transversely from samples using glass knives mounted on a Porter-Blum MT-2B ultramicrotome. Sections were stained with uranyl acetate and lead citrate, and observed with the electron microscope. Thick sections of about 1 μm were cut and stained with 0.1% toluidine blue in 1% sodium borate for 10 minutes, and were observed under a light microscope.

Breeding females of the virus-free vector were allowed to lay eggs on ragged stunt-diseased plants for 1 day. Hatching started 7 days thereafter and nymphs were kept on the diseased plants for about 2 weeks. Infectivity of the insects in the infectious colony thus obtained, was assayed and presumed virus carriers were determined as follows. Each insect was given an inoculation access period of 1 day on a rice seedling in a test tube. The inoculation access was repeated three times on fresh seedlings and the inoculated seedlings were grown in the greenhouse. Ragged stunt symptoms appeared about 2 weeks after inoculation[5]. The insects which transmitted ragged stunt in the assay tests were counted as active transmitters. Heads were removed from the thoraces of all assayed insects and were crushed in a drop of neutral 1% phosphotungstate. The extracts were examined for the presence of RRS associated particles with the electron microscope. For thin sectioning, thoraces of 4 active transmitters and 5 non-infectious insects were incised in a drop of 0.05 M phosphate buffer (pH 7) containing 0.8% sodium chloride and 3% glutaraldehyde, and heads were then excised with the salivary glands and remained in chilled fixative for about 20 minutes. After washing with cold buffered saline solution, heads were post fixed with 1% osmium tetroxide in the same solution. Fixed samples were embedded, and thin sections of the salivary glands and posterior side of the heads were cut so as to include the cephalic parts of the fore-gut and brain. The sections were stained and examined as described previously.

Results

Dip preparations of leaf and gall tissues Full and empty virus-like particles 55-60 nm in diameter occurred in dip preparations of the ragged stunt infected tissues stained with phosphotungstate (Fig. 1A). Particles were abundant and often in a cluster in the preparations from galls, but occurred less frequently in those from the non-gall leaf tissues. Filaments of about 10 nm in diameter occurred in glutaraldehyde or paraformaldehydefixed preparations (Fig. 1B). In some glutaraldehyde-fixed preparations, 1-6 filaments of 150-500 nm in length extended from particles (Fig. 1C). Particles without filaments appeared full, but those with 1 or 2 filaments were partly penetrated with stain and those with 5 or 6 filaments appeared empty. Glutaraldehyde-fixed particles were about 50 nm in diameter with 6 knobs ("spikes") around their periphery (Fig. 1B). Occasionally, unfixed or paraformaldehyde-fixed preparations also revealed "spikes" on particles. In uranyl acetate stain, particles were 50-55 nm in diameter with "spikes" in rare occasion. No virus-like particles were found in dip preparations from healthy leaf tissues.

Extracts of infectious insects Similar virus-like particles to those observed in the dip preparations from plants occurred in extracts from insects (Fig. 1D). Larger particles of about 70 nm in diameter were also observed in insect extract preparations, although the smaller virus-like particles were far more abundant (Fig. 1D). Fifty and 35% of insects in the carriers of smaller particles transmitted ragged stunt in this experiment (Table 1). All active transmitters contained smaller

Table 1. Active transmitters and particle carriers of rice ragged stunt in an infectious colony of *Nilaparvata lugens*. The infectious insects were given inoculation access periods of 1 day on rice seedlings for 3 times and then extracts of the insect heads were examined for the presence of virus-like particles.

Insect colonies	Sex	Number of insects	Particle carriers	Active transmitters
Infectious	male	20	18	9
	female	24	17	6
Non infectious	male	13	0	—
Non infectious[a)]	male	10	0	—

a) Insects were given an acquisition access period of 1 day and then were held on healthy seedlings for 1 day.

particles and none of non-carriers of smaller particles transmitted the disease. Non-infectious insects contained larger particles in a low frequency but no smaller particles. The percentage of virus carriers in infectious colonies obtained in other experiments were variable, ranging from 30 to 90% of the populations examined.

Virus-like particles in sectioned plant tissues

Virus-like particles in plant tissues were about 65 nm in diameter and had electron dense cores of about 45 nm in diameter surrounded with outer shells of about 10 nm in width (Fig. 2). Empty particles also occurred in the cells.

Virus-like particles occurred in the phloem tissues of ragged stunt-diseased rice leaves. They were scattered in the cytoplasm of phloem parenchyma cells or were embedded in viroplasm-like inclusions composed of fine filaments, 7 to 10 nm in width

(Fig. 3.) Virus-like partlices also occurred in the sieve tubes. Necrotic cells in the phloem tissues often contained particles. Neither virus-like particles nor inclusions were found in the healthy leaf tissues.

The gall tissues occurred at the abaxial side of the vascular bundles (Fig. 5A). Apparently, the gall tissues resulted from hyperplasia of the phloem tissues. Amorphous inclusions were observed by light microscopy in the gall cells (Fig. 5B). By electron microscopy, the gall cells contained virus-like particles in viroplasm-like inclusions in the cytoplasm (Fig. 4). Virus-like particles and filaments, which might originate from inclusions were scattered in the degenerated gall cells (Figs. 2 and 4). Degenerated gall cells also contained few cellular organelles, membrane structures, and intact plasmalemma. Cryasalline arrangement of virus-like particles in gall cells was rarely observed. Parenchymatous cells surrounding xylem elements in the gall tissues were free of particles.

Virus-like particles in insect cells

Virus-like particles were embedded in viroplasm-like inclusions in the cytoplasm of the cells from infectious insect vectors. The particles and inclusions were similar to those observed in diseased rice plant cells.

Inclusions containing abundant virus-like particles occurred frequently in the salivary glands. Particles were scattered or aggregated in crystalline arrangemants in the periphery of inclusions (Fig. 6). Inclusions often occupied large spaces in cells of salivary glands. Occasionally, particles were arranged on the surface of the secretory granules and some of these granules contained virus-like particles (Fig. 10). Inclusions with particles also occurred in salivary duct cells.

Inclusions in fat-body cells often contained crystalline aggregates of virus-like particles (Fig. 8). Tubules containing particles occurred in the cytoplasm of these cells, often adjacent to crystals, or in or around inclusions (Figs. 8 and 9). Tubules were about 110 nm in diameter and their walls were 10–15 nm in thickness. Virus-like particles in tubules were similar to those in inclusions (Fig. 10). Particles in phagocytic vesicles of fat-body cells had no outer shells (Fig. 8).

Inclusions with virus-like particles also occurred in the cells of nerve tissues, muscles, and fore-gut. No virus-like particles were found in non-viruliferous insect tissues.

Discussion

Virus-like particles observed in cells of ragged stunt diseased rice plants and infectious insect vectors were similar in morphology to reolike plant viruses : maize rough dwarf virus (MRDV), pangola stunt virus (PSV), rice black-streaked dwarf virus (RBSDV), rice dwarf virus, sugarcane Fiji disease virus (SFDV), and wound tumor virus[7]. These viruses induce "viroplasm"[3,9,11,13,15,17] and tubules with virus particles [1,2,4,13,14] in plant and insect vector cells, as did RRS in these experiments. Relatedness of the RRS associated particles to reolike plant viruses is supported by the similarity of RRS agent and reolike viruses in relationships to insect vectors[5,7]. Occurrence of "viroplasm" in infectious insect cells suggests multiplication of the particles in the insect tissues.

RRS associated particles was phloem-restricted and gall tissue apparently resulted from hyperplasia of phloem tissue. The particles might have specific affinity with phloem cells, since MRDV, PSV, RBSDV, and SFDV have also been reported in phloem tissues or in the neoplastic phloem cells[3,11,13,17]. These reolike viruses are transmitted by planthoppers, induce galls along leaf veins, and cause stunting of

plants and leaf distortion, as does RRS. Recently, these reolike viruses were included in one subgroup-ACANTHOVIRUS[7]. ACANTHOVIRUS particles are about 70 nm in diameter, have outer shells with external spikes and cores of 45-50 nm in diameter with internal spikes[7]. In the case of RRS, virus-like particles in the cells were about 65 nm in diameter, had an outer shell and a core of about 45 nm in diameter, however, particles in dip preparations stained with phosphotungstate or uranyl acetate were smaller. Outer shells of ACANTHOVIRUS particles are stripped in the presence of phosphotungstate, and complete virions are obtained when particles are first adsorbed to a support film, and then they are fixed with glutaraldehyde prior to treatment with phosphotungstate or are stained with uranyl acetate[7]. In our experiments, particles were not fixed and stained on a support film, as crude extracts were used for electron microscopy. Particles in dip preparations might correspond to cores of virus-like particles in the cells, may be with partly degraded shells. Glutaraldehyde-fixed particles were similar in morphology as spiked cores of ACANTHOVIRUS particles[7].

In ACANTHOVIRUS group, only RBSDV naturally infects rice and causes stunting of plants and tumors along the veins, whereas MRDV has been transmitted experimentally to rice[7]. RBSDV causes white waxy tumors along the veins, later they become dark brown[13], whereas RRS caused galls of distinct appearance; smooth, flat, narrower, and white to brown in color. RBSDV were not transmitted by *N. lugens*[16]. None of known viruses in the ACANTHOVIRUS group have been transmitted by *N. lugens*[7]. It seems that RRS is a new disease caused by a virus agent belonging to the ACANTHOVIRUS group. Further experiments including serology are required to elucidate the position of the RRS agent in the group.

The filaments that extended from glutaraldehyde-fixed particles were similar as filaments in inclusions. Moreover, the particles with 1 or 2 filaments had reduced amount of contents and those with 5 or 6 filaments appeared empty. These facts indicate that the filaments may be structural units of particles, although their chemical properties were not examined in these experiments. Similar filaments were observed in extracts of PSV and MRDV infected leaves crushed in glutaraldehyde[4].

The larger particles observed in extracts of insects had nearly the same morphology as virus-like particles in the RRS infected cells, however, the particles were also found in the extracts of non-infectious insects. Apparently, the larger particles are not pathogen of RRS. The larger particles were not observed in sectioned cells of non-infectious insects, which might be due to low concentration of the particles in the insect tissues. Our current investigations indicate possible transmission of the larger particles through brown planthopper eggs, while RRS was not transovarially transmitted in the vector nor seed transmitted in rice (Hibino *et al.*, unpublished data).

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和 文 要 旨

Rice ragged stunt 罹病イネおよび媒介昆虫細胞内のレオウイルス様粒子

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イネの ragged stunt 罹病株は萎縮し；葉は短かく，先端がまき，葉縁が切れこみ，葉脈上に gall を生じ，茎は分枝し，穂は異常となり，空籾を生ずる。

Ragged stunt 罹病イネの葉および gall 組織の dip 法観察により，直径55-60nm の reovirus 様粒子を認め，同様の粒子は保毒した媒介虫 ***Nilaparvata lugens*** のまさい液中にも認められた。グルタルアルデヒド固定した組織から dip 法によって得た粒子は spike を持ち，1－6本のひも状構造物が粒子から伸びていた。Gall は篩部組織の異常増生により生じ，gall の細胞内には光顕観察により封入体が認められた。超薄切片観察によればウイルス様粒子は篩部および gall 組織内の細胞にのみ認められ，直径約 65nm で，直径約 45nm の core と電子密度の低い外被からなり，viroplasm 様封入体内および細胞質内に散在していた。罹病株上で飼育したトビイロウンカの 30—90%はウイルス様粒子を持ち，これらの保毒虫の½—⅓が病気を伝搬した。病気を伝搬した媒介虫を超薄切片法により観察したところ，ウイルス様粒子がだ腺，神経組織，筋肉，脂肪体，および前腸細胞内の viroplsma 様封入体中に認められた。粒子はだ腺および脂肪体細胞に多く，結晶配列をしていた。一列に並んだウイルス様粒子の入ったさや状構造物が脂肪体細胞で認められた。

Explanation of plates

Figs. 1. Electron micrographs of virus-like particles in phosphotungstate stain. (A). A cluster of particles in a dip preparation of a gall tissue. (B). Particles with "spikes" and filaments in a dip preparation of a glutaraldehyde-fixed gall tissue. (C). Particles with 1 or 6 filaments, or without filaments in a dip preparation of a glutaraldehyde-fixed gall tissue. Note difference in electron density of these particles. (D). A cluster of particles in a extract of an infectious insect *Nilaparvata lugens*. Note larger particles in and outside the cluster (arrows).

Fig. 2. Virus-like particles and filaments (arrows) in a degenerated gall cell of a ragged stunt-diseased rice plant.

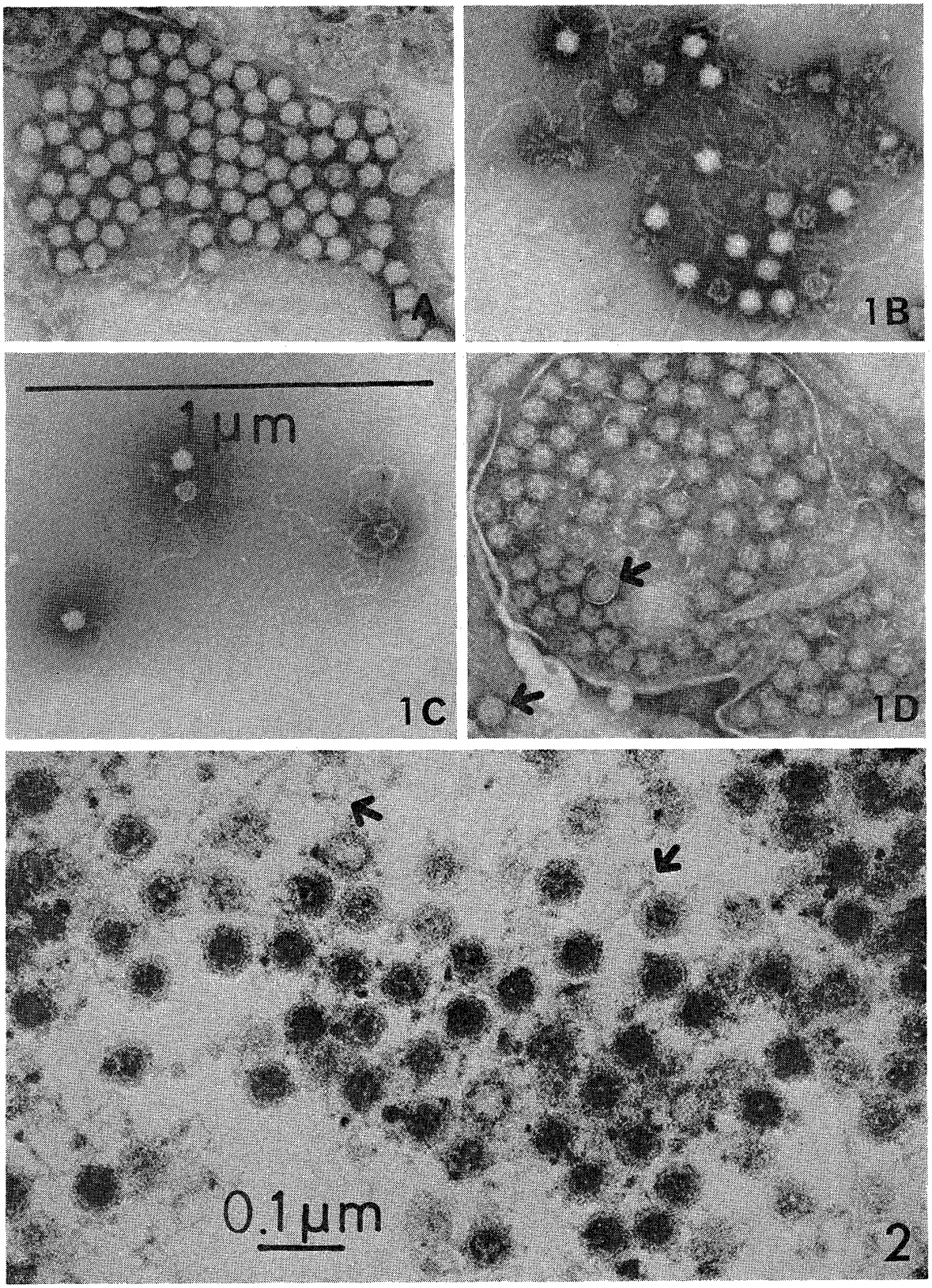
Fig. 3. Part of a phloem parenchyma cell of a ragged stunt-diseased rice leaf showing virus-like particles embedded in a viroplasm-like inclusion (I) or aligned between membranes next to a nucleus (N).

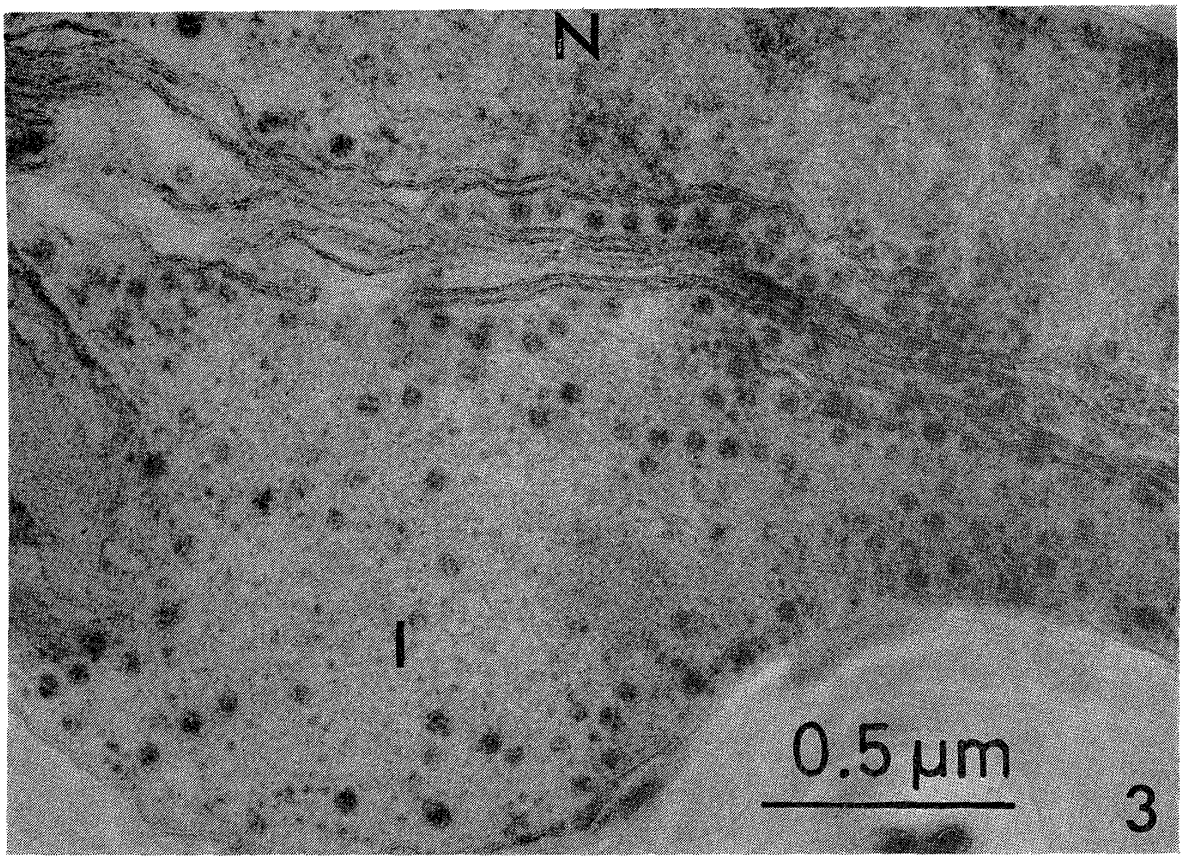
Fig. 4. Parts of gall cells of a ragged stunt-diseased rice leaf showing virus-like particles embedded in viroplasm-like inclusion (I) or scattered in the degenerated cytoplasm.

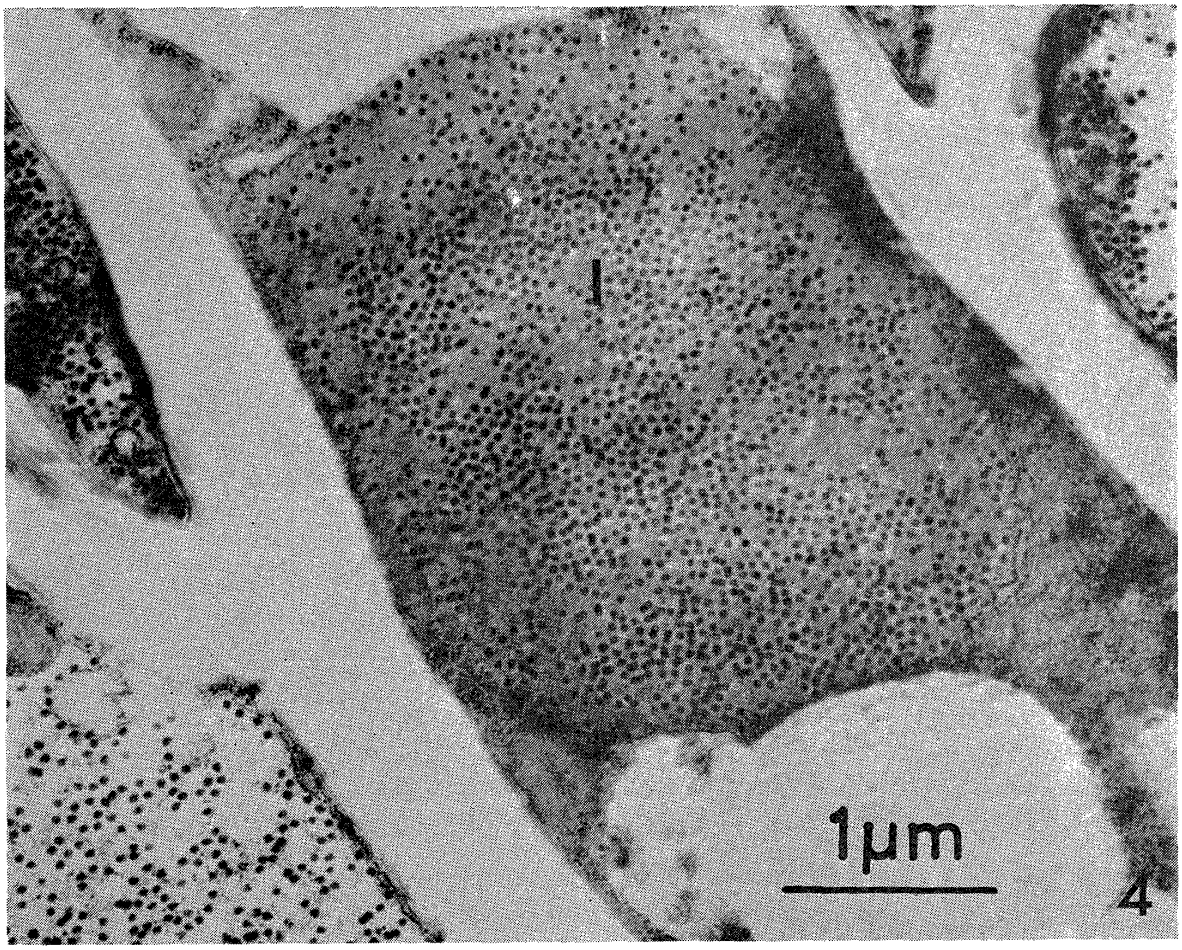
Fig. 5. Light micrographs of a cross section of a gall on a ragged stunt-diseased rice leaf. (A). A gall tissue resulted from hyperplasia of a phloem tissue (P) on a leaf vein. X, xylem element. (B). Enlargement of the enclosed area in Fig. 5A showing inclusions (arrows) in the cells.

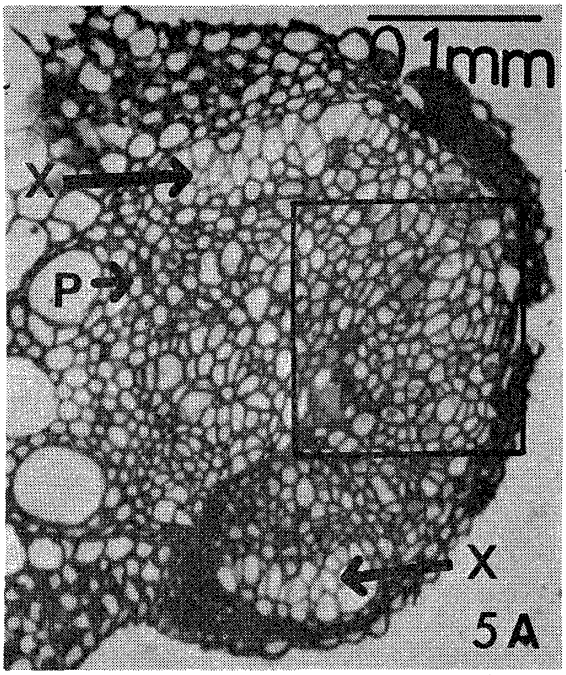
Figs. 6 and 7. Parts of salivary gland cells of infective vectors *Nilaparvata* lugens. Fig. 6. A crystalline aggregate of virus-like particles in a viroplasm-like inclusion (I). Fig. 7. Virus-like particles arranged on the surface of secretory granules (G) and in a granule (arrow).

Figs. 8–10. Parts of fat-body cells of infective vectors. Fig. 8. A viroplasm-like inclusion (I) containing a crystalline aggregate of virus-like particles and tubules with particles (arrows), and a phagocytic vesicle (P). Fig. 9. Long and short tubules with virus-like particles in the cytoplasm. Fig. 10. Tubules with virus-like particles in a vesicle.









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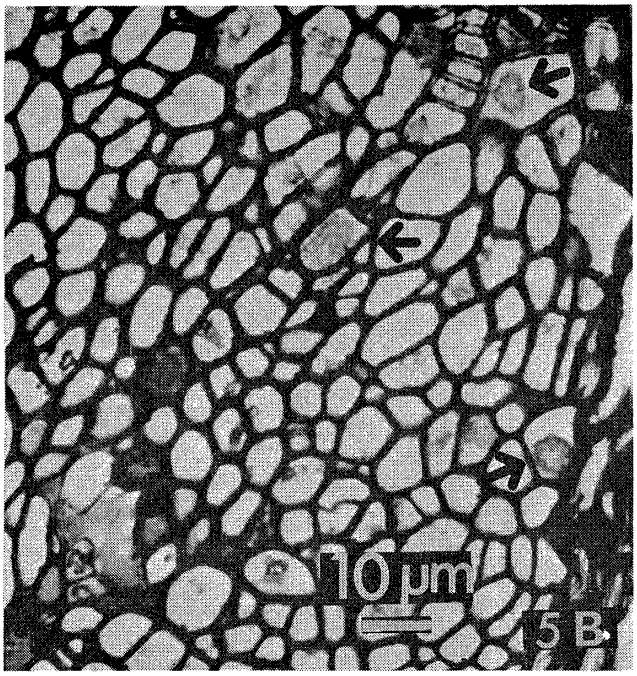

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