Agronomie (1992) 12, 651-659
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Immunogold labelling of beet necrotic yellow vein virus particles inside its fungal vector, *Polymyxa betae* K

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(Received 3 february 1992; accepted 4 June 1992)

Summary — Many aspects of the association of *Polymyxa betae* with beet necrotic yellow vein virus during transmission have not been elucidated. In order to study the specificity of virus acquisition by the vector and its transmission to the host, thin sections were made through sugar beet roots infected both by the virus and the fungus. Immunogold labelled virus was identified at different stages of the fungus (plasmodia, zoosporangia and mature zoospores). However, specifically labelled clusters of virions have not yet been observed in mature resting spores.

beet necrotic yellow vein virus / *Polymyxa betae* / immunogold labelling / virus–vector association

Résumé — Marquage à l'or colloïdal du virus des nervures jaunes et nécrotiques de la betterave dans le champignon vecteur *Polymyxa betae* K. *De nombreuses questions relatives à l'association entre* Polymyxa betae *et le virus de la rhizomanie qu'il transmet restent sans réponse. Dans le but de mieux appréhender la spécificité de l'acquisition du virus par son vecteur ainsi que sa transmission à la plante hôte, des coupes ultrafines ont été réalisées dans des radicelles de betterave sucrière infectées à la fois par le virus et le champignon, puis soumises au marquage immunocytochimique. Du virus marqué à l'or colloïdal a été détecté dans différents stades du champignon (plasmodes, zoosporanges, zoospores bien différenciées). Cependant, des amas caractéristiques de BNYVV n'ont pu être mis en évidence dans les cystosores et les spores de repos, probablement en raison de la difficulté de pénétration des résines dans de tels tissus.*

marquage immunocytologique à l'or / BNYVV / virus* / Polymyxa betae / *association virus–champignon vecteur

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV) is a rod-shaped virus of sugar beets (Tamada and Baba, 1973), described in Japan, Europe, North America, China and the former Soviet Union. It is the causative agent of an important disease called rhizomania. BNYVV is transmitted by the soil-borne plasmodiophoraceous fungus *Polymyxa betae* K (Keskin, 1964) an obligate parasite of plants from the families Chenopodiaceae, Portulaceae and Amaranthaceae (Canova, 1966; Tamada, 1975; Fujisawa and Sugimoto, 1977).

More than 20 different viruses are now believed to be transmitted by lower fungi from the orders Plasmodiophoromycetes and Chytridiomycetes (Brunt and Richards, 1989; Adams, 1991). However, the details of the mechanism of transmission and association between fungally-transmitted viruses and their vectors are still unknown.

Earlier attempts to find the virus in the zoospores of *Polymyxa* failed. Giunchedi and Langenberg (1982) examined more that 220 zoospores without success. Furthermore, earlier work provided no definite proof of the presence of particles in the fungus. Tamada (1975) presented a micrograph of "virus-like particles" of BNYVV in a zoospore, but the image did not allow the fine structure of the putative virus particles to be distinguished. Other papers dealing with the subject did not present micrographs (Tamada *et al*, 1975; Stocky *et al*, 1977; Vuittenez and Stocky, 1979). Fujisawa and Sugimoto (1977) reported finding rod-shaped particles in resting-

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spores of *P betae* but the number of particles detected was too small to allow definite conclusions to be drawn.

Owing to the density of the fungal cytoplasmic contents, viral particles are difficult to see within fungal plasmodia, zoosporangia or cystosori. The association of *Polymyxa betae* with the virus it transmits is thought to be internal; according to Tamada (1975), zoospores with virus have been found and cystosori in dry BNYVV-infected roots remain able to transmit BNYVV to beet seedlings for a long period of time. Langenberg and Giunchedi (1982), found virus particles in close contact with the fungal vector in the host tissue, but they were always outside the fungal structures. Allaham and Stocky (1983), Vuittenez *et al* (1984), and afterwards Abe and Tamada (1986), published convincing electron micrographs of virus-like particles in immature zoospores of *Polymyxa betae*. Recently, Jianping *et al* (1991), using immunogold labelling, have described the presence of a bymovirus, barley mild mosaic virus, inside *Polymyxa graminis*.

Here we show the presence of immunogold-labelled BNYVV particles in young plasmodia, zoosporangial and cystogenous plasmodia, in a mature zoospore in a zoosporangium and in a free zoospore of *Polymyxa betae* outside the root cells. This paper thus demonstrates the association of *P betae* with BNYVV using immunogold labelling techniques.

MATERIALS AND METHODS

Virus, fungus and host plants

Soil from a contaminated field near Strasbourg was used as source of viruliferous *Polymyxa betae*. The soil had been shown to be essentially free of major contaminant fungi, particularly *Olpidium* sp. Contamination with *Pythium* and *Phytophthora* did not present a problem because their development time under our conditions (2–3 wk) was such that, even if present, they would not have been abundant at 8 days post-infection when root samples were generally collected. A suspension of distilled water: soil (5 : 1 (w/w)) was prepared. After thoroughly homogenizing, 1 ml of suspension was pipetted into glass test tubes (1 x 6 cm). One pregerminated seed of sugar beet, cv Monosvalof, was placed in each tube so that the seed adhered to the side above the meniscus. After 5–9 days at 22 °C, the presence of fungus was checked microscopically and virus was detected by ELISA.

To obtain older infected plants, a sand culture of plants was established in propylene centrifuge tubes. A suspension of virus-carrying cytosori from infected beet roots was added to each plant. Twice a week, the plants were watered with a complete nutrient solution. Plants were harvested 2 or 5 wk after infection. Older roots with cystosori and younger roots in which zoosporangia were prevalent were embedded separately.

Embedding

Pieces of rootlets (1.5 mm long) containing *P betae* were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h and postfixed in 1% osmium tetroxide for 1 h at 4 °C. After rinsing in distilled water, they were dehydrated in a graded ethanol series and penetrated with LR White resin or passed through propylenoxide and penetrated with a mixture of Epon–Araldite resins. The blocks were cured for 2 d at 60 °C to polymerize the resin.

Immunolabelling

Ultrathin sections silver to gold in colour were cut with an LKB Ultrotome III and collected onto Pioloform-covered nickel grids.

The procedure for immunolabelling was set up using leaf sections of *Chenopodium quinoa*, a local lesion host of BNYVV, which is present in local lesions in greater quantity and is more easily detected than in roots of beet. The optimal procedure giving a good signal/background ratio in our hands was as follows: immunolabelling was done by placing the grids face-down on drops of solutions. As a first step, sections were treated with a saturated sodium metaperiodate solution for 30 min at room temperature to restore protein antigenicity of the osmium-treated tissue sections (Bendayan and Zollinger, 1983). This step was omitted for LR White embedded tissues.

Non-specific binding of antiserum was prevented by floating the grids on 1% bovine serum albumin in PBS (PBS–BSA) for 15 min. One ml of anti-BNYVV antiserum raised in rabbit was absorbed with a healthy sugar beet preparation (1 g tissue in 1 ml 0.1 M phosphate buffer) and clarified by low speed centrifugation (Langenberg and Van der Wal, 1986). This serum was then used diluted with PBS–BSA buffer 1:500 for Epon–Araldite-embedded tissues and 1:5 000 for LR White-embedded tissues.

As controls, rabbit preimmune serum and antitobacco mosaic virus (anti-TMV) serum were used. After an incubation for 4 h at 37 °C, the grids were thoroughly rinsed on PBS–BSA drops. Then, a conjugate of protein A with colloidal gold (diameter 15 nm, Biocell ref: EM GAR 15) diluted with PBS–BSA 1 : 10 for Epon–Araldite embedded tissues and 1 : 50 for LR White embedded tissues, was applied for 2 h at 37 °C. After another stringent wash with Millipore-filtered water, the

sections were stained with uranyl acetate and lead citrate and examined in the electron microscope (Hitachi HU – 11C – S) at 75 kV.

RESULTS

Labelling specificity

With the exception of a very low background, non-specific labelling of non-viruliferous *Polymyxa betae* was observed at any stage of its life cycle, either with the BNYVV antiserum or with the preimmune serum used as control.

Furthermore, using the anti-TMV serum, no specific reaction between viruliferous fungus and antiserum was seen and in particular the clusters of visible BNYVV particles present in the plant cytoplasm and the fungus were not labelled. Finally, the very characteristic appearance of BNYVV in infected cells (particles in angle-layered clusters; Putz and Vuittenez, 1980), makes it possible to recognize the virus under its colloidal gold labelling. The specificity of the labelling for BNYVV is thus acceptable. The amount of labelling observed for tissue embedded in Epon–Araldite was low compared to tissue embedded in LR White. This may be due to incomplete restoration of antigenicity during the sodium metaperiodate treatment using the former embedding medium.

Life cycle of *P betae* and presence of virus

In most sections we could observe several stages of the fungus life cycle.

Free zoospores

Occasionally, in close proximity to roots free zoospores were observed containing virus in their vacuoles or vesicles (fig 1). We do not know if these are primary zoospores issued from cystosori in the soil or secondary zoospores because in such roots we have also observed labelled virus in zoospores of mature zoosporangia.

Young plasmodia

Young plasmodia of different dimensions were frequently present in the infected roots. Being usually very electron-opaque, they contained large amounts of fibrous material, which rendered it very difficult to identify virus if it was not

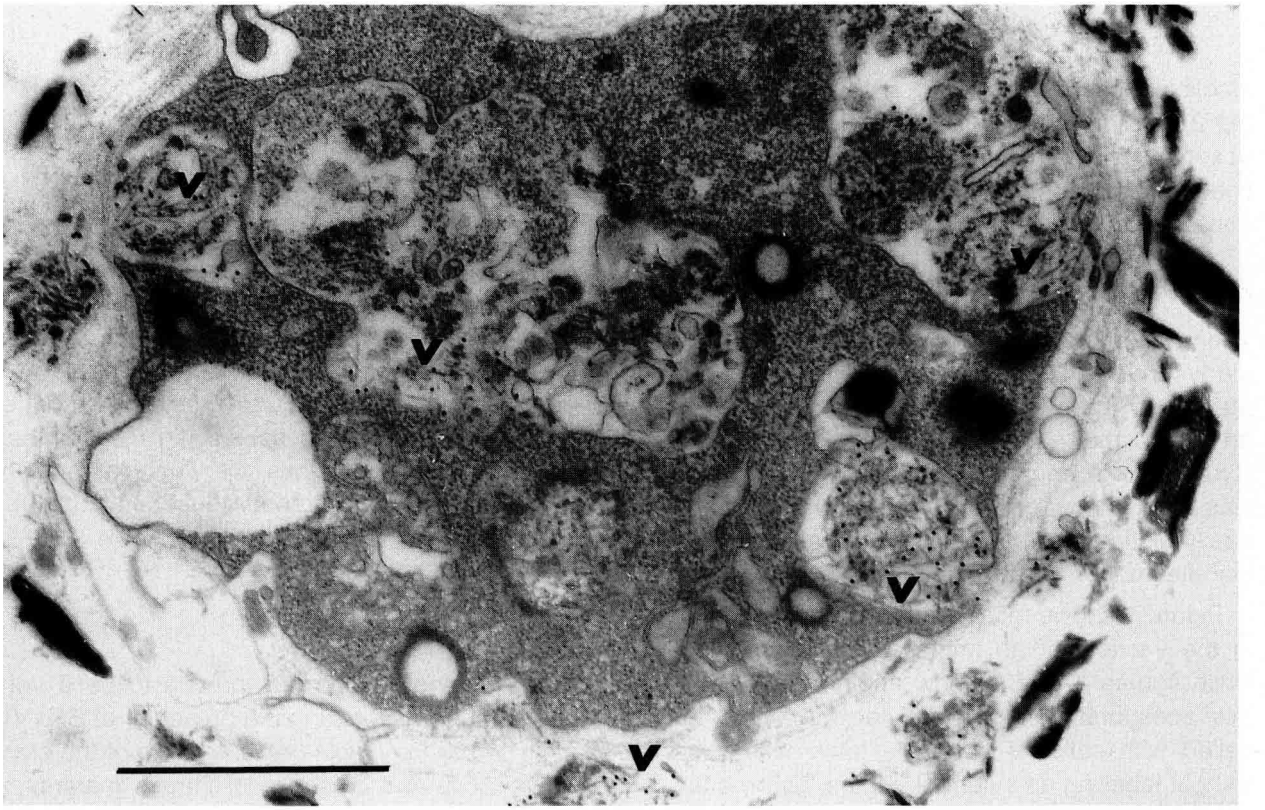


Fig 1. Free zoospore found in the proximity of a root hair. Several vacuoles and pinocytotic vesicles contain immunogold-labelled virus (v). Labelled virus is likewise found outside the zoospore. Bar = 1 μm.

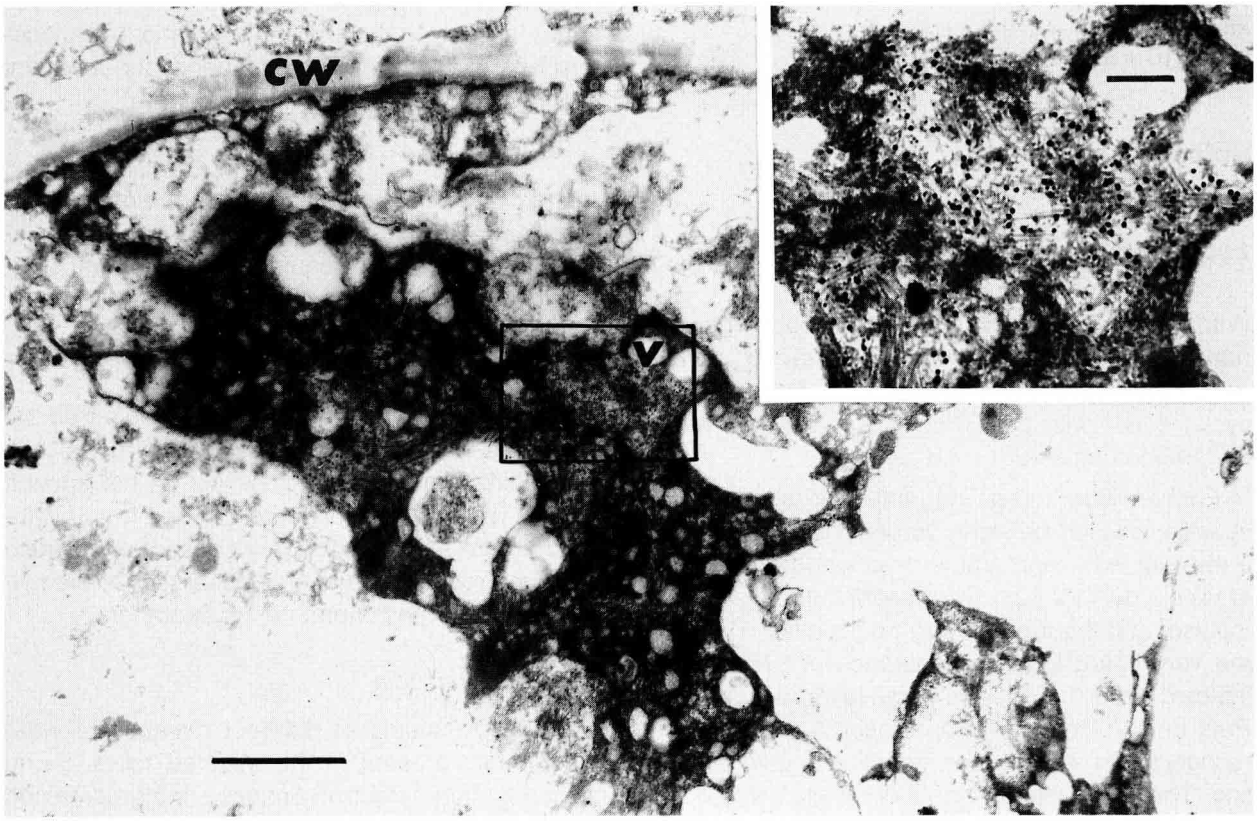


Fig 2. A young dense plasmodium with labelled virus particles in a less dense zone. Bar = 500 nm. The insert shows detail of the virus-containing zone. Bar = 100 nm.

labelled. Virus was often observed at this stage (fig 2) but it was not possible to determine from observation whether the plasmodium was destined to evolve into a zoosporangial or cytogenous structure.

Zoosporangial plasmodia

The immature zoosporangia were usually irregularly shaped. Osmophilic vesicles were numerous in the fungus and virus particles located in small clusters were observable. Cytoplasm of immature zoosporangium (fig 3) was electron-opaque but flagella were already easily distinguishable. Virus was present in this cytoplasm.

Figure 4 shows more clearly the labelled virus in the vacuole of an immature zoosporangium while figures 5 and 6 show another vacuole of this zoosporangium, the former with virus labelled with colloidal gold, the latter with virus without labelling as a control. Figure 7 shows the virus inside separated zoospores after cleavage of the zoosporangium.

Cystogenous plasmodia

We have found several putative cystogenous plasmodia with BNYVV inside. There may be a tendency to exclude the virus during the cleavage of resting spores inside the plasmodium. Alternatively, virus may be present but more difficult to detect in the interior of plasmodia. We could always observe virus near the internal cleavage sites, but it would not be possible with our observations to be sure whether it would remain inside the resting spores (fig 8) after their maturation. In other cases, the virus was present in an area that would probably degenerate (fig 9).

DISCUSSION

Gold immunolabelling has proven to be a very useful tool for searching the presence of BNYVV in its vector *Polymyxa betae*. In spite of the electron-opacity and presence of fibrous material in the fungus, virus was rapidly and clearly distinguished by association with its gold label. All the

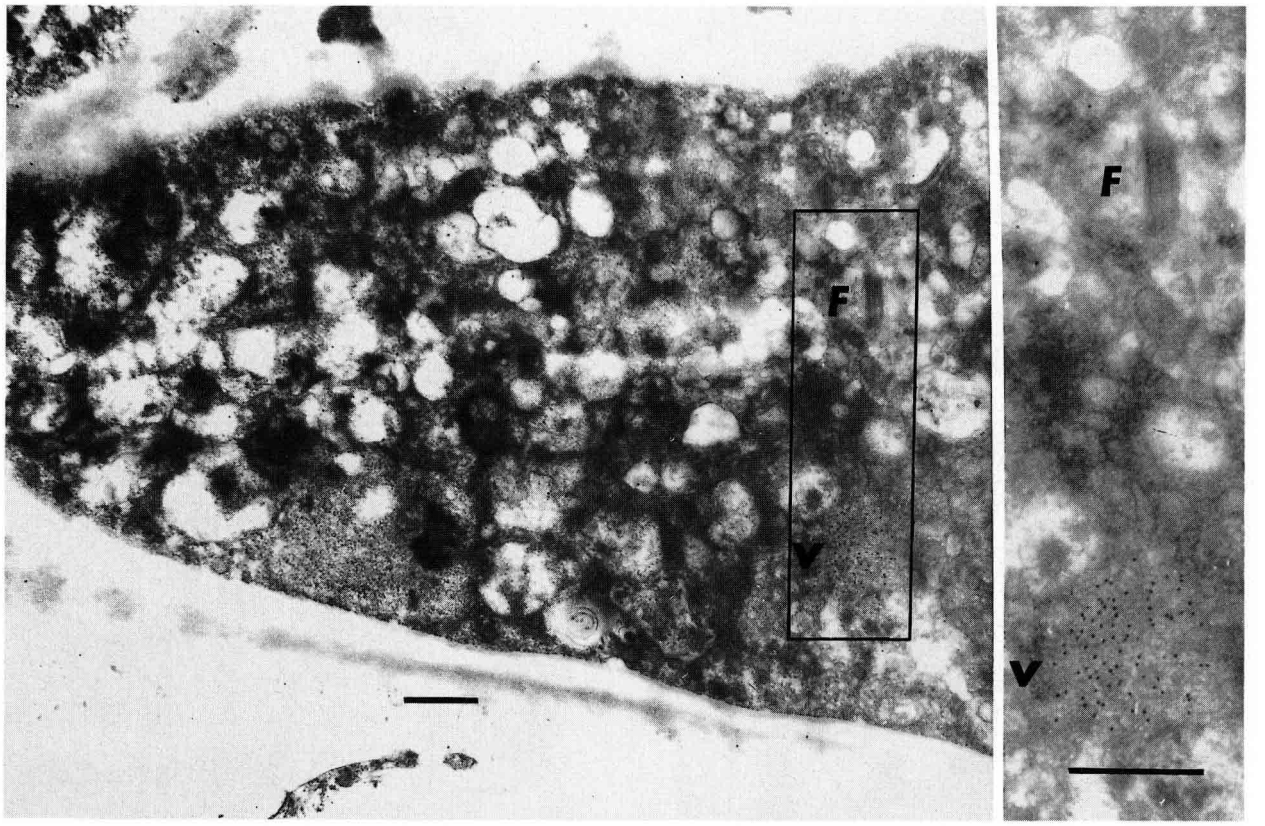


Fig 3. Longitudinal section in a root hair completely filled with a zoosporangial plasmodium. A gold-labelled region (v) and a flagellum (F) are visible. Bar = 500 nm.

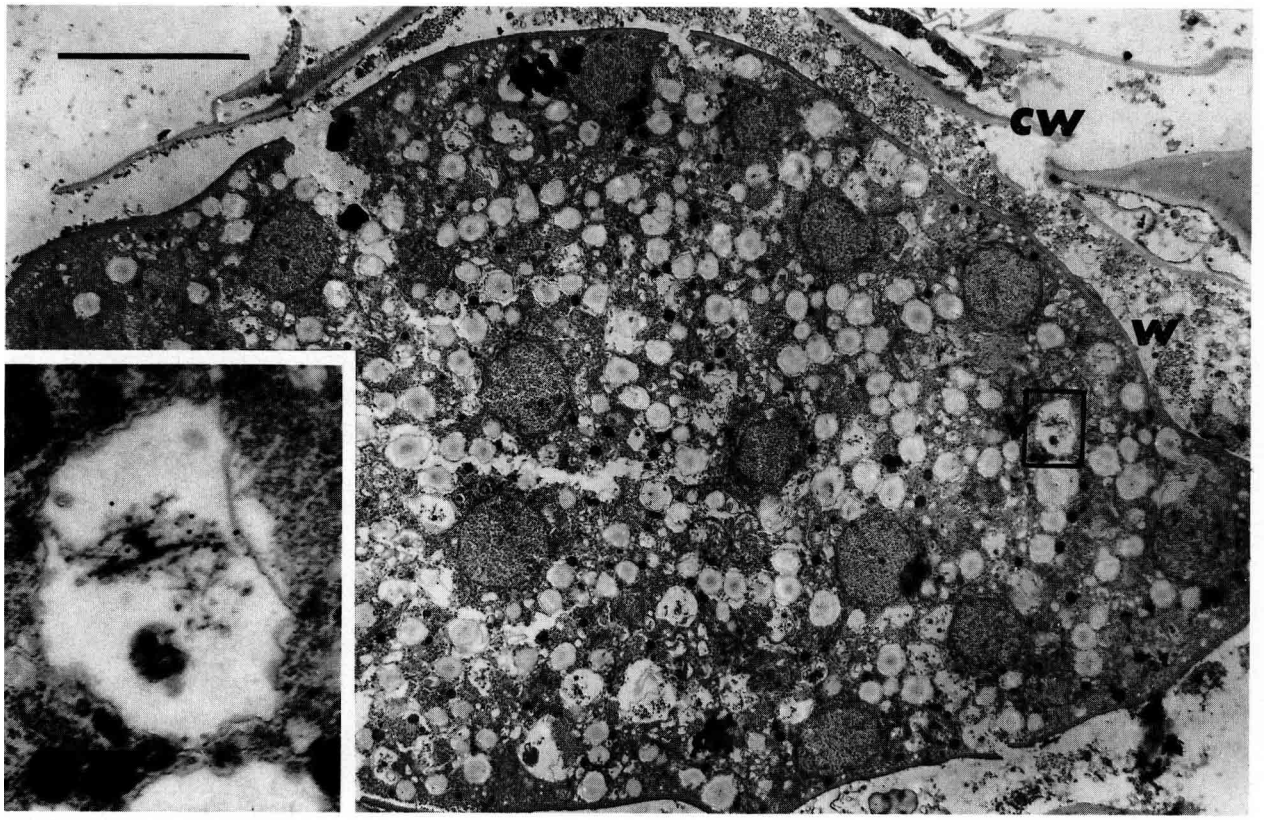
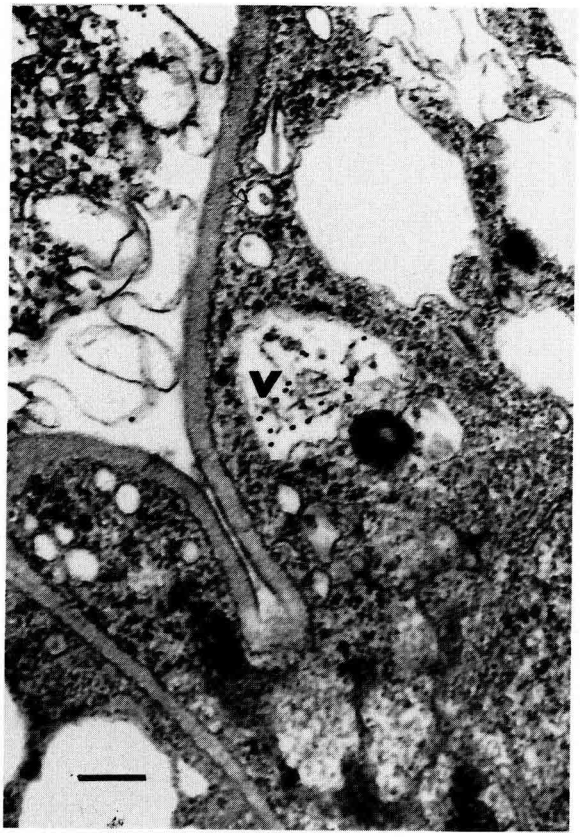
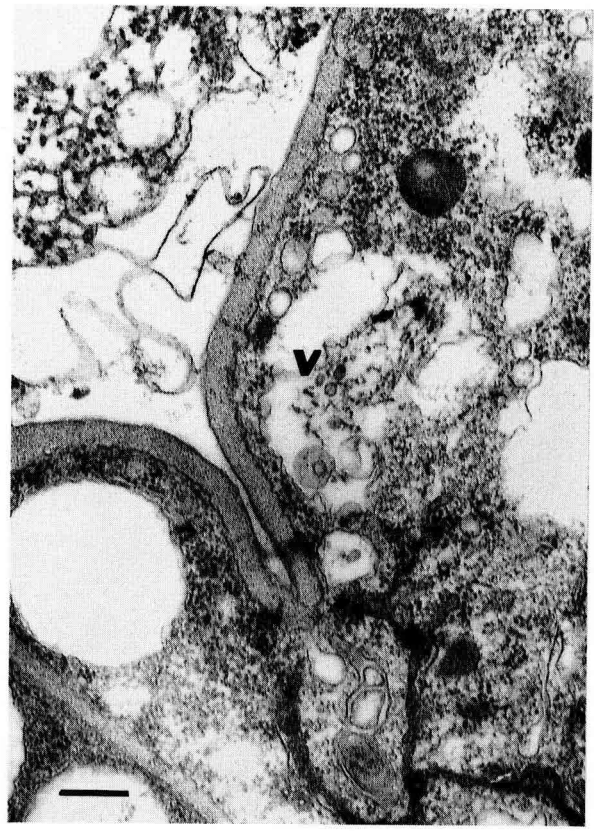


Fig 4. Immature zoosporange containing labelled virus within numerous vacuoles (w = fungus wall: cw = cell wall). Insert shows one such vacuole. Bar = 200 nm.





Figs 5–6. Zoosporangial plasmodium. **5.** Gold-labelled virus in the structure. **6.** A neighbouring thin section but without gold-labelling in which non-labelled virus can be distinguished. Bar = 200 nm.

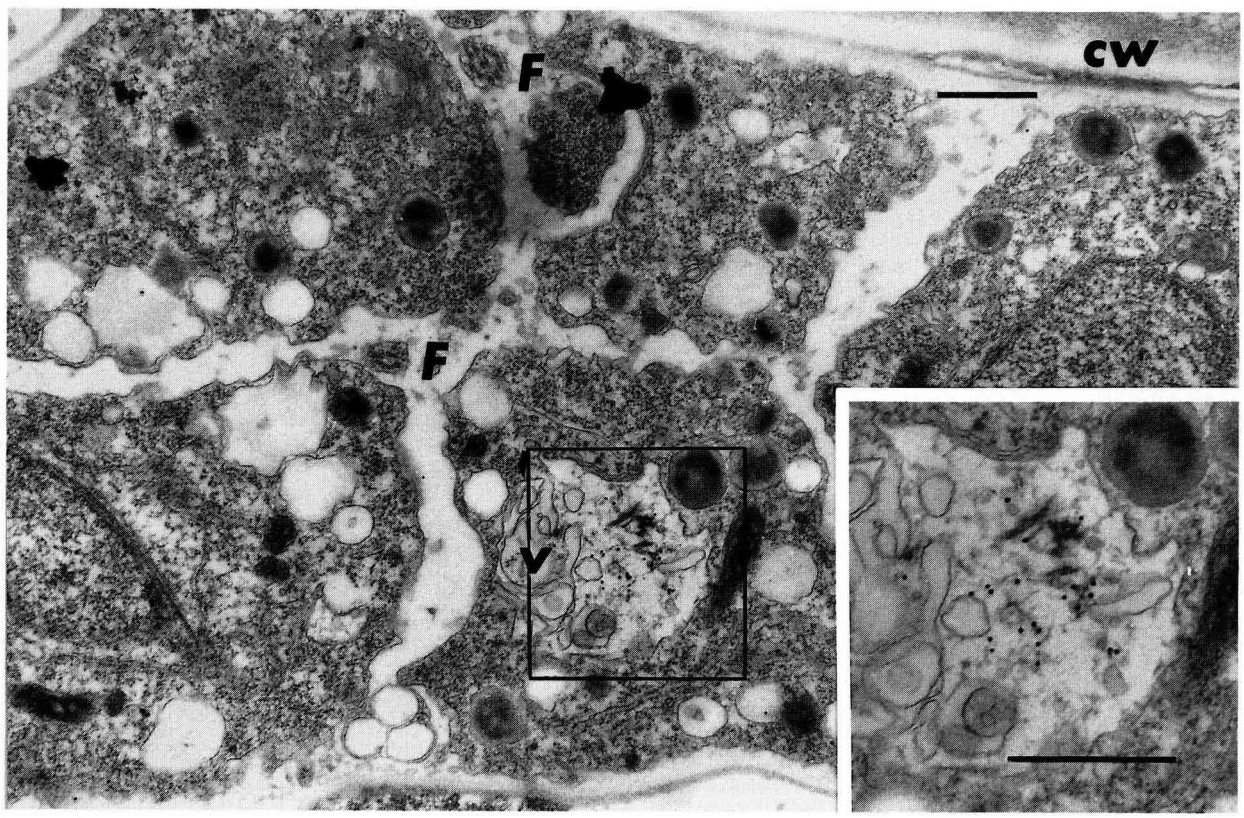


Fig 7. A zoosporangium containing individual zoospores. Gold labelling in the interior of a vacuole of one zoospore (insert) reveals the presence of virus. Bar = 500 nm.

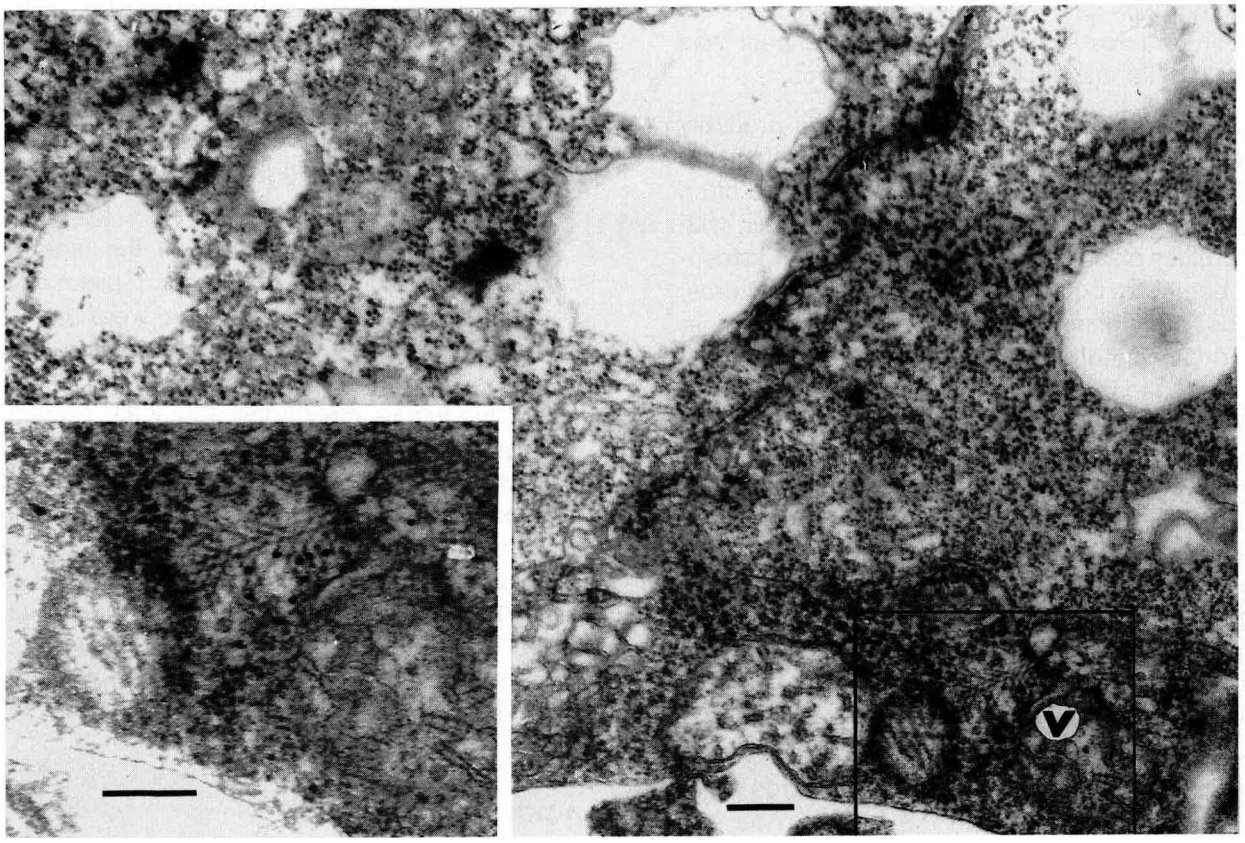


Fig 8. Cystogenic plasmodium with gold-labelled virus aggregated in chevrons. Bar = 200 nm.

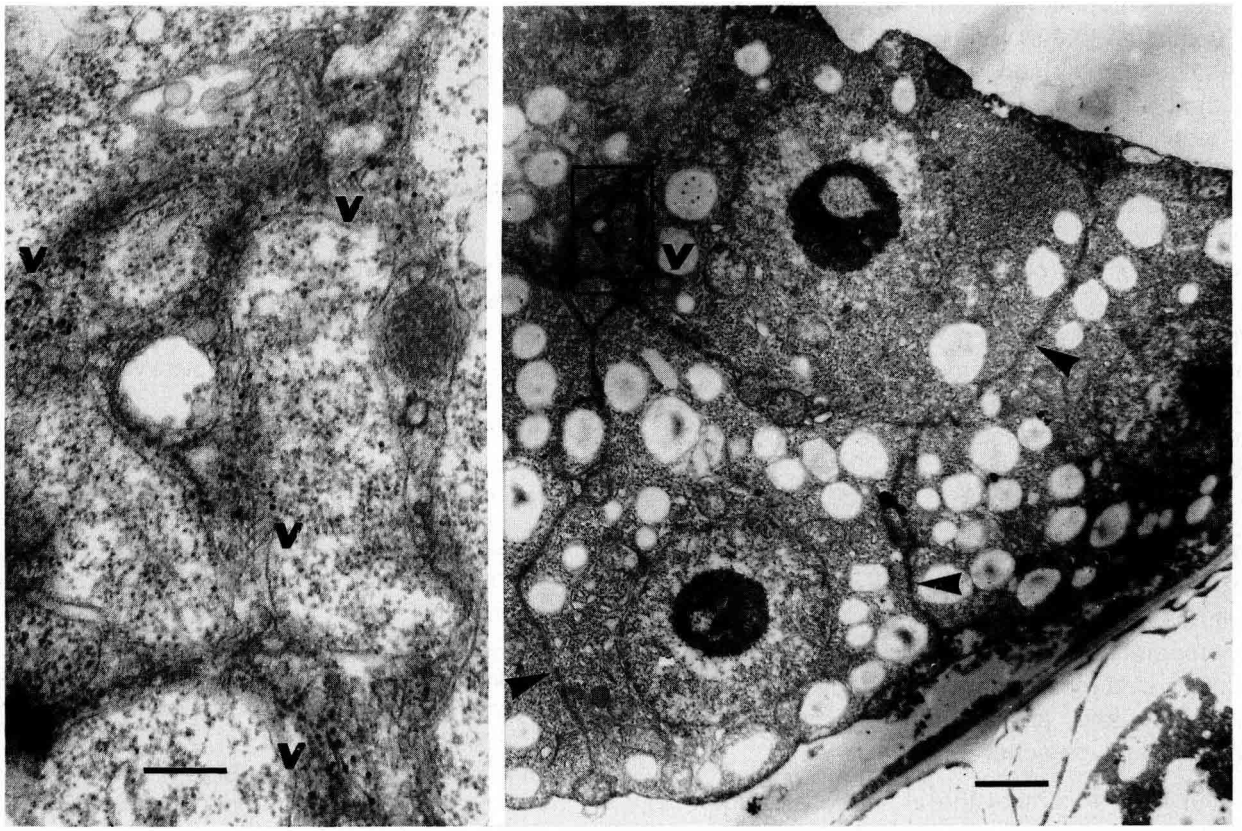


Fig 9. Cystogenic plasmodium undergoing cleavage (arrows). Bar = 1 μm. In the insert several aggregates of gold-labelled virus can be seen. Bar = 200 nm

micrographs presented here were issued from Epon–Araldite inclusions in which contrast was much better than in LR White inclusions.

The amount of virus in the fungus probably depends on the amount of virus in the host cell at the time of infection by the fungus. We often observed large amounts of virus in contact with the older plasmodia, zoosporangia and cystosori, but it was not present inside these structures, thus confirming observations of Langenberg and Giunchedi (1982). In the roots of 7-day-old plants the virus was found in the young plasmodia (in large amounts), in zoosporangial and cystogenous plasmodia and in immature zoospores (in small amounts) but not in mature zoospores. In young plasmodia we observed a number of pinocytotic vesicles that are probably responsible for liberating virus from or acquiring virus by plasmodium. The virus was often present in young plasmodia in certain areas which could correspond to vesicles generated by picnocytosis. Thus we suppose that, after some time, the acquisition of virus by plasmodium is impossible. If we assume that virus probably does not multiply in the fungus (Abe and Tamada, 1986), the very low amounts of virus injected into the cell by a zoospore would escape our methods of detection. We did not count the number of fungal structures observed, but estimate that in some cases at least 50% of plasmodia contained virus. Nevertheless, it is evident that after differentiation into zoospores or resting spores only some of them will carry the virus. The fact that we have failed to find virus in mature resting spores can probably be attributed to the low number of well-embedded cystosori of appropriate age observed. However, in spite of the apparent exclusion of virus from plasmodia during cleavage of resting spores, in view of the amount of virus observed in the free zoospores, it appears likely that the amount of virus in resting spores should be sufficient for being detected.

We did not try to pellet free zoospores of *Polymyxa betae* as did Jianping *et al* (1991), with zoospores of *Polymyxa graminis*, because pure cultures of *Polymyxa betae* were not available. In preparations of free zoospores from partially purified cultures, contaminants (chytridial fungi, amoebae and algae) were always present (Wiedemann, personal communication).

Our data show that the *in situ* localization of labelled BNYVV particles is possible at all stages of the *Polymyxa betae* life cycle except for the electron-dense mature resting spores. It is noteworthy that the virus appeared mainly in vacuoles of the zoosporangia, but not in those of the cystogenous plasmodia. The viral clusters found mainly at the external side of cystogenous plasmodia, were smaller and less numerous than in the zoosporangial plasmodia.

Our results clearly indicate that the virus is carried inside zoospores by young plasmodia and by zoosporangia; however, we were unable to explain whether the fungus acquires and transmits BNYVV by pinocytosis or by passage across the fungus membrane. Recently, Tamada and Kusume (1991) indicated that the 75-K readthrough protein encoded by RNA 2 is essential for transmission of BNYVV by *P betae*. The transmission efficiency is also enhanced by the 31-K protein encoded by RNA 4 (Lemaire *et al*, 1988; Tamada and Abe, 1989). It will be interesting to localize these proteins by the same gold-labelling technique in order to determine their role in the transmission mechanism.

ACKNOWLEDGMENTS

This work was mostly carried out during a 1-year postdoctoral stay at INRA Colmar by P Rysanek; we thank the Centre International des Étudiants et Stagiaires (CIES Paris) for financial support during this period and Ken Richards (IBMP Strasbourg) for precious help with the manuscript.

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