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Mushroom virus disease in the Netherlands: symptoms, etiology, electron microscopy, spread and control

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Abstract

During the 1960s, Dutch mushroom farmers suffered severe losses from an
infectious disease. Three types of virus particles were associated with the
disease: isometric particles 25 and 34 nm in diameter and bacilliform parti-
cles 19 nm wide and 50 nm long. Symptoms were highly variable. Two, possibly
three types of particle, were demonstrated in ultrathin sections of diseased
fruiting bodies; one type, the 34-nm particle, was observed in sections of
virus-infected mycelium from a nutrient medium and of basidiospores from
diseased mushrooms.

The disease spread with viable mycelium and spores from infected mush-
rooms. The time of infection governed loss of yield: earlier infection con-
siderably reduced yield, whereas later infection did not. Results of the
trials were used in drawing up control measures, which have been implemented
among Dutch growers and have considerably reduced national losses, as shown
by annual returns from the growers.

Contents

1 Introduction

Mushroom cultivation has expanded enormously in the Netherlands during the last twenty years: the number of growers increased from fifty in 1950 with a total production of about 250 000 kg mushrooms (Bels, 1962) to over a thousand, with an annual production of nearly 30 million kg mushrooms, in 1970 (van de Pol, 1971). Mushrooms used to be grown in caves in the South of the country (Bels-Koning & Bels, 1958), but are nowadays grown in specially constructed mushroom-houses.

In the 1960s the Dutch mushroom farmers suffered severe losses from a highly infectious disease, which proved to be due to virus. Growers could not eradicate the disease once their farms were contaminated. Yield was seriously reduced and the crop was of poor quality. A survey we held among Dutch growers showed that in 1967 and the first half of 1968, one in three mushroom farms was contaminated; on these farms average loss of yield was 15%. Thus in 1967 in the Netherlands, 4.5% or about 800 000 kg of mushrooms were lost, total yield being 17.5 million kg.

Little was known about spread of the disease, though English and American literature gave some hints (Gandy, 1960; Schisler et al., 1967). Reports on control were unsatisfactory (Last et al., 1967).

Most results of my research have been published in nine articles and are here collected by subject rather than in chronological order. Literature on mushroom virus disease has been reviewed in each paper.

Virus disease of cultivated mushrooms was the first clear example of a fungus infected by virus (Gandy & Hollings, 1962); later Blattný & Králík (1968) described a virus disease of the wild basidiomycete *Laccaria laccata* (Scop. ex Fr.) Cooke. There is considerable presumptive evidence that other fungi are subject to attack by viruses. Many reports describe virus-like particles in the fungi *Alternaria tenuis* Nees (Isaac & Gupta, 1964), *Penicillium stoloniferum* Thom (Ellis & Kleinschmidt, 1967), other *Penicillium* spp. (e.g. Banks et al., 1968, 1969), *Aspergillus foetidus* Thom & Raper (Banks et al., 1970), *Ophiobolus graminis* Sacc. (Lapierre et al., 1970), *Sclerotium cepivorum* Berk. (Lapierre et al., 1971), *Piricularia oryzae*

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2 A virus disease of cultivated mushrooms in the Netherlands

2.1 Etiology and symptomatology

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I   A virus disease of cultivated mushrooms in the Netherlands
    Neth. J. Pl. Pathol. 74: 48-51 (1968).

II A virus disease of cultivated mushrooms in the Netherlands
    Mushr. Sci. 7: 213-220 (1969).

A virus disease of cultivated mushrooms in The Netherlands

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Gandy (1960) was the first to demonstrate that a serious disease of the cultivated mushroom, *Agaricus bisporus* (Lange) Sing., could be transmitted be means of mycelium. Hollings (1962) isolated three types of virus particles from diseased fruit bodies; isometric particles with a diameter of 25 and 29 mμ respectively, and elongated particles with rounded ends, size 19 × 50 mμ. By injecting a small quantity of a cell-free virus preparation into the base of young carpophores he could prove its infectivity. Some weeks after injection a pure culture was made of mycelium from mushrooms growing on the inoculated trays. It showed the slow, abnormal growth, characteristic of the disease (Gandy and Hollings, 1962). At spawning mycelium from the injected trays was added to trays with healthy mycelium. The mushrooms produced were mis-shapen and cropping was greatly reduced (Hollings et al., 1963). The demonstration of infectivity, however, was not completed by means of re-isolation of virus particles from carpophores harvested from inoculated trays.

In the United States of America particles were also found to be connected with a transmissible mushroom disease. The particles observed had a diameter of 25 mμ (Hollings, 1965; Schisler et al., 1967). Infectivity of these particles was not demonstrated.

The present study was carried out in order to investigate the possible correlation between a transmissible, crop-reducing disease of cultivated mushrooms in The Netherlands which showed certain similarities with Mushroom die-back (Gandy and Hollings, 1962), and the three kinds of virus-like particles which could be isolated from affected mushrooms.

Samples of fruit bodies, collected at mushroom farms where the disease was reported, were subjected to a combination of purification procedures based on those described by Hollings et al. (1965) and by Kitano et al. (1961). The latter method was originally applied to ECHO 7 virus, and was found to give very good results when used for purification of potato leafroll virus from its vector *Myzus persicae* (Peters, 1967).

The purification procedure was as follows: either fresh carpophores, or carpophores which were stored at 4°C, were rinsed under running tap water and blotted with filter paper. After removing the lower parts of the stipes the fruit bodies were ground for 2 min in a Waring blendor in 30 ml of 0.033 M phosphate buffer containing 0.1 % thioglycollic acid, adjusted to pH 6.8, per 10 g of tissue. Fifty ml portions of the homogenate were subjected to ultrasonic treatment (Hollings et al., 1965) with a

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Kerry Vibrason cell disruptor (probe diameter 0.9 cm, output 50 W) for 10 min. During the treatment the thick-walled glass tube with an inner diameter of 3.5 cm, which contained the homogenate, was kept at 4°C. A slightly modified version of the method of Kitano et al. (1961) was applied to the sonicated homogenate. To one volume of homogenate, an equal volume of 2.5 M potassium phosphate buffer pH 6.8 and 0.8 volume of a mixture of 2-butoxyethanol and 2-ethoxyethanol in the proportions 1:2 were added. The phosphate buffer was prepared by mixing hot solutions of 2.5 M KH_2PO_4 and 2.5 M K_2HPO_4 until a pH of 6.8 was reached.

The components were mixed gently by hand. Centrifugation at 1000 g for 5 min resulted in the partition of an aqueous bottom phase, an organic top phase and a gelatinous interphase. After decantation of the liquids the interphase was resuspended in 20 ml 0.033 M phosphate buffer pH 6.8 per 100 ml of crude homogenate. Centrifugation at 5000 g for 10 min gave a clear supernatant which was subjected to ultracentrifugation at 105,000 g for 60 min.

The pellet was resuspended in 0.5 ml 0.033 M phosphate buffer pH 6.8 per 100 ml crude homogenate. Centrifuging at 5000 g for 10 min gave a clear, slightly opalescent supernatant. The virus-like particles could be observed, after negative staining with 2% phosphotungstic acid pH 6.0, in a Siemens Elmiskop1 electron microscope. In most samples of diseased mushrooms three types of virus-like particles were detected in varying concentrations:

1. Isometric particles, diameter about 25 mμ (identical to Hollings's Mushroom virus 1?). Fig. 2 and 3.
2. Elongated particles with rounded ends, size 19 × 50 mμ (identical to Hollings's Mushroom virus 3?). Fig. 2.
3. Isometric particles with a distinct hexagonal outline, diameter about 34 mμ (Fig. 1). The diameter of these particles differed markedly from that of Hollings's Mushroom virus 2 (diameter 29 mμ).

No virus-like particles were present in healthy mushrooms.

To test the infectivity of the cell-free preparations containing all three types of particles carpophores were grown in steam-sterilized 30 × 30 × 25 cm trays on an approximately 20 cm high layer of horse manure-straw compost which was covered with a casing layer of soil with a depth of 5 cm. The trays were kept at 15 to 17°C; the relative humidity was 70–80% and care was taken to keep the soil moist. The first crop (flush) of fruit bodies appeared five weeks after spawning.

Very young mushrooms of the first flush were, according to Gandy and Hollings (1962), inoculated with a purified preparation containing all types of particles by injection in the bases of the stipes with a hypodermic syringe and fine needle (27 G × 5/8″, Becton, Dickinson Cie.). The inoculated trays were observed daily and compared with the control trays, and once a week samples were collected from the trays which were tested for the presence of virus-like particles.

A few days after injection no particles could be detected in the inoculated mushrooms. Fruit bodies of the third flush, however, appearing two weeks after inoculation, showed long stipes and the off-white colour typical of the disease. These mushrooms contained the three types of virus particles, predominantly the 34 mμ "sphere" which also prevailed in the inoculum used in this experiment. Cropping ceased almost completely in the infected tray. Carpophores of the uninoculated control trays did not contain any particles.

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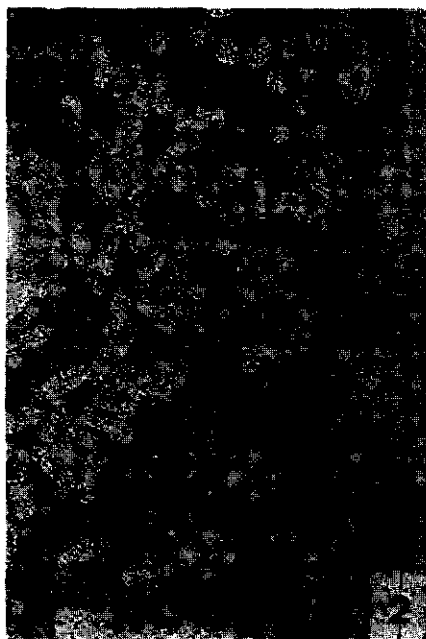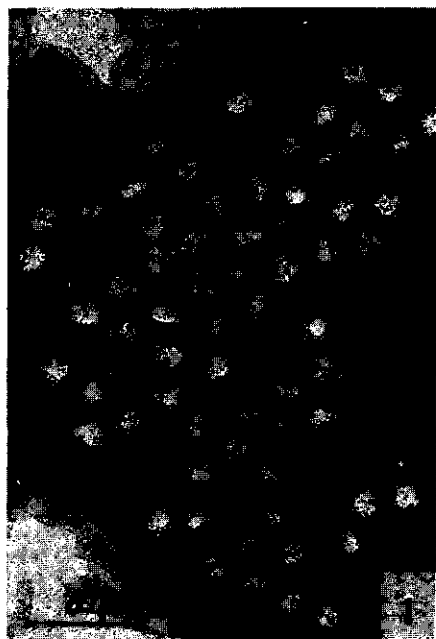
Fig. 1–3 are all at the same magnification ($\times 160,000$)
Fig. 1. Virus particles isolated from cultivated mushrooms, diameter 34 mμ. Some 25 mμ particles are visible. The preparation was negatively stained with phosphotungstic acid.
Fig. 2. Elongated virus particles with rounded ends, size 19 $\times$ 50 mμ, and 25 mμ particles
Fig. 3. Mushroom virus particles with a diameter of 25 mμ

Fig. 1–3 hebben dezelfde vergrotingsfactor (160.000 $\times$)
Fig. 1. Uit champignons geïsoleerde virusdeeltjes met een diameter van 34 mμ. Enkele 25 mμ-deeltjes zijn zichtbaar. Negatief contrast met fosforwolfraamzuur.
Fig. 2. Langwerpige virusdeeltjes met afgeronde einden, grootte 19 $\times$ 50 mμ, en 25 mμ-deeltjes
Fig. 3. Champignon-virusdeeltjes met een diameter van 25 mμ

Electron micrographs were taken by Mr. S. Henstra of the Service Institute for Applied Mechanics and Technical Physics in Agriculture at Wageningen.
De elektronenmicroscopische opnamen werden gemaakt door de heer S. Henstra van de Technische en Fysische Dienst voor de Landbouw te Wageningen.

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Thus it was demonstrated that a purified preparation containing the three kinds of virus particles was able to infect healthy mushroom cultures and that all types of virus particles could be re-isolated. The infection procedure did not always lead to success, probably due to the age of carpophores used in virus purification. Nevertheless, mechanical transmission will be an important tool in future infection experiments in which the infectivity of the three types of virus particles will be investigated separately and which may lead to the correlation of symptom expression with type of virus particle(s) involved.

Samenvatting

Een virusziekte van champignons in Nederland

In Nederland treedt een ziekte op in de champignoncultuur, die gelijkenis vertoont met de "Die-back disease" in Engeland. Uit zieke champignons werden drie soorten virusdeeltjes geïsoleerd: bolvormige met een diameter van 25 mμ (Fig. 2 en 3), respectievelijk 34 mμ (Fig. 1), en langwerpige deeltjes met afgeronde einden van 19 × 50 mμ (Fig. 2).
Inoculatie met een celvrij preparaat, waarin naast enige virusdeeltjes van 25 mμ en 19 × 50 mμ voornamelijk deeltjes van 34 mμ voorkwamen, bracht voor de ziekte karakteristieke symptomen teweeg. Herisolatie van de drie soorten virusdeeltjes uit deze kunstmatig geïnfecteerde culture bleek mogelijk te zijn. Mechanische inoculatie kan een belangrijk hulpmiddel zijn bij het onderzoek naar infectievermogen van de verschillende soorten virusdeeltjes afzonderlijk.

Acknowledgments

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A virus disease of cultivated mushrooms in the Netherlands

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Abstract

For the last couple of years the yield on many Dutch mushroom farms was reduced by a mysterious disease which showed certain similarities to 'Die-back disease' in England. Various symptoms of this disease are described.

The disease-causing agent was transmissible to healthy mushroom cultures by means of diseased mycelium, which showed a very slow and declining growth as compared to healthy mycelium. A great number of samples was taken from various mushroom farms where the unknown disease had been reported. A special purification method was applied and led to the observation of three types of virus particles in the samples under study: 1. 'spherical' particles, diameter 25 nm (Hollings's Mushroom virus 1?); 2. elongated particles with rounded ends, size 19 × 50 nm (Hollings's Mushroom virus 3?); 3. 'spherical' particles, diameter 34 nm, with a distinct hexagonal outline. These particles differ considerably from Hollings's Mushroom virus 2 (diameter 29 nm).

No virus particles were observed in samples of apparently healthy mushrooms.

Mostly we found a combination of the three types of virus particles in varying concentration ratios. The mixture has been shown to be infectious by mechanical inoculation of healthy mushrooms using a cell-free virus preparation.

Introduction

In 1948 a very serious infectious disease of cultivated mushrooms (*Agaricus bisporus* (Lange) Sing.) was observed in the United States of America; this disorder of unknown cause was called 'La France disease' (Sinden & Hauser, 1950). During the following years a wide variety of names was given to mushroom disorders having some characteristics in common, but all of uncertain origin (Brown disease, Watery Stipe, X-disease). In 1957 devastating crop losses occurred in Britain due to a similar disease. Gandy (1960) found that it was caused by an infectious agent, which could be transmitted by hyphal anastomosis. Experiments by Gandy & Hollings (1962) demonstrated the presence of three types of virus particles associated with the disease under investigation. It was named 'Die-back disease' to distinguish it from any other possible disorder and to refer to the loss of crop and the degeneration of the mycelium, which phenomenon appeared to be more characteristic than the symptoms of the fruit bodies. These symptoms are highly variable, probably depending upon environmental conditions mainly.

Disorders of this type were not reported in the Netherlands until 1964,

when a heavy outbreak occurred. Considerable losses of crop were incurred. In 1966 investigations into this disease were started.

Symptoms

The various symptoms going with the disease under investigation have been extensively described by several authors, and on the whole we can agree with them (Gandy, 1962; Schisler *et al.*, 1967). Under Dutch conditions the following symptoms are observed, often in combination:

1. Locally the mycelium does not permeate, or hardly permeates, the casing layer, or it disappears from the casing layer after initial normal growth. In these areas no fruiting occurs (bare zones, fig. 1); the immediate result is, of course, a serious loss of crop. All around these areas mushrooms are found in dense clusters, maturing too early. The barren zones are often marked by such competitors as *Botrytis crystallina* (Bon.) Sacc. or *Sporendonema purpurascens* Bon.

2. Mycelium isolated from diseased sporophores on agar shows a slow and degenerated growth as compared with healthy mycelium, as was already reported by Gandy in 1960. In Petri dishes with peak-heated ground, and sterilized compost similar differences are very clearly visible after the relatively short time of one week or less (A.P.A. Oversteyns, pers. comm.).
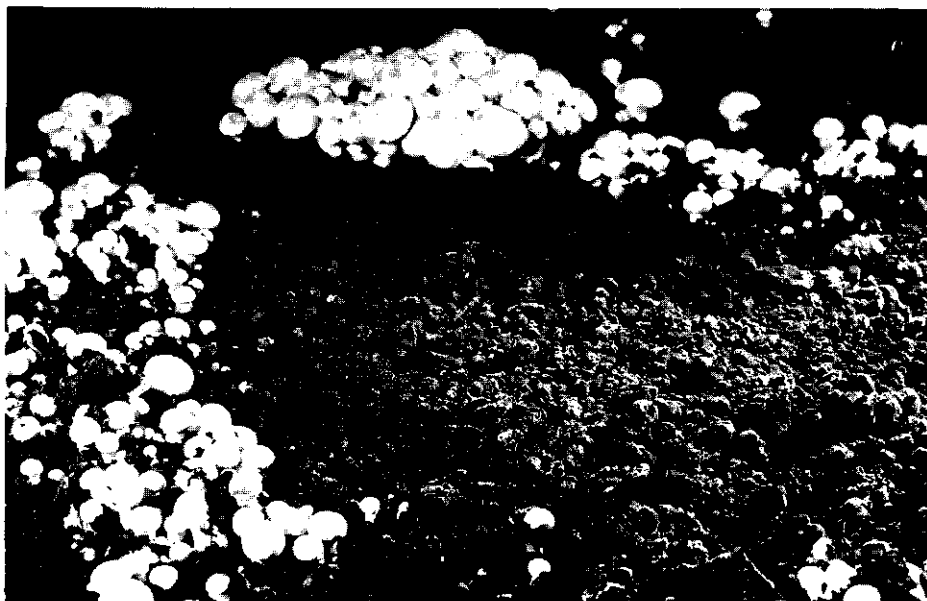
Fig. 1. Bare zone (photograph: P. J. C. Vedder).

3. The delayed appearance of the pinheads of the first flush can be an important indication of the disease, as well as the formation of the fruiting primordia below the surface of the casing layer. As soon as these mushrooms appear above the casing soil, their pilei are already opened.

4. Symptoms of sporophores are highly variable. The following abnor-

Fig. 2. A: Elongated stipes with small, early maturing caps. B: 'Drumsticks' (photographs: P. J. C. Vedder).

Fig. 3. A: Thickened, barrel-shaped stipes with small, flat pilei. B: Brown, slimy caps. The stipes are tapering downwards (photographs: P. J. C. Vedder).

malities can be found, separately or together:
— off-white colour of the caps; early maturity.
— slow development of the pinheads; dwarfing.
— elongated, slightly bent stipes; sometimes with small, early maturing pileus

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(fig. 2A). The stipes can be very thin ('drumsticks', fig. 2B).
— the mushrooms are loosely attached to the substrate: at the slightest touch they are pushed over.
— watery stipes; streaking in the stipes.
— stipes are spongy. They quickly turn brown on cutting and show an abnormal structure.
— thickened, barrel-shaped stipes; the veil is attached to the thickest part of the stipe, thus lower than usually. Pilei are small and flat (fig. 3A).
— brown, slimy caps occur owing to a secondary bacterial rot; stipes are sometimes tapering downwards (fig. 3B); during the first flush sometimes a few light-brown caps can be observed.
— abnormal or absent veils; 'hard gill'.
   5. A specific, musty smell can be perceived in a diseased growing room.

Isolation of virus particles

   Samples of fruit bodies, collected at mushroom farms where the disease was reported, were subjected to a combination of purification procedures described by Hollings *et al.* (1965) and by Kitano *et al.* (1961). The former method includes ultrasonic treatment, the latter is based on an organic solvent phase system. The virus particles are retained in the interphase, from which they can be se-
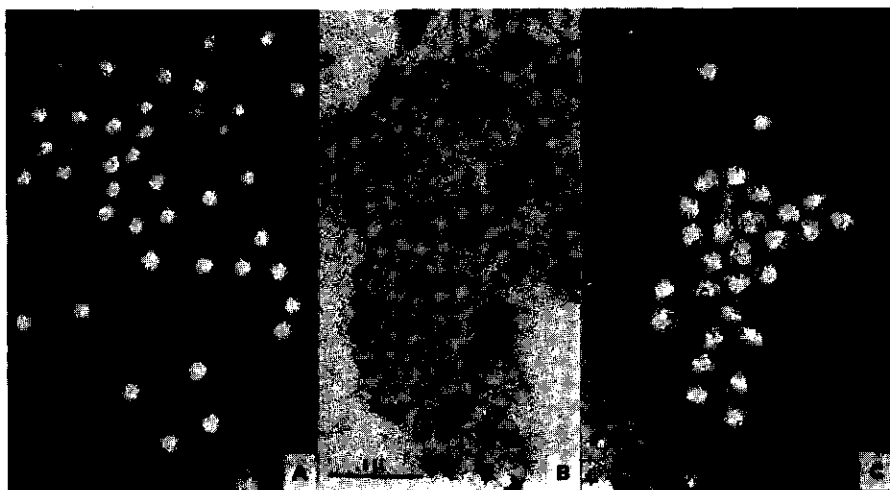

Fig. 4. Mushroom virus particles, negatively stained with phosphotungstic acid (electron micrographs: TFDL, Wageningen). A: isometric particles, diameter 25 nm. B: elongated particles with rounded ends, size 19 × 50 nm. C: isometric particles, diameter 34 nm.

parated by ultracentrifugation. The particles could be observed, after negative staining with phosphotungstic acid, in an electron microscope.

In most samples of diseased mushrooms three types of virus particles were detected in varying concentrations (Dieleman & Temmink, 1968):

1. isometric particles, diameter about 25 nm (identical to Hollings's Mushroom virus 1?) (fig. 4A).

2. elongated particles with rounded ends, size 19×50 nm (identical to Hollings's Mushroom virus 3?) (fig. 4B).

3. isometric particles with a distinct hexagonal outline, diameter about 34 nm. The diameter of these particles differed markedly from that of Hollings's Mushroom virus 2 (diameter 29 nm) (fig. 4C).

Usually no virus particles were present in seemingly healthy mushrooms; rarely some 25 nm particles were observed.

Infection experiments; re-isolation of virus particles

The disease is easily transmitted with the aid of infected mycelium. The supply of a small quantity of diseased mycelium, which was grown on sterilized compost, to a healthy mushroom tray leads to the development of symptoms of the disease.

Infection experiments with cell-free virus preparations are far more difficult to perform. Gandy & Hollings (1962) proved the infectivity of a cell-free virus preparation by injecting a small quantity into the base of sporophores. The demonstration of infectivity, however, was not completed by means of re-isolation of virus particles from sporophores harvested from inoculated trays.

We have tested the infectivity of the cell-free preparations containing the three types of particles mentioned before. For that purpose mushrooms were grown in steam-sterilized 30 $\times$ 30 $\times$ 25 cm trays on an approximately 20 cm high layer of horse manure-straw compost which was covered with a casing layer of soil with a depth of 5 cm. The trays were kept at 15 to 17°C; the relative humidity was about 80%. The first flush appeared five weeks after spawning.

Very young mushrooms of the first flush were, according to Gandy & Hollings (1962), inoculated with a purified preparation by injection into the bases of the stipes with a hypodermic syringe and fine needle. The inoculated trays were observed daily and compared with the control trays; once a week samples were collected from the trays which were tested for the presence of virus particles, by means of the previously indicated purification procedure.

A few days after injection no particles could be detected in the inoculated mushrooms. Fruit bodies of the third flush, however, appearing two weeks after inoculation, showed long stipes and the off-white colour typical of the disease. These mushrooms contained the three types of virus particles, predominantly the 34 nm 'sphere' which also prevailed in the inoculum used in this experiment. Cropping ceased almost completely in the infected tray. Sporophores of the uninoculated control trays did not show any symptoms or contain any particles.

However, this infection procedure did not always lead to success.

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Experiments to investigate the importance of the three types of virus particles observed are being performed. This research is greatly hampered by the lack of a simple and reliable test method. The want of such method is also felt in the study of the epidemiological aspects of the disease. In recent years, however, our knowledge of this disease has made considerable progress.

Résumé
Une maladie à virus chez le champignon de couche cultivé aux Pays-Bas

Depuis quelques années, on constatait dans beaucoup de champignonnières hollandaises une diminution de rendement causée par une maladie inconnue qui avait une certaine ressemblance avec le 'Die-back disease' en Angleterre. Différents symptômes sont décrits.

Cette maladie pouvait être transmise aux cultures saines de champignons de couche au moyen de mycélium infecté, qui présentait une croissance très lente et retardée par rapport au mycélium sain. De nombreux échantillons furent prélevés dans des champignonnières où la maladie inconnue s'était manifestée. Une méthode de purification spéciale a été employée et a conduit à l'observation de trois types de particules de virus dans les échantillons étudiés:

1. des particules 'sphériques', diamètre 25 nm (Hollings' Mushroom virus 1?);

2. des particules allongées avec des bouts arrondis, 19×50 nm (Hollings' Mushroom virus 3?):

3. des particules 'sphériques', diamètre 34 nm, avec des contours hexagonaux distincts. Ces particules se distinguent considérablement de Hollings' Mushroom virus 2 (diamètre 29 nm).

Aucune particule de virus n'a été observée dans des champignons apparemment sains.

Le plus souvent, on a trouvé les trois espèces de particules de virus présentes ensemble et à des concentrations variées. Le mélange s'est montré transmissible par l'inoculation mécanique de champignons sains en utilisant une préparation de virus acellulaire.

Zusammenfassung
Eine Viruskrankheit beim kultivierten Champignon in den Niederlanden

Seit einigen Jahren wurden in vielen holländischen Champignonbetrieben die Erträge verringert durch eine unbekannte Krankheit, die gewisse Ähnlichkeit mit 'Die-back disease' in England zeigte. Verschiedene Symptome werden beschrieben und illustriert.

Diese Krankheit wurde durch verseuchtes Mycel in gesunde Champignonkulturen übertragen. Das kranke Mycel zeigte im Vergleich zu gesundem Mycel ein sehr langsames und verzögertes Wachstum.

Eine grosse Anzahl Proben wurde aus verschiedenen Betrieben genommen, bei denen diese unbekannte Krankheit aufgetreten war. Es wurde eine spezielle Reinigungsmethode angewendet, die zur Feststellung von 3 Arten von Viruspartikeln in den untersuchten Proben führte:

1. 'kugelförmige' Partikel, Durchmesser 25 nm (Hollings' Mushroom virus 1?).

2. längliche Partikel mit abgerundeten Enden, 19×50 nm (Hollings' Mushroom virus 3?)

3. 'kugelförmige' Partikel, Durchmesser 34 nm, mit einem deutlichen sechseckigen Umriss. Diese Partikel unterscheiden sich beträchtlich von Hollings' Champignon-Virus-2 (Durchmesser 29 nm).

In Proben von gesund aussehenden Champignons wurden keine Viruspartikel gefunden.

Meistens wurde eine Kombination der 3 Typen von Viruspartikeln in wechselnder Zusammensetzung gefunden. Die Kombination war übertragbar durch mechanisches Beimpfen gesunder Champignons mit einem zellfreien Viruspräparat.

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2.2 Additional information and discussion

Mushroom virus disease has now been recognized in many mushroom-growing countries all over the world, and has probably been present as long as mushrooms were grown. It was successively reported from the United States (Sinden & Hauser, 1950), England (Anonymous, 1957), Denmark (Hansen & Block, 1967), the Netherlands (Section 2.1, 1968) and Australia (Paterson, 1968).

The most plausible explanation of its late detection (1964 in the Netherlands) is that the disease had been present for a long time but was not recognized earlier because of the variable and often unclear symptoms; in poor mushroom cultures, any bare zone or misshapen fruiting body is not conspicuous. The occurrence of virus disease was established, by isolation of virus particles from suspected mushrooms, in caves of the St Pietersberg near Maastricht, a rather isolated former centre of mushroom growing. Local growers did not consider these infected mushrooms abnormal, although they had long stipes and were very loosely attached to the substrate. Virus-infected spores may have been present in the caves for years; once established, it would be extremely difficult to control the disease.

Symptoms varied. Usually there were bare zones, where the casing soil was devoid of mycelium, surrounded by mushrooms with long stipes and small, early maturing and off-white caps. The fruiting bodies were often loosely attached to the substrate and inferior. But bare zones and long stipes may have other causes. Isolation of virus particles from suspected mushrooms is decisive, if mushrooms are correctly sampled, e.g. from around bare zones.

Usually characteristic, but likewise variable, is the weaker growth on agar of mycelium isolated from infected fruiting bodies than of healthy mycelium. Some isolates hardly grow, whereas others show moderate to almost normal growth rates. The individual growth rates are maintained even after repetitive transfer to fresh media, although some, especially moderately growing cultures sometimes recover slowly. Because of the wide variation and because some infected isolates grow almost normally, this interesting feature is no reliable test. Moreover, an aberrant growth on agar may be caused by other factors such as composition of the nutrient medium (Fritsche, 1969).

Apart from the method of Kitano et al. (1961) at pH 6.8, none of the many clarification procedures tested yielded cell-free virus preparations

clean enough to be examined by electron microscope. At higher pH virus particles were severely damaged; at lower pH virus particles were even completely lost, especially particles 34 nm in diameter. Hence the butanol-clarification method, which Hollings (1962) used, was unsatisfactory: it did not yield clean virus preparations and obviously destroyed the 34-nm particles. Hollings & Stone (1971) had similar experience.

The change in incidence of the different types of mushroom virus particle in the United Kingdom in the past five years, as suggested by Hollings et al. (1971), is unlikely. They state that the 34-nm virus particles are now the most prevalent, whereas these particles were seldom observed in 1967. By then, however, they had introduced another test for mushroom virus (in crude juice) instead of those used previously, which probably caused more damage (Hollings et al., 1967). With crude juice, the unstable 34-nm particles could now survive.

In the method of Hollings et al. (1967), mushrooms are squeezed with a hand-press through cheese cloth, and the expressed juice is mixed with phosphotungstic acid and viewed directly in the electron microscope. Though very convenient, I found that the method was not a good test for virus; it often yielded preparations with too much debris, in which virus particles could not easily be detected. Although more laborious, the method of Kitano et al. (1961) at pH 6.8 proved more satisfactory. As a rule portions of 30 g of mushrooms were ground; a quantity of 50 ml was treated with ultrasound and further processed. Several steps of the procedure are critical, for instance the molarity of the potassium phosphate buffer must be precisely 2.5, or else it does not work. The method is limited because of the extremely high, perhaps damaging, salt concentration and the 'corrosiveness' of the organic solvents, and of the ultrasonic treatment before clearing. For complete purification and isolation of the different types of mushroom virus particles, probably a much milder procedure is required for clarification.

Although Hollings et al. (1971) stated that the mushroom virus particles 34 nm in diameter were seldom observed in 1967, I have isolated such particles from most samples of diseased mushrooms since 1966, usually with one or two of the other types in varying concentrations. Cell-free preparations from severely diseased fruiting bodies from a highly contaminated farm sometimes only contained a few virus particles, whereas almost normal mushrooms now and then contained vast numbers of particles; the reverse sometimes occurred too. So far, no relationship has been encountered between symptoms and types or amount of virus particles. Mushrooms were considered infected

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if electron-microscopic preparations contained either a few clusters of 34-nm particles, or many 25-nm particles. Both 34-nm and 25-nm particles have been found separately, but the other particle types may not have been noticed or may have been absent from the cell-free preparations through poor performance of the clarification technique or through minute concentrations in the fruiting bodies. In cell-free preparations, there could be more of virus particles 34 nm in diameter than of the other two particle types.

The concentration of the individual particle types might be affected by factors like climatic conditions (temperature; relative humidity; nutritional value or condition of the compost), pathogenicity of viral strains, and genetic properties of the mushroom host. Further research is needed to elucidate these aspects. Another question is, the frequent occurrence of a few particles 25 nm in diameter in cell-free preparations from apparently healthy mushrooms; such mushrooms were taken to be healthy.

So far I have not managed to isolate virus particles from the tiny bit of material (some dozens of milligrams) that mushroom mycelium, and especially infected mycelium, provides on agar. Hollings et al. (1965) suggested direct electron-microscopy of mycelium treated with ultrasound. I found this method unsatisfactory. Attempts to isolate virus particles from larger amounts (up to a few grams) of infected mycelium, grown in a shaken liquid malt medium, by ultrasonic treatment followed by the method of Kitano et al. (1961) at pH 6.8, failed. The resulting cell-free preparations were extremely dirty. Probably virus particles must be isolated from mycelium by another clarification method than for fruiting bodies.

As the three types of mushroom virus particles often occur together, a mutual relationship, though unlikely, could not be excluded. To test this possibility, however, the three types of virus particles must be completely purified and isolated from each other, in order to inoculate them separately into mushroom cultures. After disappointing results for purification, particularly with the 34-nm particles, we considered whether the virus particles might be membrane-bound or attached to some cell constituents, rather than free in the cytoplasm of mushroom cells. Consequently more information was needed on the intracellular appearance of mushroom virus. Because of the special character of virus-infected mycelium, research started with a very slowly growing infected culture. Chapter 3 deals with this and other aspects of ultrastructure.

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3 Electron microscopy

3.1 Intracellular appearance of mushroom virus

Papers:

III   Intracellular appearance of mushroom virus
Virology 39: 147-152 (1969).

IV   Intracellular appearance of mushroom virus in fruiting bodies and
basidiospores of *Agaricus bisporus*
Virology 47: 94-104 (1972).

Intracellular Appearance of Mushroom Virus

Virus particles have been found associated with "Die-back disease" of cultivated mushroom, *Agaricus bisporus* (Lange) Sing. (*1, 2*). Recently, it has been proved that this disease is caused by one or more viruses (*3*). In cell-free preparations extracted from diseased mushrooms and stained with phosphotungstic acid, usually three types of virus particles were observed: isometric particles with a diameter of 25 mμ and 34 mμ, and elongated particles with rounded ends, 19 × 50 mμ (*3*).

Since the disease under investigation is the first clear case of a fungus attacked by virus, it was interesting to know whether the particles could be detected in the fungus cells with the aid of common procedures, and in which part of the fungus cell mushroom virus occurs. Moreover such an approach might elucidate the role of the three kinds of virus particles associated with "Die-back disease." The present report deals with the detection of mushroom virus in diseased mycelium of *Agaricus bisporus*.

Preliminary attempts to demonstrate virus particles in tissue of mycelium and fruit bodies, embedded in styrene methacrylate resin, were unsuccessful. Therefore a suitable working procedure was developed by comparing several methods with pelleted virus. For this purpose virus-diseased mushrooms were ground in 0.033 *M* phosphate buffer containing 0.1 % thioglycollic acid, pH 6.8, and subjected to a combination of two purification procedure
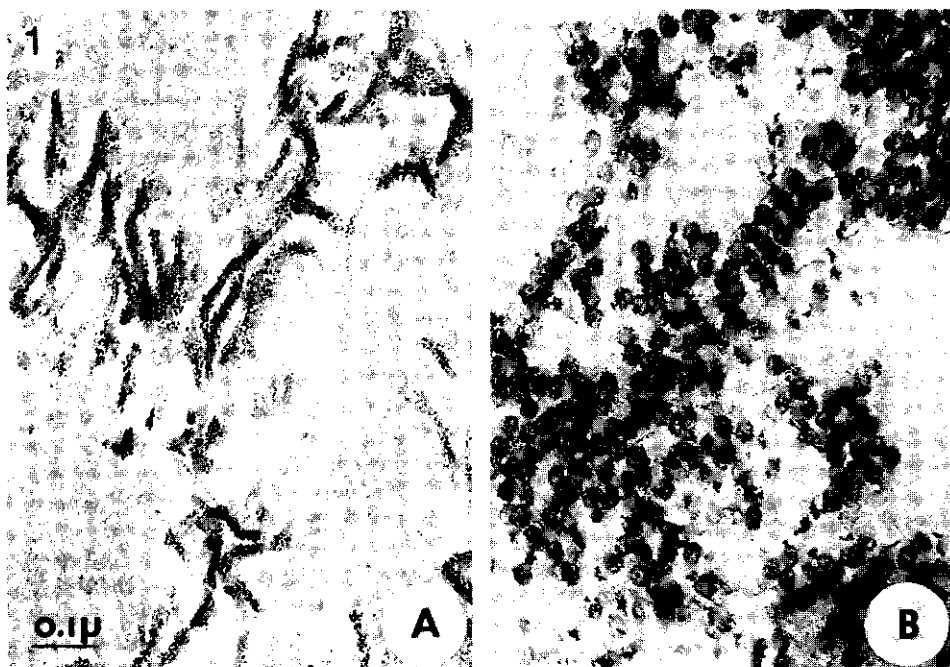
Fig. 1. Ultrathin section of pelleted mushroom virus. A. Without pre-staining. B. Pre-stained with 0.5% uranyl acetate overnight after fixation. Note the dark centre in some of the particles. Electron micrograph: Laboratory of Virology, State Agricultural University, Wageningen.

including ultrasonic treatment (4), the other based on an organic solvent phase system (5), as was described previously (3). The virus particles are retained in the interphase, from which they can be separated by ultracentrifugation at 105,000 g for 60 min. Immediately after removing the supernatant liquid the pellets were pre-fixed in the centrifuge tubes for 1 hour in 6% (v/v) glutaraldehyde buffered to pH 6.8 with 0.1 M phosphate buffer. The pellets could then easily be detached from the tube wall. They were washed in three changes of the buffer and post-fixed for 1 hour in 1% (w/v) osmium tetroxide in the same buffer. The whole fixation procedure was carried out at 4°. After washing, one pellet was left overnight in 0.5% uranyl acetate in water at 4° (6, 7). The other pellet immediately after fixation, was subjected to dehydration in a graded series of ethyl alcohol and propylene oxide, and embedded in a 1:3 mixture of

Epon 812 and Araldite 6005 (8). The following day the pre-stained pellet was treated in the same way. Sections were cut with a glass knife on an LKB Ultrotome III and picked up on Formvar-coated 150 mesh copper grids. The sections were post-stained for ½ hour in 2% uranyl acetate and for 5 min in Reynolds's lead citrate (9), and examined with a Siemens Elmiskop 1 or a Philips EM-300 electron microscope. Figure 1 shows that pre-staining of the material overnight in uranyl acetate was essential for clearly demonstrating the mushroom virus particles. Consequently this method was applied to the fungus tissue.

Mycelium, isolated from healthy and from virus-diseased mushrooms, was grown on 2% Biomals agar in petridishes for 10–14 days at 25°; virus-diseased mycelium usually showed a slow and degenerate growth as compared to healthy mycelium (10, 11).

Abbreviations used in Figures: ER endoplasmic reticulum   NP nuclear pore   N nucleus   NU nucleolus   NM nuclear membrane   M mitochondrion

FIG. 2. Section of virus-diseased mushroom mycelium on agar. A. Fungus cell with an aggregate of virus particles close to a nucleus. B. Enlargement of the virus area. C. Enlargement of some particles. Note the dark centre. Electron micrograph: Technical and Physical Engineering Research Service (TFDL), Wageningen.

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Intracellular Appearance of Mushroom Virus in Fruiting Bodies and Basidiospores of *Agaricus bisporus*

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Virus particles of 34 nm diameter were detected in ultrathin sections of cap, stipe, and basidiospores of *Agaricus bisporus* (Lange) Sing. These particles formed dense aggregates in vegetative mycelium, but occurred dispersed and often abundantly in the cytoplasm and sometimes in vacuoles of fruiting bodies. In basidiospores 34 nm virus particles were found grouped together in small vacuoles and occasionally in the spore cytoplasm. Virus particles of 19 × 50 nm were seldom observed in cytoplasm of the stipe. Isometric particles of 25 nm, resembling ribosomes, were found in disarranged cells of the cap of mushrooms known to contain many 25 nm virus particles. These isometric particles occurred in vacuoles, free or clumped together and membrane bound, or were aggregated into membrane-limited electron-dense bodies. In such cells many 34 nm particles were also observed. In cap and stipe cells of the same mushrooms helices, thought to consist of ribosomes, were often found.

Virus particles of 34 nm diameter were observed in dolipores, which implies cell to cell translocation. The means of spread of mushroom virus disease by hyphal anastomosis and by spores from diseased mushrooms were confirmed.

No viruslike particles, electron-dense bodies, or helices were found in tissue or spores from healthy mushrooms.

INTRODUCTION

The virus nature of an infectious disease of the cultivated mushroom, *Agaricus bisporus* (Lange) Sing., was established by Hollings (1962), when he isolated virus particles of 25, 29, and 19 × 50 nm from diseased mushrooms. The disease, first noticed in 1948 (Sinden and Hauser, 1950) and having various names including "La France disease" and "Die-back disease," is spread by viable mycelium (Gandy, 1960) and by spores from infected mushrooms (Schisler *et al.*, 1967). Symptoms are reviewed by several authors (Gandy, 1962; Schisler *et al.*, 1967; Dieleman-van Zaayen, 1969).

In cell-free preparations from diseased mushrooms, stained with phosphotungstic acid, usually three types of virus particles were observed (Dieleman-van Zaayen and Temmink, 1968): isometric particles with diameters of 25 and 34 nm, and elongated particles with rounded ends, 19 × 50 nm. The 34 nm particles had a distinct hexag-

onal outline. When a cell-free virus preparation was injected into the stipes of young mushrooms on a growing-tray, symptoms developed a few weeks later in mushrooms of a subsequent crop; from these mushrooms, the three types of virus particles, predominantly the 34 nm particles, could be reisolated (Dieleman-van Zaayen and Temmink, 1968). Then Hollings (1968) also observed the 34 nm virus particles, so far overlooked probably because of their unstable nature.

Mushroom virus was earlier detected in ultrathin sections of virus-diseased vegetative mycelium of *A. bisporus* on agar (Dieleman-van Zaayen and Igesz, 1969).

The ultrastructure of healthy tissue of this basidiomycete fungus was briefly reviewed by Manocha (1965); Scannerini (1967) and Thielke (1967, 1969) described some aspects such as lomasomes, and basidia, in greater detail.

The present report deals with the detection of two, possibly three, types of mush-

room virus particles in ultrathin sections of fruiting bodies, and of one particle type in basidiospores.

MATERIALS AND METHODS

Diseased fruiting bodies were collected from contaminated mushroom farms. Healthy mushrooms were obtained from the Mushroom Experiment Station at Horst (L.), the Netherlands.

Fruiting bodies. Cap and stipe tissue of fresh, young mushrooms was prepared and sectioned as described by Dieleman-van Zaayen and Igesz (1969). As they stated, prestaining *en bloc* with 0.5 % uranyl acetate in water overnight after fixation (Strugger, 1956; Hess, 1966) is essential for clearly demonstrating the mushroom virus particles. This method was employed throughout the present work.

Mushroom virus in pellets obtained by ultracentrifugation was treated as indicated by Dieleman-van Zaayen and Igesz (1969).

Basidiospores. Mushroom spores are about $7 \times 5 \mu m$ in diameter, more or less ovoid, and have a thick, hardly permeable wall. To bypass the difficulties encountered in fixing dormant spores (Hawker, 1965; Bracker, 1967) these were induced to germinate prior to fixation. The techniques used in spore collection, germination, and preparation of ultrathin sections are described elsewhere (manuscript in preparation).

RESULTS

Fruiting Bodies

Sporophore tissue consists of loosely arranged hyphae. Most of our observations are pertinent to cap cells, which are usually smaller than cells of the stipe and appear more or less circular in cross section; they are rich in cytoplasm.

Occurrence of 34 nm diameter particles. Rather high concentrations of virus particles were observed throughout the fruiting body, in cytoplasm of base, stipe, and cap. The particles were of the same size as those occurring in vegetative mycelium and also showed a dark center. The particles, however, were not 25 nm in diameter as mentioned before (Dieleman-van Zaayen and Igesz, 1969), but represented the larger

mushroom virus particles of 34 nm, as will be clear from comparison of sectioned virus pellets (Fig. 1B) with negatively stained virus particles (Fig. 1A). In Fig. 1B the dark center is clearly visible in the large (34 nm) virus particles. Measuring these particles in ultrathin tissue sections yielded an average diameter of 30 nm.

As was indicated earlier, in vegetative mycelium the virus particles usually occur in dense aggregates, often near a septum (Fig. 2) or close to a nucleus. In the mushroom cap, virus particles of 34 nm are observed either in loose aggregates in some part of the cell (Fig. 4), or throughout the cell (Fig. 5) in the cytoplasm. Although the cap tissue mainly consists of loosely arranged hyphae, the cells in the lamellar trama of the gills are arranged in a regular fashion (Manocha, 1965) and are closely connected. Septal pores are abundant in this region. The septal pore apparatus of *A. bisporus* is of the dolipore type, described by Girbardt (1958) and by Moore and McAlear (1962). Dolipores connect the cytoplasm of two adjacent cells. Figure 6 shows a median section through a septum, revealing virus particles on either side of the dolipore; a movement of the virus particles in a certain direction is suggested. Figure 7 shows 34 nm particles in a dolipore, which implies cell-to-cell translocation.

Occasionally virus particles were found in vacuoles, in both cap and stipe tissue (Fig. 8). Particles were sometimes observed in association with an osmiophilic substance which nearly filled the vacuole (Fig. 9).

Cells invaded with 34 nm particles showed no definite signs of deterioration as compared to cells from healthy mushrooms.

Occurrence of $19 \times 50 nm$ particles. In cytoplasm of the stipe occasionally some particles of $19 \times 50 nm$ were observed (Fig. 3). Detection of these virus particles is difficult for the following reasons: (1) Their amount in diseased mushrooms generally seems to be minute as judged by amounts in cell-free virus preparations; (2) these particles are often inadequately stained in ultrathin sections (see Fig. 1B, the pelleted virus); (3) they are difficult to distinguish in cross section, since their diameter of 19 nm

resembles that of small ribosomes. The particles may very well be present in cap tissue but remain undetected.

Occurrence of 25 nm particles. In cap tissue of a mushroom sample, known to contain a large amount of 25 nm virus particles in addition to virus particles of 34 and 19 × 50 nm, particles with the same diameter as ribosomes (about 22 nm) were observed in vacuoles, either dispersed or clumped to-
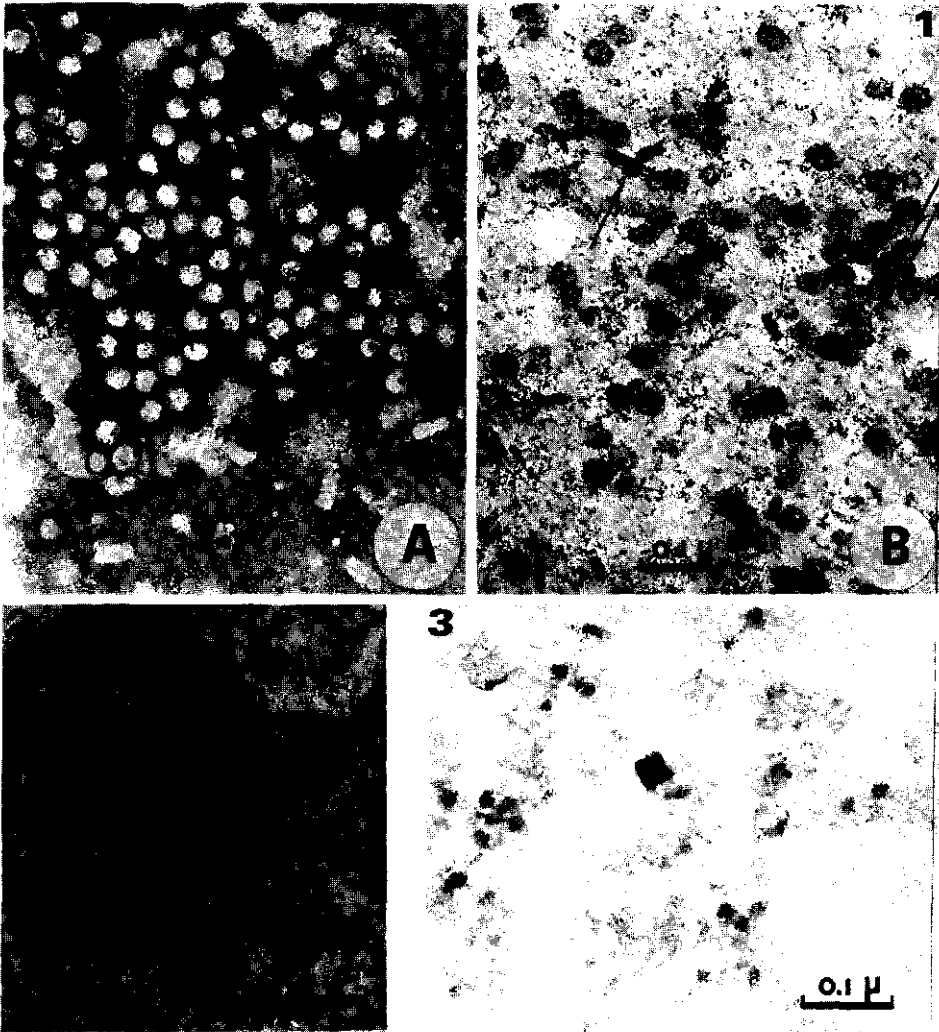
FIG. 1. Virus particles extracted from diseased mushrooms: isometric particles with diameters of 25 and 34 nm, and elongated particles with rounded ends, 19 × 50 nm. (A) Cell-free preparation, negatively stained with phosphotungstic acid. (B) Ultrathin section of pelleted mushroom virus, stained with uranyl acetate and lead citrate. Arrows indicate the deeply stained 25 nm particles. Note the dark center in the 34 nm particles.

FIG. 2. Ultrathin section of diseased vegetative mycelium, grown on 2% Biomalsagar. Dense aggregate of 34 nm virus particles near a septum. *PA*, septal pore apparatus (dolipore). Marker indicates 500 nm.

FIG. 3. Section of mushroom stipe tissue. Three virus particles of 19 × 50 nm, aggregated side to side, in the cytoplasm.
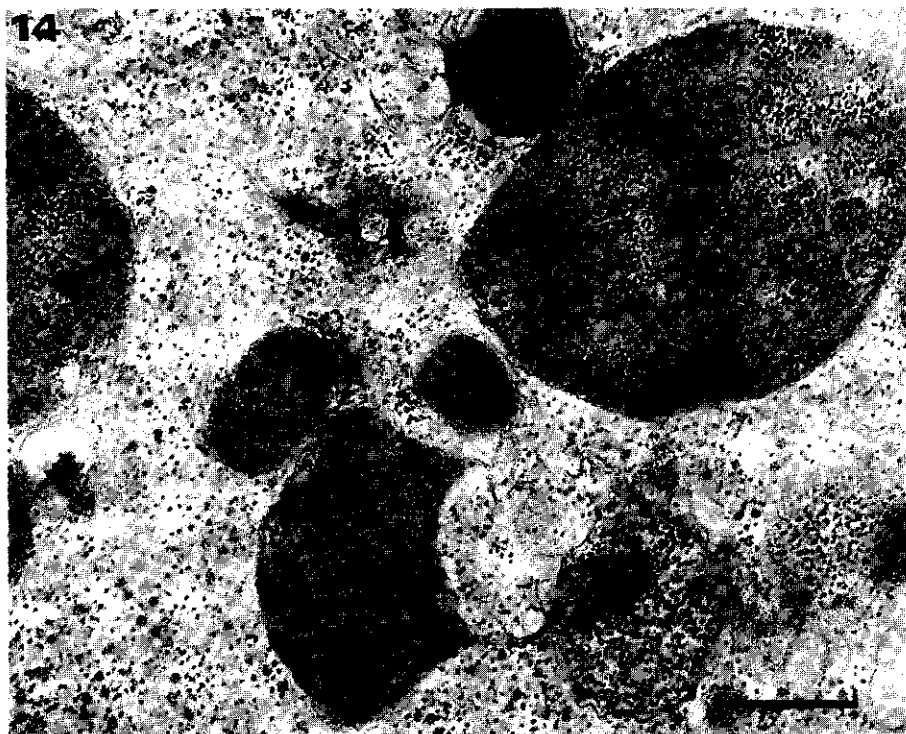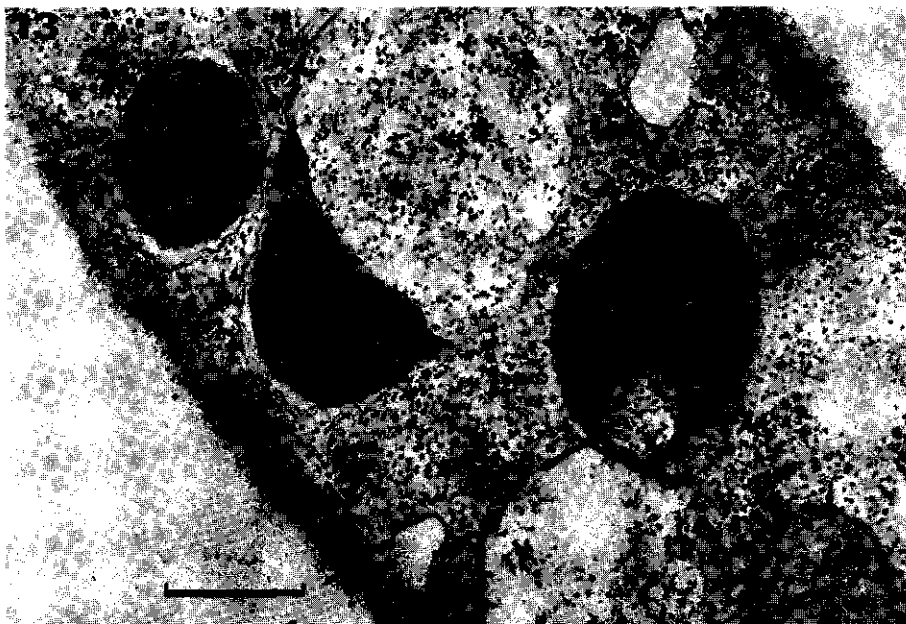
FIGS. 13 and 14. Ultrathin sections of diseased mushroom cap, from a mushroom sample known to contain a large amount of 25 nm virus particles in addition to particles of 34 and 19 × 50 nm. Membrane-bound, electron-dense bodies of various sizes and shapes, possibly composed of 25 nm virus particles, occurring in rather disarranged cells. The cells contain a large number of 34 nm virus particles. The bodies show some inner structure. Markers indicate 500 nm.
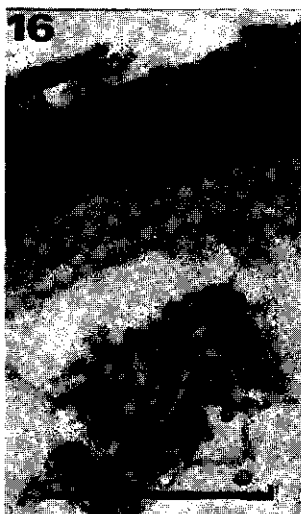
Figs. 15–17. Ultrathin sections of basidiospores from virus-diseased mushrooms. Virus particles of 34 nm are grouped together in small vacuoles. The spores are enveloped by a thick wall. Markers indicate 500 nm.

Fig. 15. Section of a spore containing lipid bodies (*L*) and virus particles (*V*).

Fig. 16. Detail of basidiospore with 34 nm virus particles.

Fig. 17. Germinating basidiospore with 34 nm virus particles (*V*), ribosomes and mitochondria, and a "typical membrane complex" (*MB*), which is often found in fungus spores. *GT*, germ tube.

hundreds per section. They usually were grouped together in small vacuoles, and sometimes occurred in the spore cytoplasm.

The germinating spores otherwise showed normal spore constituents (Fig. 17). Spores from healthy mushrooms did not possess viruslike particles in their small vacuoles. Further differences between spores from diseased and healthy individuals were not revealed. The walls of "healthy" spores may be slightly thicker than those of "diseased" spores, as suggested by Schisler et al. (1967). However, the observed differences in wall thickness could also be due to changes during germination.

In "diseased" spores, no other types of virus particles were observed. They could, however, readily be missed or overlooked.

In germ tubes from "diseased" spores, large numbers of 34 nm virus particles were found. They occurred in dense aggregates, as in the vegetative mycelium. In germ tubes from "healthy" spores no viruslike particles were detected.

DISCUSSION

Virus particles of 34 nm diameter have been shown to occur in large amounts in all kinds of mushroom tissue and in basidiospores. The means of spread of the disease via hyphal anastomosis (Gandy, 1960) and by spores from diseased mushrooms (Schisler et al., 1967), have now been confirmed. Methods to prevent or control the disease, based on the means of spread, are described elsewhere (Dieleman-van Zaayen, 1970).

The micromorphology of fungus cells closely resembles that of higher plant cells. Similarly, the ways of spread of mushroom virus resemble those of some plant viruses transmissible by grafting or seed, and the process of cell to cell translocation is comparable. Passage from cell to cell of 34 nm mushroom virus particles most probably takes place through dolipores, as that which occurs in higher plants through plasmodesmata (Esau et al., 1967; DeZoeten and Gaard, 1969).

Germinating spores of A. bisporus sometimes appear to be partly empty. This can be caused by consumption of storage products during germination. Another explana-

tion may be that the principal storage product in fungi, glycogen (Zalokar, 1965; Wells, 1965) has been washed out by the procedure employed, i.e., en bloc staining with aqueous uranyl acetate. This technique implies disappearance of the greater part of glycogen, and clumping of the residual glycogen into irregular masses (Manasek, 1969; Vye and Fischman, 1970). The method was required to detect 34 nm virus particles (Dieleman-van Zaayen and Igesz, 1969).

The application of this en bloc prestaining with aqueous uranyl acetate implies that the electron-dense, membrane-bound bodies consisting of numerous particles cannot be made up of glycogen. The particles composing the dense bodies definitely do not represent ferritin, which was seldom found as small, regularly arranged particles in healthy germinating tubes. The dense bodies may be made up of ribosomes, which are very deeply stained by the procedure employed. However, aggregation of ribosomes into membrane-bound bodies is improbable. Electron-dense bodies similar to those consisting of 25 nm particles have been reported by Gerola et al. (1966) to occur in the young sieve elements of the leaf secondary veins from Brassica chinensis L. infected with turnip yellow mosaic virus (TYMV). The roundish bodies contained a number of osmiophilic particles, believed to be virus particles. This similarity is accompanied by a remarkable morphological resemblance; the structure of 25 nm mushroom virus particles is the same as that of TYMV, i.e., a $T = 3$ structure with hexamer-pentamer clustering of subunits (J. T. Finch, personal communication).

Unidentified, membrane-bound, dense bodies were reported by Thielke (1967) to occur in the maturing basidium of A. bisporus. In a later publication (Thielke, 1969), the presence of vacuoles with an electron-dense material which was membrane-bound, was mentioned instead of the dense bodies. A probable analogy with secondary vacuoles in growing cells of the stipe was assumed. In that case, the phenomenon described by Thielke could be related to the vacuole containing an osmiophilic substance, shown in Fig. 9.

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3.2 Morphological resemblances of fungus viruses to those of higher plants

Mushroom virus particles 25 nm in diameter are remarkably similar in structure to particles of turnip yellow mosaic virus (TYMV). Centrifuging in a sucrose (10-40%,w/v) density gradient often yielded a top component, most probably consisting of empty protein shells.
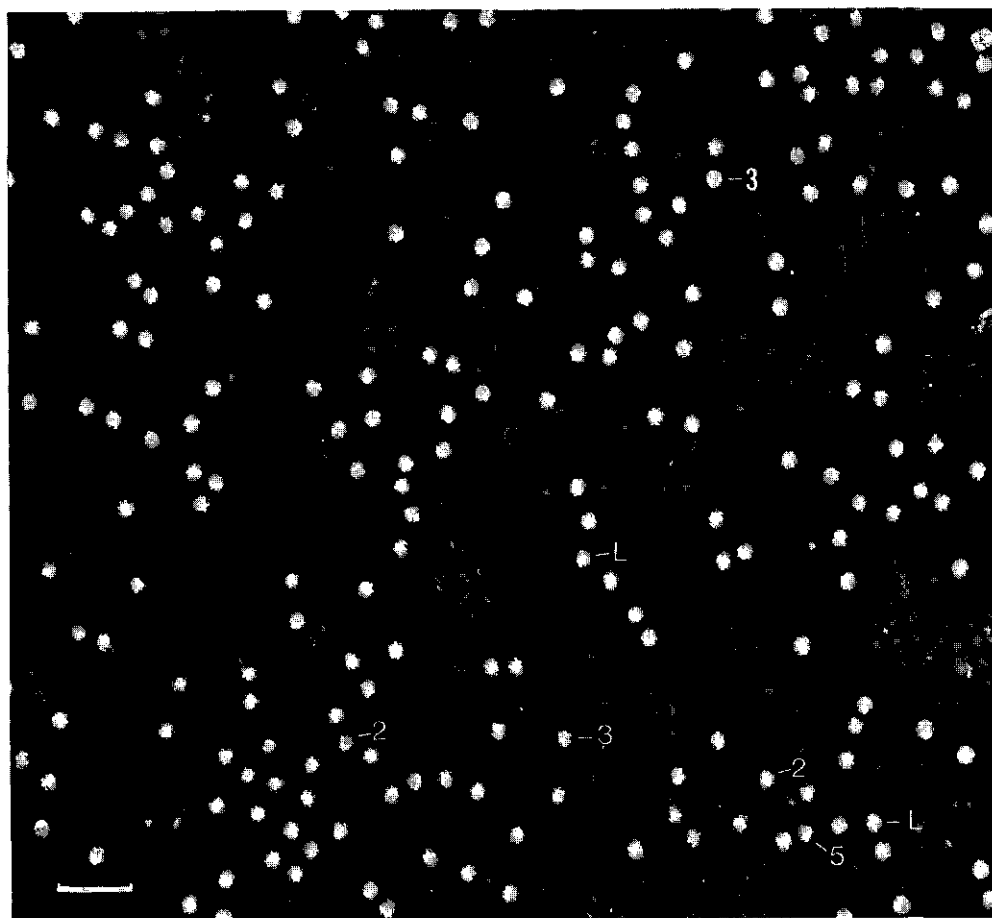

Fig. 3.2.1. Mushroom virus particles 25 nm in diameter, clarified, centrifuged in a sucrose density gradient (10-40%) and negatively stained with phosphotungstic acid. Some particles show 2-fold views (2), others are close to 3-fold views (3), 5-fold views (5) or are close to the local 2-fold direction (L). The marked scale is 100 nm.
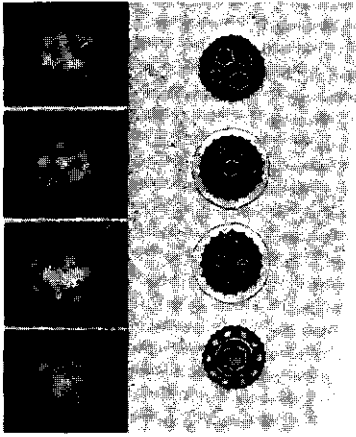
Fig. 3.2.2. Mushroom virus particles 25 nm in diameter, enlarged from Fig. 3.2.1, showing a 2-fold view, being close to a 3-fold view, to the local 2-fold direction and to a 5-fold view (from top to bottom, left row). The right row is a sketch of corresponding views of a model (after Finch & Klug, 1966).

Figs 3.2.1 and 3.2.2. show that the particle is the same in structure as TYMV, which is composed of 32 large morphological units arranged in the $T = 3$ icosahedral surface lattice, where T is triangulation number, with hexamer-pentamer clusters of 180 structure units (Finch & Klug, 1966). Some mushroom virus particles obviously show 2-fold views (a view of the particle down a 2-fold axis, labelled 2), others are close to 3-fold views (3) or 5-fold views (5). Particles marked L are close to the local 2-fold direction.

Thus the 25-nm particles have 180 protein subunits in obvious hexamer-pentamer clusters, with accessory particles which are probably empty protein shells, so that they look closely related to viruses of the turnip yellow mosaic virus group, described by Harrison et al. (1971).

Cell-free preparations with 25-nm mushroom virus particles did not react with antiserum against TYMV. No beetles are known to feed on culti-vated mushrooms, so that a beetle vector of the 25-nm particles is highly improbable. Although the virus does not necessarily have to share all prop-erties with the type member of the turnip yellow mosaic virus group to merit inclusion in the group, some properties will be decisive. For instance, the nature of the viral nucleic acid will be of importance for classification. So far this factor is not known.

Similar striking resemblances and differences between a fungal virus and a plant virus were encountered after isolating rod-shaped virus-like particles from apothecia of *Peziza ostracoderma* Korf (syn. *Plicaria fulva* R. Schneider). The particles closely resembled those of tobacco mosaic virus. During research on mushroom virus disease they were observed several times

45

Intracellular Appearance and Some Morphological Features of Viruslike Particles in an Ascomycete Fungus

Peziza ostracoderma Korf [synonym *Plicaria fulva* R. Schneider] is a fungus frequently occurring as a contaminant in mushroom nurseries. Fruitbodies (apothecia) of this Ascomycete collected from nurseries occasionally contain rod-shaped viruslike particles which closely resemble tobacco mosaic virus (TMV) (*1*). From such apothe-

rod-shaped viruslike particles present in a fungus, it seemed worthwhile to find where these particles were in the cells of the apothecia of *P. ostracoderma*. The way of preparing and sectioning the tissue was identical to that applied to mycelium of the cultivated mushroom *Agaricus bisporus* (Lange) Sing. (*3, 4, 5, 6*). Ultrathin sections were ex-
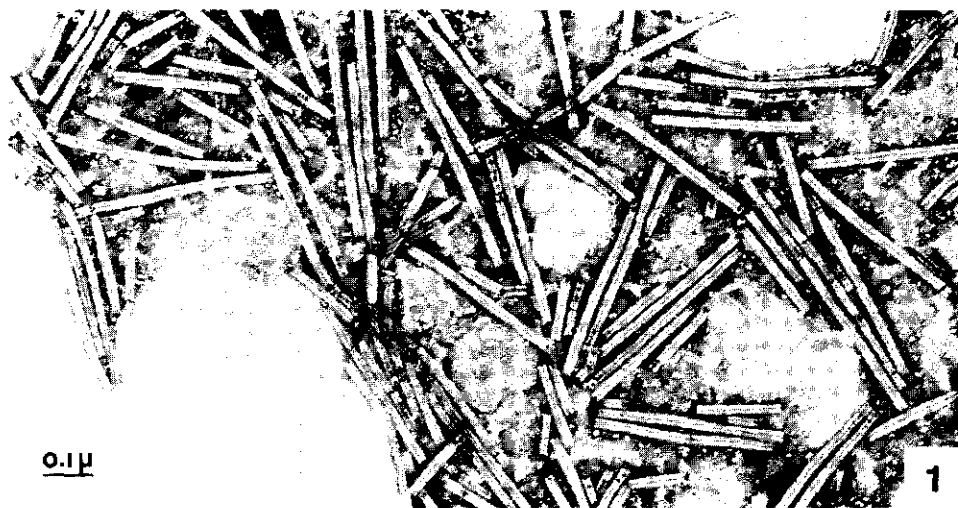
FIG. 1. Rod-shaped viruslike particles isolated from *Peziza ostracoderma* Korf. Negative stain with phosphotungstic acid (pH 5.5). Electron micrograph: Technical and Physical Engineering Research Service (TFDL), Wageningen.

cia, cell-free preparations with the rod-shaped particles were obtained by a purification method devised for isolating virus from cultivated mushrooms (*1, 2*). Particles negatively stained with phosphotungstic acid, pH 5.5, averaged 17 × 350 nm in size and had a clear axial canal (Fig. 1). Apothecia that contained such particles did not differ macroscopically from those without particles (*1*). Since this is the first reported case of

amined with a Siemens Elmiskop 1 or a Philips EM-300 electron microscope.

In apothecia from samples known to contain the viruslike particles, crystallike arrangements of the particles were found in cells just below the asci. They were often observed in vacuoles (Fig. 2) and sometimes in the cytoplasm. The crystallike aggregates consisted of rods arranged crosswise (Fig. 3). Sectioning these aggregates at different an-

gles and at different levels, revealed particles alternately in transverse and in longitudinal array, as well as in crosshatched patterns (Fig. 2).

Similar crystalline arrangements have been demonstrated to occur in plant cells infected with a strain of TMV (7).

Cross sections of the rods occasionally showed a central core. In cells of apothecia from samples without viruslike particles, no particles or particle aggregates as illustrated in Fig. 2 and 3 were found. Vacuoles have been observed in some cells of these fruitbodies, but the vacuoles containing particles typically also contained cell debris (Fig. 2).

It was not possible to inoculate preparations of the rod-shaped viruslike particles into *P. ostracoderma*, because apothecia of the fungus could not be cultivated. Low concentrations of similar particles occasionally have been found in cell-free preparations from cultivated mushrooms (1). Attempts to infect mushrooms with the rods failed. Mechanical inoculation of cultivated mushrooms, however, is difficult even with the mushroom virus (2, 8).

To check for possible identity of the observed particles with TMV, indicator plants for this virus were inoculated with prepara
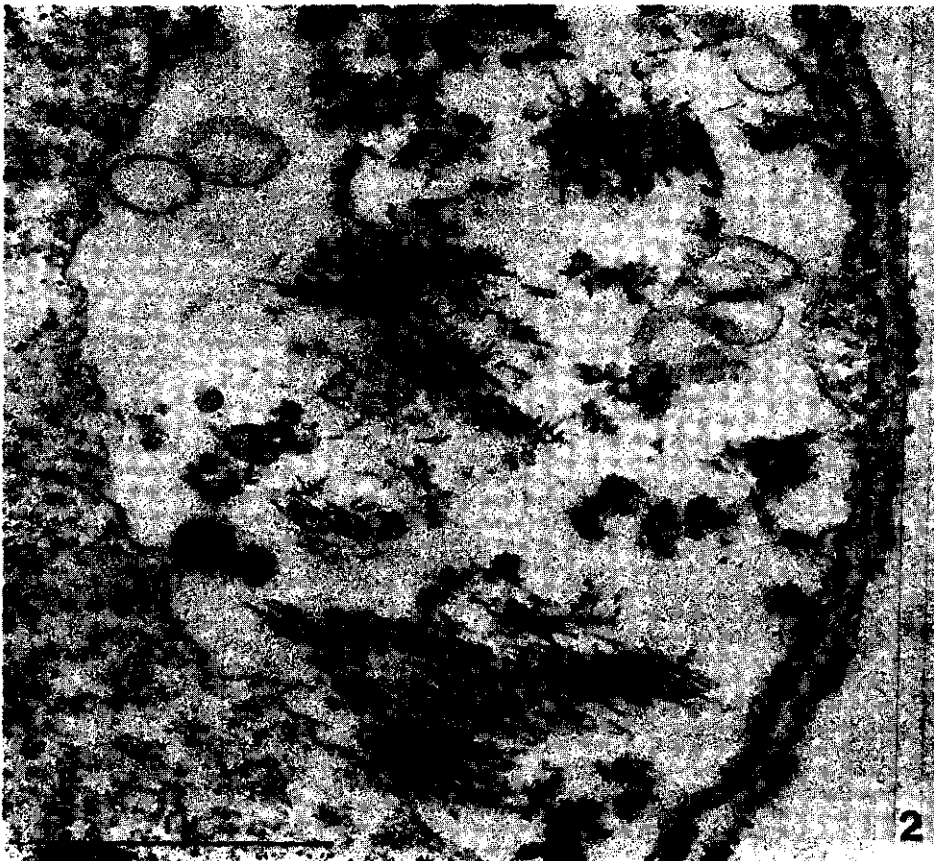
FIG. 2. Ultrathin section of fruitbody of *Peziza ostracoderma*. Fungus cell with aggregates of viruslike particles in vacuole. Electron micrograph: Laboratory of Virology, State Agricultural University, Wageningen, and TFDL, Wageningen.
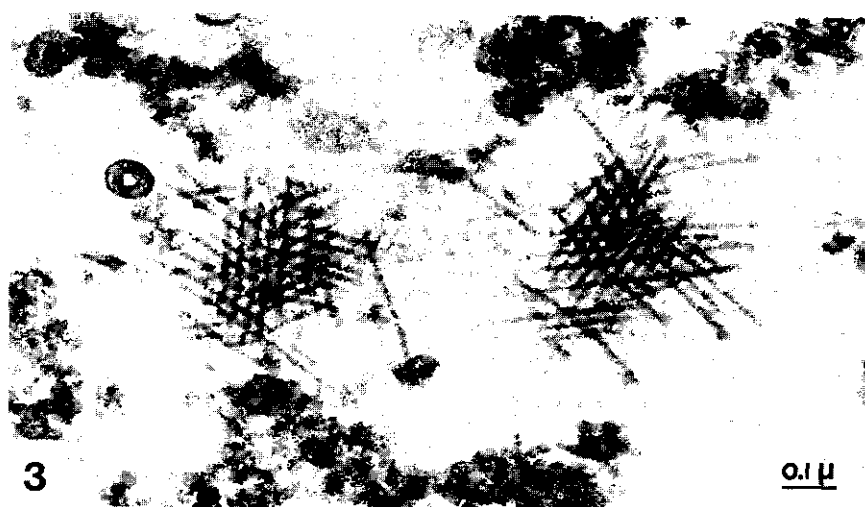
FIG. 3. Crosswise arrangement of rod-shaped viruslike particles in vacuole of a cell of *Peziza ostracoderma*.
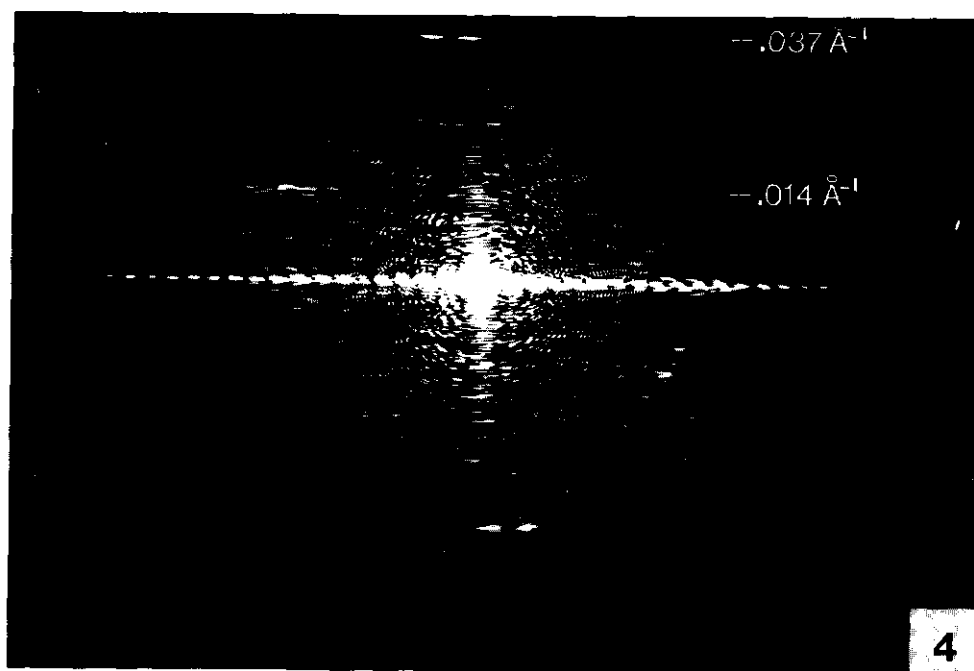
FIG. 4. Optical diffraction pattern of viruslike particles, negatively stained with uranyl formate. Near-meridional intensity occurs on a layer line at $0.037\ \text{Å}^{-1}$ corresponding to a periodicity of about $27\ \text{Å}$ in the particle image. Traces of a layer line at $0.014\ \text{Å}^{-1}$ can also be seen. Photograph: MRC Laboratory of Molecular Biology, Cambridge.

tions of the viruslike particles (1), but no symptoms developed.

Cell-free preparations containing the rods did not react with antiserum against TMV in microprecipitation tests. Nor did the particles in a preparation that had been incubated with TMV-antiserum, mounted in negative stain and examined in the electron microscope, differ in appearance either from other particles that had been incubated with normal serum, or from particles that had been incubated in saline. TMV particles treated with antiserum became clumped and covered with a layer of antibody molecules. These results, and lack of symptoms in inoculated indicator plants indicate that the observed particles are not identical with TMV. Moreover, optical diffraction patterns of electron micrographs of the particles, negatively stained with uranyl formate (9), revealed that their structure differed appreciably from that of TMV (10). In Fig. 4 the near-meridional intensity on the layer line at 0.037 Å^{-1} shows that the particle structure is helical with a basic pitch of about 27 Å which is larger than the 23 Å pitch of TMV. The layer line at 0.014 also is not exactly one-third of 0.037 as it would be in TMV, thus the structure does not repeat after three turns as precisely as that of TMV. Apart from a superficial resemblance, the fungal viruslike particles differ distinctly from TMV.

Whether *P. ostracoderma* can be artificially infected and whether the fungus will show symptoms after infection, is currently under study, as well as the significance of the rods in *A. bisporus*.

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lected from diseased mushrooms known to contain mainly an abundance of 34-nm particles. Infection trials showed, however, that virus particles of 25

3.4 Additional information and discussion
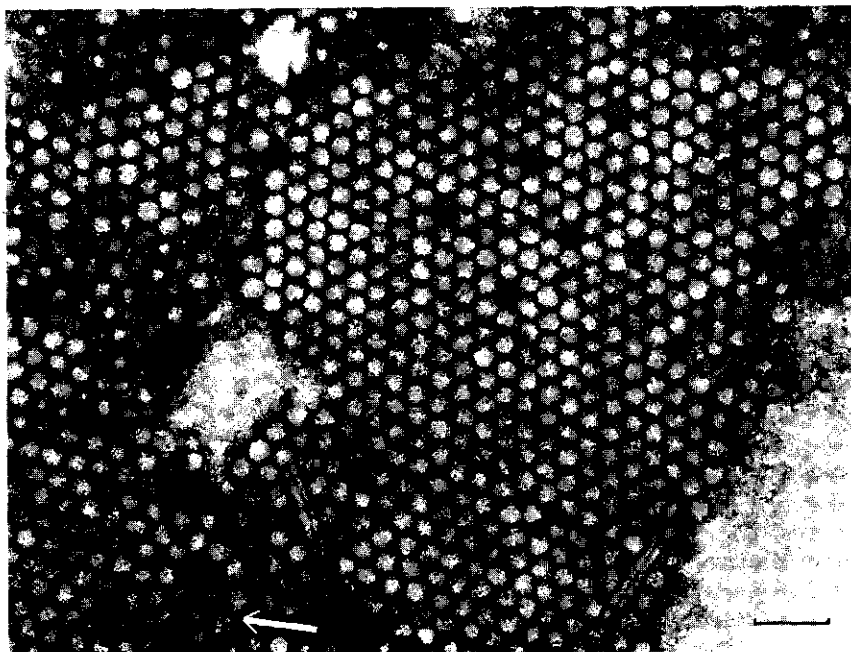
Fig. 3.4.1. Electron microgram of mushroom virus particles 25 nm in diameter; some 34-nm particles (arrow) and a few rod-shaped virus-like particles 17 x 350 nm are visible. Note that the aggregated 25-nm particles look angular and hexagonal in outline. Negative stain with phosphotungstic acid. The marked scale is 100 nm.

and of 19 x 50 nm can also be transmitted by spores; such particles still have to be demonstrated in infected spores. Also, in ultrathin sections of infected mycelium, only 34-nm virus particles have been found; they occurred in dense aggregates, but still in much smaller amounts than may occur in fruiting bodies. Both virus particles of 25 nm and of 19 x50 nm are difficult to detect: 25-nm particles resemble ribosomes, 19 x 50-nm particles are often inadequately stained. If their concentration in mycelium be lower than that in fruiting bodies, as with the 34-nm particles, detection is extremely difficult.

It would still be possible to purify 25-nm particles and to study their properties, to find out if they are serious applicants for the turnip yellow mosaic virus group in most properties, which would be of interest to virus classification. However, these particles are not always numerous enough for

purification trials. Moreover, cultivation of virus-infected material (fruiting bodies) has proved to be complicated: either the cultures do not produce any mushrooms, or the mushrooms formed contain too few particles or none of the desired type at all. For purification trials, I usually obtained diseased mushrooms from commercial farms.

The role played in mushroom virus disease by the rod-shaped virus-like particles, frequently isolated from *Peziza ostracoderma*, should be examined. Unpublished later studies indicate, that they differ distinctly from TMV, not only from the common strain but also from the aucuba strain, which forms the angled-layer aggregates in Turkish tobacco (Warmke, 1967, 1968). Recently the structure of this strain, kindly provided by Dr M.K. Corbett (Maryland, United States) was examined by Dr J.T. Finch (Cambridge, England). Optical diffraction patterns and X-ray diffraction studies pointed out that its structure is very like that of the common TMV-strain both in pitch and in helical parameters (3-turn repeat). Aggregation of particles into a specific arrangement apparently has nothing to do with the construction and the pitch of their helix; in fact, similar angled-layer aggregates have been reported to be formed by detached bacterial pili of 7 x 500-2000 nm with a rigid helical structure and a pitch of 24 Å (Brinton, 1965).

The rod-shaped particles associated with *P. ostracoderma* and sometimes observed in cell-free mushroom virus preparations, definitely differ from the TMV-particles found associated with some Erysiphaceae (Yarwood, 1971; Nienhaus, 1971). Their low concentration in *P. ostracoderma* and especially in *A. bisporus* is a great hindrance for further research.

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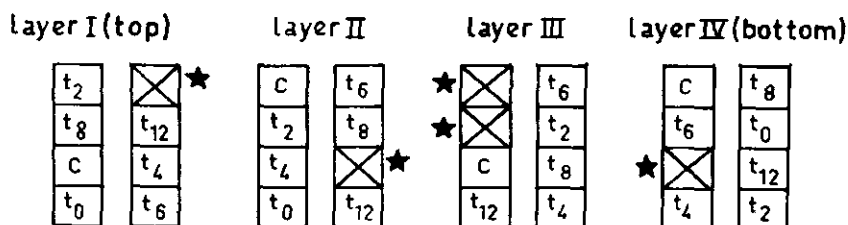
layer I (top)   layer II   layer III   layer IV (bottom)

★ These plots were used for other experiments.
For the same reason, from layer III, t_0 had to be omitted.

Fig. 4. Scheme of treatments for the experiment concerning the importance of time of infection

between the layers at the Experimental Station. Because of this a different treatment was applied in each plot of one layer, so that each treatment was done once in each layer. Lots were drawn to decide how to divide up the various treatments over the plots of a layer to ensure that any effect of one treatment on another was merely arbitrary; infections will sometimes spread from one plot to another (see **Fig. 4** for the scheme of treatments). Infection took place each time in the middle of a plot with 2.5 gram self-prepared, diseased spawn. For the spawning, 375 gr. spawn per m² was used.

All infected plots were obviously diseased: all showed symptoms of the virus disease and virus particles could be isolated from the mushrooms.

The yield of each plot was ascertained separately (uncut mushrooms). After a good three weeks of harvesting, the growing-room was cooked out as is usual with virus experiments. The test was planned in four repetitions so that the yields could be processed mathematically, but this proved completely superfluous: the differences were obvious enough. The yields of four plots at a time that had received the same treatment were now added (see **Table I**). The last column shows the yields per m² if the mushrooms had been cut ($= -20\%$). For the missing yield of plot t_0, which was withdrawn, the expected yield was determined mathematically from the other yields.

The figures recorded in the last column of **Table I** are plotted in **Fig. 5**.

Table I
Yields after various times of infection

Inoculation (days after spawning)	Total yield (kg)	kg/m²	kg/m² — 20% (cut mushrooms)
0	3.630	0.9	0.7
2	25.560	3.2	2.6
4	24.170	3.0	2.4
6	43.555	5.4	4.3
8	54.755	6.8	5.4
12	100.400	12.5	10.0
Control (uninoculated)	160.150	20.0	16.0

68

Mushroom yields
(kg/m²)
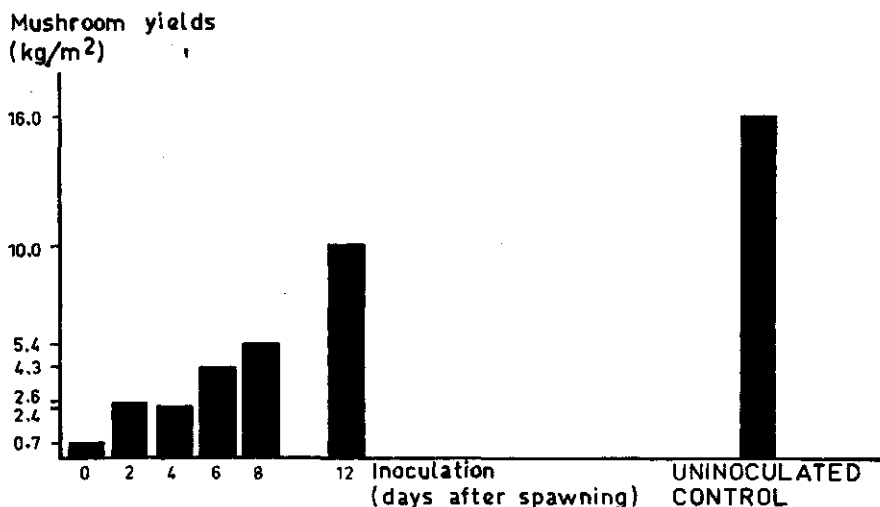
Fig. 5.   Mushroom yields in kg/m² (cut mushrooms) at various times of infection

Note that the control plots showed a yield of 16 kg/m² (cut mushrooms) in a good three weeks of picking, which may certainly be considered a very high yield. From the remaining figures and from the graph, it is obvious that,

(a) using a *constant* quantity of infectious matter, the time of infection plays a very important part in the virus disease;

(b) infection during or shortly after spawning has the most serious effect;

(c) even 8 days after spawning, however, infection will cause a 66% loss in yield;

(d) early infection will have even more disastrous consequences if it takes place in more than one spot at once, as will be the case in practice, and not in one spot only, in the middle of the plot such as here.

Infection during spawning or shortly afterwards can happen, for instance, because the spawning machine has been inadequately disinfected, but more likely because there is still viable, diseased mycelium present in the wood of shelves and trays. Another possible source of infection is the presence of 'diseased' spores on the premises, which fall on the compost during or after spawning and germinate there. 'Diseased' spores often germinate better and faster than healthy ones, as was noted by Schisler *et al.* (1967), and which can be read also from **Table 2**. The percentages in this table were obtained in the following way: Spores of diseased and healthy mushrooms, respectively, were collected on filter paper and afterwards transferred to a nutrient medium (2% Biomalsagar) in test tubes. For Experiment I, 48 tubes were provided with 'diseased' spores, and 48 with healthy ones; for Experiment II, the number was 60 in each case. It was decided each day, by a visual inspection, whether the spores had germinated. Spore germination is expressed in percentage of test tubes in which germination was observed.

As can be seen from the table, spores of diseased mushrooms had already germinated for the greater part after eight days on agar; in the spawned compost this will probably happen even more quickly and completely.

Table 2

Germination of healthy and 'diseased' spores on an artificial nutrient medium (2% Biomalsagar) in test tubes

No. days after transfer to agar	Experiment I (No. tubes: 2 × 48) % tubes with germinated healthy/'diseased' spores		Experiment II (No. tubes: 2 × 60) % tubes with germinated healthy/'diseased' spores	
7	0	0	0	40
8	0	43.7	8.3	71.6
9	32.2	60.3	13.3	90
10	54.8	74.9	26.6	90
15	67.6	83.3	66.6	93.3
20	67.6	83.3	73.3	93.3
25	*no longer considered*		80	93.3

When 'diseased' spores fall during spawning, for instance, on the compost, this is comparable with infection eight days after spawning (see **Table I** and **Fig. 5**). As mentioned earlier, the yield loss then is no less than 66% in relation to the control, at least with infection in one place only. But spores especially will turn up in the compost in various places at once and this can cause a real disaster.

From this too, it is once more apparent that *infection during or shortly after spawning*, whether this occurs with viable, diseased mycelium or with 'diseased' spores, will have most serious consequences.

3. The part played by used baskets

By growing mushrooms in baskets which came from an infected mushroom farm it was proved that multiple-use baskets (especially wooden picking baskets, but also cardboard boxes lined with plastic) can play their part in spreading the virus disease. The mushrooms showed symptoms of the disease and virus particles were found.

In the wooden baskets and in the boxes, remains of mycelium and spores were found; the latter especially can be conveyed into the growing-room by ventilation air. The same baskets will reach various farms.

The solution to this problem is to use non-returnable baskets; used baskets could, if necessary, be disinfected (disinfection of wooden baskets: cook-out in a growing-room after completion of crop, thus 12 hours at 70°C. (158°F.); disinfection of cardboard boxes; heat for 6 hours at 70°C.). A farm, however, can be infected before an opportunity has arisen to disinfect the baskets on the premises, as we have observed a few times. The use of multiple-use baskets, therefore, must be discouraged, especially in countries where there are many spores in existence, due to the preference for large, open mushrooms.

4. The isolation of the virus growing-room itself

After nearly two years of experiments on the virus disease in the isolated growing-room of the Experimental Station, not one of the other growing-rooms has been infected with the disease. Although such a well-isolated growing-room, attended to by only one person, cannot be compared

with a mushroom-growing business, it still gives an indication of the measures to be taken in practice to control the disease.

Control

A. The effect of sodium pentachlorophenate*

One of the principal ways in which the virus disease is spread is by viable, diseased mycelium. When the wood of trays or shelves is cooked out before emptying, it is highly probable that even at a temperature of 70°C. in the compost for a period of 12 hours, not all parts of the wood will reach a sufficiently high temperature to destroy the remains of mycelium contained in it. New wood is usually impregnated so that mushroom mycelium cannot grow into it, but this is not repeated later.

Sodium pentachlorophenate (SPCP) was tested on its qualities as a means of preserving wood, partly because of the favourable results which seemed to have been obtained abroad (principally in Denmark). A preliminary test with several trays in the virus growing-room of the Experimental Station showed early promising results. A test on a larger scale was carried out at a commercial mushroom farm, in which 159 (not disinfected) trays were immersed in a solution of 2% SPCP, to which soda (Na_2CO_3) had been added to enable the solution to spread more efficiently over the wood. Ten trays were left untreated as control. The immersion consisted of wetting the trays thoroughly for some seconds. After drying the trays were hosed down with a considerable quantity of water to prevent possible subsequent damage to the mushrooms. After that, mushrooms were grown in the trays.

The previous crop from these trays had shown violent symptoms of the virus disease; in mushrooms from untreated trays now too, many virus particles were to be found. This, however, was not the case with mushrooms from the trays which had been treated. A visual judgement of the trays led to a percentage of 40% healthy for the ten untreated trays, and 86% healthy for the 159 trays treated with SPCP. The latter percentage could have been higher, but a number of the trays were attacked by *Verticillium sp.*; only a very small number of the treated trays was perhaps affected by the virus. Immersion in 4% SPCP would certainly have resulted in a completely healthy crop. Through this treatment the yield from this farm rose by 37.5%, which is by no means the maximum possible increase.

SPCP is now being used throughout the country with very good results. Applying this method means that growing-rooms need never be cooked out *after* emptying any more (but still *before!*), not even if virus disease is present. Mushroom mycelium only grows to within a few mm. of the wood treated with SPCP and can thus not penetrate into it (**Fig. 5**), which means that the wood can be cleaned much more simply after the crop.

It only remains to say that ten other trays from this same grower underwent gassing with methyl bromide (600, 700 and 800 CTP), but the results were very unsatisfactory. After a great number of experiments with methyl bromide and both virus-diseased and healthy mushroom material, it is my opinion that this gas is inadequate as an after-crop sterilant (Dieleman-van Zaayen, in preparation).

*This chemical is available in the United Kingdom as SANTOBRITE or DOWICIDE G.
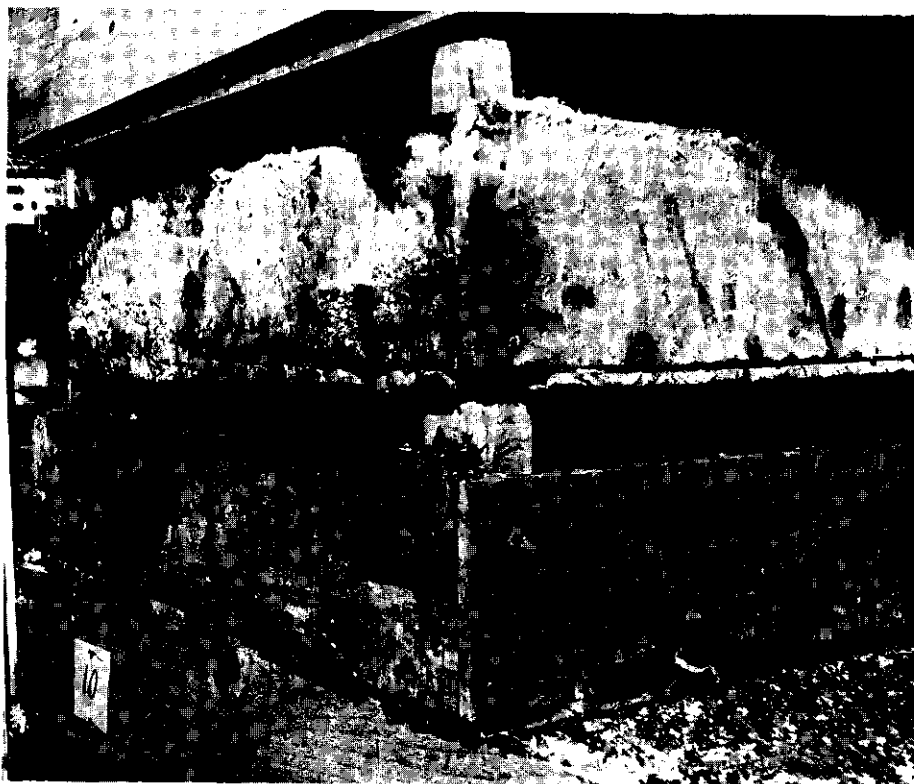
Fig. 6. The effect of sodium pentachlorophenate
The upper tray is the untreated control (mycelium grows on the wood), the lower tray
was dipped in a 4% solution of pentachlorophenate: mycelium does not grow on the
wood

B. *Measures to prevent/to control the virus disease*

Summarizing with regard to the virus disease, the following important points have been brought up:

1. **The disease is spread by viable mycelium and spores of diseased mushrooms.**
2. **Early infection is dangerous, especially an infection simultaneous with or shortly after spawning. Up to the time of casing compost and mycelium must be protected.**

With these facts in mind, and on the basis of the information obtained with the aid of the isolated virus growing-room, a meeting was held at the Experimental Station, attended by scientists, extension officers and educational staff. During this meeting, a number of measures to prevent or if necessary, to control the disease were drawn up. This led to a list being compiled, which was issued to Dutch growers in November 1968. This list is shown in **Table 3**, the left column giving preventive measures, and the right showing ways and means of controlling the disease after it has been diagnosed on the premises. The methods are not set out in their order of importance, but in chronological order according to the growth of the crop. They are, of course, applicable to Dutch conditions, which means to small farms where cultivation takes place on shelves following the one-zone system. In the majority of such farms, growing-rooms are situated side by side and they lead out into a covered working-corridor that can be a gathering-place for spores (which explains the measure concerning plastic

partitions in this corridor; this is an imitation of the isolating chamber in front of the virus growing-room in Horst).

Table 3

Mushroom Experiment Station — Horst (L), The Netherlands
Institute of Phytopathological Research, Wageningen

WHAT TO DO ABOUT VIRUS

When the disease is not yet present

Steam the compost for 12 hours at a temperature of 70°C. (158°F.). At emptying take away the compost quickly.

Spray the wood with 2% sodium pentachlorophenate to which 0.5–1.0% soda (Na_2CO_3) has been added. After drying spray with water.

Disinfect doors, little holes in the floor, shutters, racks, floors and walls with formaldehyde (*not* with sodium pentachlorophenate!). Also clean the manure yard and adjacent patches of ground with formaldehyde (we recommend 4% pure formaldehyde or 10% of the 40% commercial solution).

Before filling, fit spore filters; during growing time these spore filters should be replaced once or twice according to the amount of dust in the air. Use a fan for extracting air.

Immediately after spawning use a pesticide against flies, then cover the compost with paper. Keep the paper moist. Wet the paper twice a week with a 2% solution of the 40% commercial formaldehyde (2 litres commercial formaldehyde on 100 litres of water). Repeat till a few days before casing. *Never* use sodium pentachlorophenate here! Moisten the paper before removing it carefully.

Quickly remove cuttings and litter and destroy.

The entire farm and its surroundings should be very clean and stay so. In the working corridor formaldehyde should be sprayed. Machines (spawning machine, press, etc.), refrigerator and other utilities should be disinfected with a formaldehyde solution.

When the disease is already present

Steam the compost for 12 hours at a temperature of 70°C. (158°F.). At emptying take away the compost quickly.

Immerse the wood in a 4% sodium pentachlorophenate solution to which 0.5–1.0% soda (Na_2CO_3) has been added. After drying spray with water.

Disinfect doors, little holes in the floor, shutters, racks, floors and walls with formaldehyde (*not* with sodium pentachlorophenate!). Also clean the manure yard and adjacent patches of ground with formaldehyde.

Before filling fit spore filters; during growing time these spore filters should be replaced once or twice according to the amount of dust in the air. Use a fan for extracting air.

Place partitions made out of a synthetic material (plastic) in the working corridor between the rooms. If this is not possible, enter the growing rooms from the back end till picking starts.

Use mats with formaldehyde before the rooms to disinfect shoes.

Immediately after spawning use a pesticide against flies, then cover the compost with paper. Keep the paper moist. Wet the paper twice a week with a 2% solution of commercial formaldehyde (2 litres commercial formaldehyde on 100 litres of water). Repeat till a few days before casing. *Never* use sodium pentachlorophenate here! Moisten the paper before removing it carefully.

Pick the mushrooms when still closed. •

At a first contamination the disease can be controlled best by immediately steaming out the concerned room.

After that, act according to right column.

spawn too quickly, actually only when you cannot pick anything anymore.

The entire farm and its surroundings should be very clean and stay so. In the working corridor formaldehyde should be sprayed twice a week. Machines (spawning machine, press, etc.), refrigerator and other utilities should be disinfected with a formaldehyde solution.

Quickly remove cuttings and litter and destroy.

Keep each room as a separate entity with separate clothes, shoes, steps, buckets, picking knives, picking racks, fans, etc. Kill off diseased patches with salt and cover with plastic; make the limits of the patches rather big. First pick from the healthy parts, then from the diseased patches. Wash hands often.

Admit as few visitors in the diseased rooms as possible and keep the door towards the working corridor closed. Kill off pests, etc., in particular.
Have a short picking period only (not more than 4 weeks).
Do not change over to brown strains of

Dutch concerns which had been infected with the disease since 1964 were found to be completely free of the virus within a few months (checks for virus particles!) after applying these methods. The yield of one growing-room from such a concern rose from $\pm$ 0.5 kg/m^2 to $\pm$ 13 kg/m^2 in the following crop.

For English conditions, only slight adaptation of these measures will be needed. Stress must be laid once again on the following points:

(a) **at the first sign of the virus disease, the mushrooms should be harvested as *closed* mushrooms as much as possible;**
(b) **spore filters should be used (2 cm.-thick glass fibre is suitable for this purpose);**
(c) **spawn quickly with a disinfected spawning machine; then cover the compost immediately with paper and spray this twice a week with a diluted formaldehyde solution;**
(d) **treat wood after emptying with SPCP; before emptying, cook-out for 12 hours at 70°C.;**
(e) **apply strict hygiene (e.g. treat working-corridor with formaldehyde, etc.).**

It is not sufficient to perform one or two control measures only; the whole lot of measures should be applied to free a farm completely of the virus disease.

Is there a cure?

The measures described are first and foremost intended to prevent and protect and are a matter of strict hygiene. Are there any possible measures that might be taken which would have a curative effect? As far as chemical means are concerned, the answer at present is 'No'.

Gandy (1960b) did report that treatment of virus-diseased mushroom mycelium — which grows slowly and badly on an artificial nutrient medium such as agar — for a period of two weeks at 33°C. (91°F.) resulted in most cases in a better and faster growth. It was presumed that the temperature treatment freed the fungus from the virus.

From mushrooms supplied by many different Dutch growers, mycelium was isolated on agar; in this way a collection of more than 60 different virus-diseased mycelia was made. The complete collection, together with

several healthy cultures, was subjected to the above-mentioned temperature treatment; half the collection even more than once. The results were very indecisive; some of the mycelia, however, did show a distinct improvement in growth. Nevertheless, some of the controls (healthy mycelium) showed a similar trend. Inoculation material was prepared from some of the most promising mycelia from both treated colonies (2 weeks at 33°C.) and untreated ones. Small growing trays, filled and spawned in the usual way, were inoculated with this material. The trays containing the controls, consisting of treated and untreated healthy mycelia respectively, remained healthy; the other inocula, which included the temperature-treated cultures, produced virus-diseased mushrooms. It is superfluous to say that mushrooms from all trays, as far as any came up, were tested for the presence of virus particles. Only in one case was there any doubt as to whether or not the treated culture was diseased; this is being investigated further. The temperature treatment, however, is not very promising; improvement in the growth of a treated colony certainly does not mean that it has become virus-free or carried virus beforehand. Such a method, for that matter, even if it was found to be effective, would not be of direct interest to mushroom growers.

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4.2 An investigation of spawn

Spawn usually consists of mushroom mycelium aseptically grown on sterilized cereal grains. Dutch growers use imported spawn. In theory, spawn could be virus-contaminated, but severe infection would soon be detected by the slow growth and deformity of the mycelium.

Spawn could hardly be examined directly because of the tiny bit of mycelium on the grains. First trials indicated, that the mycelium scraped from grains yielded too little material to be tested for the presence of virus. Another solution was, to grind grains and mycelium together, in a Waring Blendor in 30 ml of 0.033 M phosphate buffer containing 0.1% (v/v) thioglycollic acid, final pH 6.8, per 10 g of spawn. Occasionally the mixture had to be diluted. The crude homogenate was then treated in the same way as fruiting bodies (Section 2.1). However this procedure often resulted in preparations that contained too much debris, so that virus particles could hardly be seen with the electron-microscope. Thus the method of detecting virus was poor. Besides, the chance of finding virus-infected spawn may be very small; thousands of litres of spawn were be tested in the laboratory for the presence of virus. Therefore, it was decided to handle the investigation differently. Help was sought from mushroom growers, of whom over 100 assisted. Only virus-free farms were allowed to take part in the test, which began in January 1968.

Samples were collected from the farms in the following way. The growers were asked to hold back a little from each bag of spawn at spawning; the bags were not mixed and spawned per growing-room, but per shelf. Growers could spawn their usual variety. The portions of spawn held back from all bags used for one shelf, were collected into a plastic bag. The bags were labelled with name and address of grower, date of spawning, spawn variety and number of shelf. Thus at Wageningen we received a parcel with 10 bags from each standard growing-room with 10 shelves. The parcels were stored in a cold room at about $4^{O}C$.

If disease symptoms were observed in a growing-room, the grower informed us. A sample of mushrooms from that growing-room was then tested for virus particles in the usual way (Section 2.1). If virus disease was diagnosed, the corresponding stored spawn was indirectly examined for virus in the following way.

A small sterilized tray of 26 x 22 x 5.5 cm^3 was filled to a depth of about 4 cm with spawn (the spawn to be tested sometimes had to be supplemented with fresh commercial spawn of the same variety) and covered with a 3-4 cm casing layer of soil. The tray was kept in an incubator at 25^oC and occasionally moistened. Once the casing soil was permeated with mycelium, the temperature was slowly reduced to $15-17^oC$ and usually after a few weeks some mushrooms appeared. They were tested for virus particles in the usual way (Section 2.1). Control trays only contained fresh commercial spawn.

Presumably because of the long storage of the spawn, some trays got contaminated with, for instance, *Trichoderma* spp., and mushrooms did not always form. Spawn was then tested for virus as before.

The results did not yield any clear indication on possible virus infection of spawn. Such an infection, however, cannot be excluded: the mycelium grown on the grains usually originates from spores; both spores and mycelium have been found to spread the virus. Virus-infected mycelial cultures do not always show a slow and degenerated growth, but they sometimes have almost normal growth rates so that they may look uninfected (Section 2.2). Anyhow, to answer the question whether spawn can be virus-contaminated or not, detection methods for virus in spawn need improvement.

Although some spawn may be virus-infected, spread of mushroom virus in other ways, for instance with basidiospores, seems more important, especially in the Netherlands where some mushroom farms are built close together, and where returnable, non-disinfected picking boxes may be used. For these reasons, further cooperation with mushroom farms in routine testing of spawn seems unadvisable.

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4.3 Methyl bromide fumigation and control

Paper:

VIII Methyl bromide fumigation versus other ways to prevent the spread of mushroom virus disease

Neth. J. agric. Sci. 19: 154-167 (1971).

Methyl bromide fumigation versus other ways to prevent the spread of mushroom virus disease

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Summary

A virus disease has been a considerable threat to the rapidly growing mushroom industry in the Netherlands. Intensive research has been done to control this disease, which spreads by mycelium and spores from infected mushrooms.

The action of methyl bromide in the gas phase was studied, and compared with current chemicals and control methods. Mushroom spores appeared to be destroyed at relatively low dosages of methyl bromide; they were also readily killed, however, by a short treatment with formaldehyde vapour. It was already known that this can be achieved by a short heat treatment (1 h at 70°C). The results of methyl bromide fumigation of mycelium, spawn, contaminated growing trays and compost permeated with mycelium were disappointing. Various mycelial isolates differed in sensitivity to methyl bromide.

Thus methyl bromide fumigation cannot replace the traditional after-crop cooking-out of mushroom houses with live steam, followed by treatment of the wood with sodium pentachlorophenate.

Introduction

A virus disease of cultivated mushrooms
In recent years the Dutch mushroom farming has suffered severe losses. They are due to a virus disease of the cultivated mushroom (*Agaricus bisporus* (Lange) Sing.), first recognized in this country in 1964. Crop yield is seriously reduced and the few mushrooms formed are misshapen (Dieleman-van Zaayen, 1969).

A survey among the more than 1,000 Dutch growers showed that in 1967 and the first half of 1968, one out of three mushroom farms was contaminated; on these farms average crop loss was 15 %. Thus in 1967, in total 4.5 % (or about 790,000 kg) of the production, which amounted to 17.5 million kg mushrooms, was lost.

The disease concerned was first observed in America in 1948 (Sinden and Hauser, 1950) and further described in England by Gandy (1960). Later it was named 'Die-back disease'. Hollings (1962) showed that different types of virus particles can be isolated and are always associated with the disease. In the Netherlands, the disease is caused by the following three types of virus particles, either alone or in combination: isometric particles, diameter 25 and 34 nm, respectively, and elongated particles with rounded ends, size 19 x 50 nm (Dieleman-van Zaayen and Temmink, 1968). Recently, one particle type has· been observed in ultrathin sections of diseased mycelium (Dieleman-van Zaayen and Igesz, 1969).

The disease is spread by viable mycelium (Gandy, 1960) and by spores from diseased mushrooms (Schisler et al., 1967; Dieleman-van Zaayen, 1968).

Mushroom growing in the Netherlands
Mushrooms are grown on compost of horse manure and straw, which was subjected to outdoor composting for two weeks. After a mushroom growing room is filled with about 100 kg/m^2 compost, the compost is pasteurized (up to 60°C) for about 10 days. Cooling to 25°C is followed by spawning (spawn is mushroom mycelium grown on sterilized grains). After two weeks of mycelial growth in the compost at 25°C, a casing layer of soil (3–4 cm) is added. The temperature is then slowly reduced to 15°C and after three weeks the first mushrooms can be harvested. These appear in flushes (weekly peaks). For five to six weeks the mushrooms are picked. The crop is then finished, the growing room emptied and after some days refilled with fresh compost. One crop takes 12 to 13 weeks.

The problem
Until recently, mushroom growers were advised to cook out the growing rooms at the end of a crop for at least 24 h at 70°C to destroy all the mycelium and spores; in case of virus disease they were advised to repeat this treatment after the room had been emptied. Such a prolonged heat treatment, however, is very detrimental to mushroom houses. Besides, it is highly questionable whether the mycelium, grown into the wood of trays and shelves, will be killed (Spradling Chidester, 1939). If not destroyed it will anastomose with mycelium of the following crop and may thus cause early virus infection and severe damage (Last et al., 1967; Dieleman-van Zaayen, 1970).

This problem has recently been solved by the application, after every crop, of wood preservatives based on sodium pentachlorophenate. Cooking-out can then be reduced to 12 h at 70°C, before emptying the room, merely to kill mushroom mycelium and harmful organisms in the compost which is afterwards used for other horticultural purposes.

The growing houses, however, still suffer from this 12-h heat treatment, and steam installations are not everywhere available. In England, good results were claimed with methyl bromide in the gas phase, either during the final stages of compost preparation to eradicate pests and harmful micro-organisms (Hayes and Randle, 1968), or as a substitute for cooking-out with live steam at the end of a crop to disinfect growing houses and to kill mushroom mycelium, other micro-organisms and pests in the compost (Hussey et al., 1962; Flegg, 1968).

According to unpublished work by Dr F. T. Last a CTP (Concentration Time Product) of 600 g.h^{-1}.m^{-3} would kill virus-infected mushroom mycelium (Flegg, 1968).

Hayes (1969) described a number of experiments on methyl bromide fumigation of spores, fruiting bodies and mycelium of cultivated mushroom and of mycelium and fruitbody tissues of *Verticillium malthousei* Ware, a pathogenic fungus in mushroom culture. With respect to virus disease, he stressed the necessity to kill viable mushroom spores and mycelial fragments. His results suggested, that methyl bromide fumigation can, with advantage, replace the traditional cooking-out with steam. However he tested only healthy mushroom tissue, mycelium and spores.

From these data it was decided worthwhile to see whether methyl bromide, although very poisonous, could be used in the Dutch mushroom growing industry, particularly to control the virus disease. A second possible application was the central disinfection of picking boxes or baskets. These baskets, used a number of times by different

growers, had earlier been shown to contribute in spreading the virus disease all over the country (Dieleman-van Zaayen and Van Tilburg, 1968, 1969).

A comparison was made with formaldehyde vapour, which is easier to handle and less poisonous. It is used in the Netherlands as a general disinfectant for control of the mushroom virus disease.

Co-operation between the Mushroom Experiment Station, the Plant Protection Service and the Institute of Phytopathological Research allowed a comprehensive experiment.

Material and methods

The trials were done in an experimental fumigation chamber of the Plant Protection Service at Wageningen. It is made of iron, with oil-painted walls, of 3 m³ capacity and with a gas circulation system charging 120 m³/h. With a heating unit a temperature of 20°C was maintained throughout the experiments. Liquid methyl bromide stored under pressure in a cylinder was led into a glass measure whence by opening a tap it passed through a volatilizer via the circulation system into the fumigation chamber. The fan in the circulation system was continuously operated to maintain a homogeneous gas/air mixture.

Several objects (indicated 1–6) were successively subjected to three methyl bromide fumigation trials (Experiments I – III). Experiment IV concerns a formaldehyde treatment.

The fumigated objects will be discussed in the following sequence:
1. Wooden trays of $57 \times 110 \times 15$ cm, in which virus-diseased mushrooms had been grown commercially. At the end of the crop, the trays were emptied but not disinfected (Exp. I).
2. Compost, permeated with virus-diseased mushroom mycelium, obtained from a contaminated mushroom farm (Exp. I).
3. Spores from virus-diseased and healthy mushrooms collected on filter paper (Exp. II).
4. Various virus-diseased and healthy mycelial isolates on 2 % Biomals agar in test tubes (Exp. II and III).
5. Self-prepared spawn from various virus-diseased and one healthy mycelial isolates in glass tubes or flasks (Exp. II and III).
6. Corn weevils, to ascertain whether methyl bromide can penetrate glass tubes sealed with filter paper instead of cottonwool plugs, as employed in fumigation of objects 4 and 5 (Exp. II).

Each treatment included non-fumigated controls.

Experiment I was at atmospheric pressure; immediately before the methyl bromide dosages of Experiments II and III were applied, the absolute pressure in the chamber was reduced by a vacuum pump to about 70 cm of mercury, to stimulate penetration of the gas into the test tubes with mycelium. After dosage of methyl bromide the atmospheric pressure in the chamber was restored by allowing air to enter through a small aperture.

During Experiment I (trays and compost) the concentration of the 'free' methyl bromide in the chamber was regularly measured by means of a thermal conductivity meter ('Gow-Mac Gasmaster'), that previously had been stamped and verified for this purpose, using data of van de Pol and Mathôt (1961). The CTP's were calculated by

multiplication of the measured concentration of 'free' methyl bromide (in g/m^3) and the exposure time (in h), according to the EPPO-report on fumigation standards (1961).

In Experiments II (mushroom mycelium on agar, spawn, and spores on filter paper) and III (mycelium and spawn) the initial dosages, necessary for a given CTP, were calculated. Taken into account was a certain loss by sorption, which was gathered from earlier experimentation (Van de Pol and Mathôt, 1961). In fumigation of mycelium, spawn and spores, losses due to sorption were considerably smaller than in fumigation of wooden trays and compost (about 6 and 24 % respectively).

Methyl bromide fumigation was always carried out for about 24 (21 – 27) h. After every treatment the fumigation chamber was ventilated for about 30 min.

In the same fumigation chamber at atmospheric pressure a comparison was made with the effect of formaldehyde vapour on virus-diseased and healthy mushroom mycelium and on spores from diseased mushrooms (Experiment IV).

Further technical details will be given when describing the trials.

Experiments and results

1 Fumigation of contaminated trays (Exp. I)

The trays, obtained from a contaminated farm, had previously contained a crop with severe symptoms of virus disease. Before and immediately after fumigation the nine trays to be examined were enveloped separately in plastic to prevent mutual contamination, and were then stored at 4°C. After they had been restored to the mushroom farm concerned, the trays were filled with compost of horse manure and straw. After 12 days of pasteurization the compost was spawned with commercial mushroom spawn (Sinden). For some weeks there was mycelial growth in the compost and then a casing layer of soil was added.

About three weeks later the first mushrooms were harvested. From the same farm 159 similar, non-disinfected and non-fumigated trays were treated with a 2 % solution of the wood preservative sodium pentachlorophenate (SPCP), with sodium carbonate as a wetting agent. Ten other trays served as controls.

Table 1. Visual judgment of crop on fumigated (Exp. I), untreated and SPCP-treated trays, and detection of virus particles in the mushrooms.

Number of trays	Treatment	Estimated number of 'healthy' trays	Virus particles [1]
10	— (control)	4	Many *25, 34*, and *19 × 50 nm*
	Methyl bromide:		
3	612 CTP	2	Relatively few *25, 34*, *19 × 50 nm* (Fig. 1)
3	712 CTP	1	No particles observed
3	801 CTP	0	Relatively few *25, 34*, *19 × 50 nm*
159	SPCP 2 %	136 [2]	No particles observed

[1] The figures in italics indicate the particle type(s) prevailing in that virus preparation.
[2] This number could have been higher, but a number of the trays were infected by *Verticillium* sp.
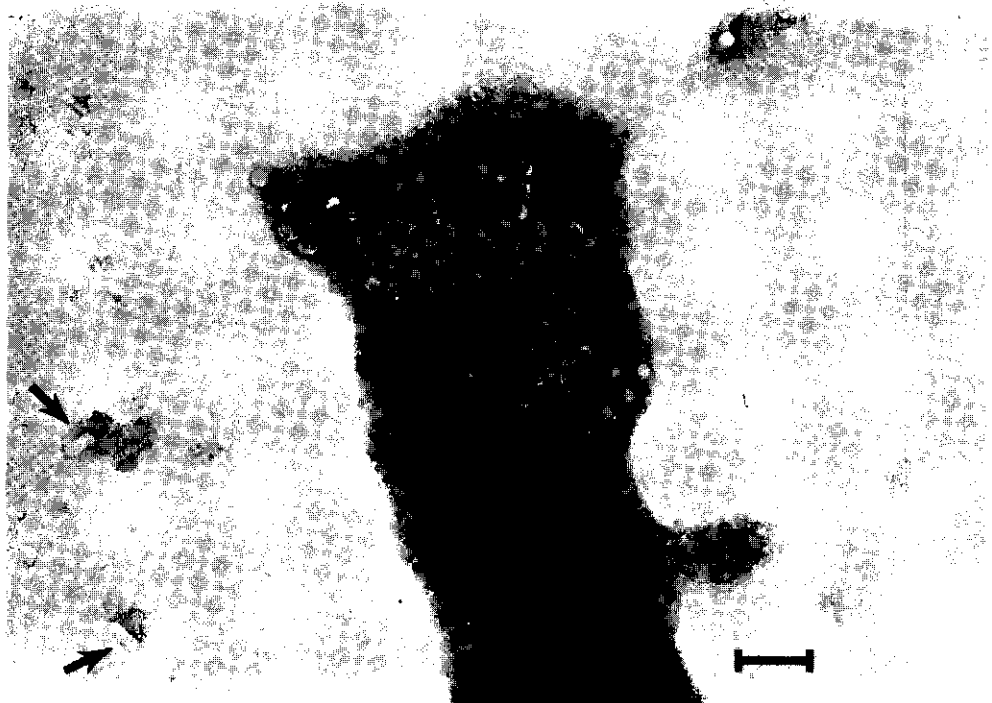
Fig. 1. Electron micrograph of virus particles of 25, 34 and 19 $\times$ 50 (arrows) nm, detected in mushrooms grown on trays, fumigated with 612 CTP methyl bromide. Marker indicates 100 nm.

The mushrooms were visually judged for symptoms when sampled and then diagnosed for virus particles according to a special method developed by Dieleman - van Zaayen and Temmink (1968). The results are given in Table 1 and Fig. 1.

The effect of SPCP is evident. Treatment with 4 % SPCP would most probably have been even more efficient.

As for methyl bromide, even a CTP of 800 seemed to be insufficient for disinfection, even though the trays were not only fumigated, but also peak-heated. The compost on this farm, however, may not have reached 60°C during peak-heating.

It is not certain whether methyl bromide is inadequate because of insufficient fungicidal action or because of insufficient penetration of the gas into the wood.

2   Fumigation of compost permeated with mushroom mycelium (Exp. 1)
A.   Portions (70 g) of fumigated and untreated compost (stored at 4°C) were put into a corner of steam-sterilized 30 $\times$ 30 $\times$ 25 cm trays, which were filled with a layer (some 20 cm) of compost of horse manure and straw and spawned with 35 g commercial spawn (Somycel 32) per tray. Inoculation with treated or untreated compost was done seven days after spawning.
B.   The above procedure (A) was repeated after some months with the same compost and mycelium, stored at 10°C. This time inoculation with portions of 80 g compost was done 11 days after spawning. Both experiments were done in duplicate. The yields (in three weeks of picking) and results of testing for virus particles of two trays were

Table 2. Yields of and virus particles in crop on small trays, inoculated with fumigated (Exp. I) compost (averages of two trays).

Treatment	A		B	
	Yields (g)	Virus particles [1]	Yields (g)	Virus particles [1]
— (inoculated control)	40	Many 25 and *34* nm	40	Many *25*, 34 and 19 × 50 nm
299 CTP	193	Relatively many *25, 34*, 19 × 50 nm	63	Many *25* and 34 nm
400 CTP	45	Rel. few 25 nm	180	Few 25 nm
499 CTP	78	Rel. few *25* and 34 nm	350	Few *25* and 34 nm
612 CTP	85	Rel. many *25, 34*, 19 × 50 mm (Fig. 4)	540	Few 25 and 34 nm
712 CTP	105	Rel. many 25 nm	495	Rel. many *25*, 34, 19 × 50 nm
801 CTP	190	Many 25 nm	350	Rel. few *25*, 34, 19 × 50 nm (Fig. 5)

[1] The figures in italics indicate the particle type(s) prevailing in that virus preparation.

averaged. Mushrooms growing on trays were tested for virus at least twice. The results are given in Table 2. These experiments were carried out at the Mushroom Experiment Station at Horst (L).

Yields of B were higher than those of A because of more favourable climatic conditions in the isolated virus growing room at that time. Yields were variable, however. Yet the higher CTP's of methyl bromide led to better yields than the lower CTP's. Obviously even a CTP of 800 cannot free compost from virus, that is kill the virus-

Fig. 2. Trays inoculated with untreated, infected compost (right) and with 800 CTP-fumigated compost (left). The right tray yielded a virus-diseased culture; the tray on the left seemed healthy.

Fig. 3. Section through compost (below) and casing soil from the trays represented in Fig. 2. Right tray: no mycelium is left in the casing soil.

infected mycelium in the compost. The CTP of 400 always showed a remarkably good result as for the amount of virus particles. However, this part of the fumigated my-celium may have been less diseased than the other compost samples.
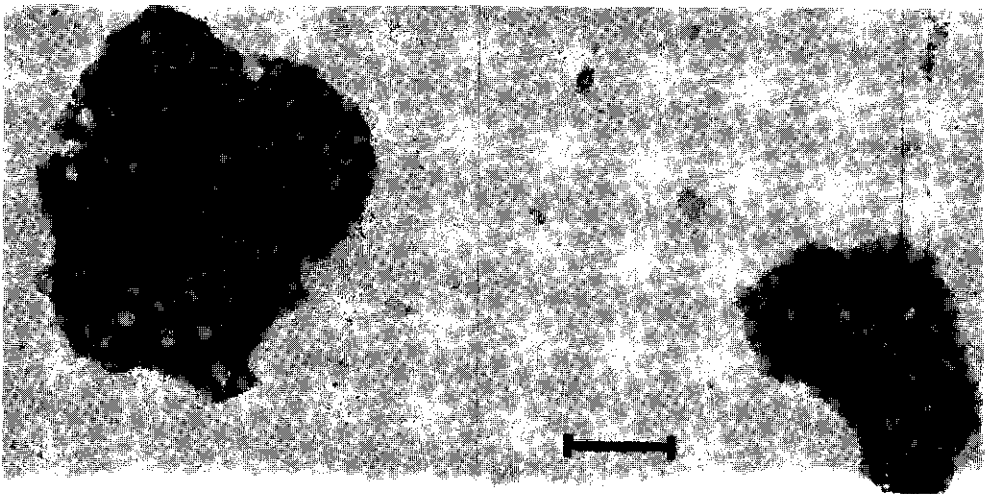
Fig. 4. Electron micrograph of virus particles of 25 and 34 nm, detected in mushrooms from trays, inoculated with 612 CTP-fumigated compost (A in Table 2). Marker indicates 100 nm.

Fig. 5. Electron micrograph of virus particles, detected in mushrooms from trays, inoculated with 801 CTP-fumigated compost (B in Table 2). Marker indicates 100 nm.

Fig. 2 (right) shows a tray, inoculated with untreated compost and virus-diseased mycelium, and a tray (left) inoculated with methyl bromide-fumigated compost and virus-diseased mycelium (800 CTP). The right tray yielded a virus-diseased culture: the few mushrooms that developed contained many virus particles. The tray on the left seemed healthy. Fig. 3 shows a section through compost (below) and casing soil from the same trays (right: untreated; left: 800 CTP methyl bromide). In the casing soil of the tray, inoculated with untreated compost, no mycelium is left, and this is characteristic of the virus disease. In the tray, inoculated with 800 CTP-fumigated compost, there is mycelium left but the mushrooms produced contained many virus particles of 25 nm (Table 2A). See also Fig. 4 and 5.

Uninoculated control trays yielded a healthy crop.

3   Fumigation of spores from virus-diseased and healthy mushrooms (Exp. II)
Spores were collected by placing virus-diseased or healthy mushrooms under a beaker on filter paper. The mushrooms had first been surface-sterilized by a 0.2 % solution of mercuric chloride (HgCl₂). The spore prints were stored in a Petri dish until

Table 3. Mycelial growth in mm on agar after germination of fumigated spores from virus-diseased and healthy mushrooms (Exp. II).

Treatment	Mycelial growth (mm)					
	'diseased' spores			'healthy' spores		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
— (control)	65	46	61	71	97	±
370 CTP	3	0	±	42	45	±
561 CTP	0	0	0	0	0	±
741 CTP	0	0	0	0	0	±

± = contaminated; 0 = no growth, no germination.

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fumigation. Just before fumigation the lid of the Petri dish was replaced by a sheet of filter paper, which was attached to the bottom of the dish. The filter paper was used because it was supposed to establish better contact with methyl bromide during treatment. Immediately after fumigation the lid was returned. Some spores were then scraped from the fumigated spore prints and transferred to a nutrient medium (2 % Biomals agar) in three test tubes per treatment. Germination was determined after 25 days by measuring the mycelial growth in mm (Table 3).

Spores, and especially 'diseased' spores, were highly sensitive to methyl bromide fumigation. A relatively low dosage of 370 CTP retarded growth of mycelium from 'healthy' spores, and nearly killed 'diseased' spores.

Table 3 also shows that healthy mycelium grew faster than virus-diseased mycelium (see untreated spores). This is another characteristic of mushroom virus disease (Gandy, 1960).

4 Fumigation of virus-diseased and healthy mycelium on agar (Exp. II and III)

The mycelial isolates No 1, 28, 52 and 92 (with 97 in Exp. III) from our 'virus collection', and a healthy isolate No 37, all maintained on 2 % Biomals agar, were fumigated. Some isolates were treated in duplicate, others in triplicate or in quintuplicate. This was done because of variation in growth rate and possibly in response to fumigation with methyl bromide. Just before fumigation, the cottonwool plugs on top of the test tubes were replaced by a cover of sterilized (30 minutes at 120°C) filter paper which was attached to the tubes by a piece of elastic. Immediately afterwards the

Table 4. Growth of fumigated virus-diseased and healthy mycelia in mm (Exp. II).

Treatment	Growth of fumigated mycelia (mm) from isolate No											
	1	28		37 (healthy)					52	92		
		I	II	I	II	III	IV	V		I	II	III
— (control)	74	63	50	75	63	67	90	65	83	51	62	58
370 CTP	82	50	39	70	56	67	83	62	36	28	0	49
561 CTP	0	41	40	0	0	0	0	0	62	0	0	0
741 CTP	0	0	0	0	0	0	0	0	13[1]	0	0	0

[1] Mycelium started to grow later on.

Table 5. Growth of fumigated virus-diseased and healthy mycelia in mm (Exp. III).

Treatment	Growth of fumigated mycelia (mm) from isolate No													
	1		28		37 (healthy)		52				92		97	
	I	II	I	II	I	II	I	II	III	IV	I	II	I	II
— (control)	52	61	63	55	58	62	58	55	52	47	64	67	33	56
692 CTP	32	±	32	33	0	32	28	25	±	42	41	56	29	39
899 CTP	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1103 CTP	0	0	0	0	0	0	0	0	0	0	0	0	0	0

cottonwool plugs were restored. Pieces of agar with mycelium were then transferred to test tubes with fresh Biomals agar. After 25 days the growth of the mycelium was measured in mm (22 days for Exp. II, 741 CTP).

The results given in Tables 4 and 5 prove, that about 600 CTP methyl bromide does not kill mycelium on agar, though growth is reduced. Some isolates, for instance number 37 (healthy), are apparently more sensitive to methyl bromide than others. A certain difference in response to fumigation can be seen between Experiments II and III, 561 and 692 CTP, respectively: though the dosage in Exp. III was higher than in Exp. II, more isolates survived the treatment in Exp. III. The age of the mycelium might be of influence.

5 Fumigation of spawn (Exp. II and III)

Spawn was prepared in the following way. Sorghum grains were boiled with water for 5 min. Then 2.33 g gypsum and 0.66 g calcium carbonate per 100 g sorghum were added and thoroughly mixed. The mixture was sterilized for 2 h at 120°C. After cooling down, pieces of agar with mycelium were transferred to the flasks or tubes with grains. After 1 – 2 weeks the grains were permeated with mycelium.

Spawn from various isolates was fumigated in:
Exp. II : wide glass tubes, diameter 3.0 cm;
Exp. III: wide glass tubes, diameter 3.0 cm; 300-ml flasks with a wide neck.

Again the cottonwool plugs were replaced by sterilized filter paper just before fumigation. After fumigation some spawn grains from the bottom of tube or flask were transferred to Biomals agar. Mycelial growth was measured after 25 days.

Various mycelia (spawn), among which one healthy (No 37b), were subjected to different fumigation treatments without duplicates (a and b indicate different strains of a mycelial isolate). The results are given in Tables 6 and 7 and those of Experiment III in Fig. 6. The excellent growth of number 112a is clearly visible, despite a dose

Table 6. Growth of mycelium (mm) from fumigated spawn (Exp. II).

Treatment	Growth of mycelium (mm) from isolate No									
	37b (healthy)	112a	7a	7b	69a	69b	40a	40b	94a	94b
— (control)	60	47								
370 CTP			13	20.5						
561 CTP					±	±				
741 CTP							23	26	30	0

Table 7. Growth of mycelium (mm) from fumigated spawn (Exp. III).

Treatment	Growth of mycelium (mm) from isolate No									
	92b	94b	97a	97b	40a	40b	112a	112b	92a [1]	94a [1]
— (control)	49	70.5							30.5	68.5
692 CTP			29	54.5					28.5	34
899 CTP					19	0			17	0
1103 CTP							+	0	+	0

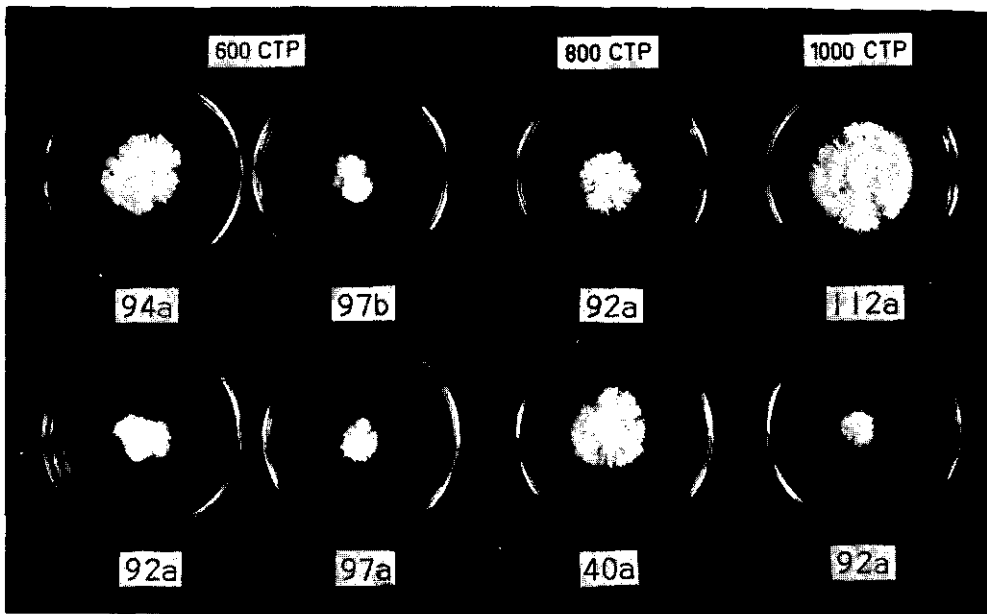[1] Flasks; + Mycelium started to grow later on.

Fig. 6. Mycelium from fumigated spawn. CTP's are rounded off downwards. For explanation, see text.

of over 1000 CTP. It is obvious from these results that 600 CTP is not sufficient to kill mycelium in the form of spawn. Even a CTP of 1103 cannot destroy all types of mycelia on grains of sorghum.

6 Fumigation of corn weevils (Exp. II)

During Experiment II, five tubes each with 50 corn weevils, were added to every treatment except that with 741 CTP. Each tube had a diameter of 3.0 cm and was sealed with filter paper. When counted immediately after fumigation, all 5×50 controls were alive, but none of the fumigated corn weevils (5×50 at 370 CTP, 5×50 at 561 CTP) survived. This indicates penetration of the gas through the cover of filter paper into the tubes.

The effect of formaldehyde vapour on spores and mycelium (Exp. IV)

For comparison various virus-diseased (No 40, 52, 92 and 112) and healthy (a 'Sinden' isolate) mycelia and spores from virus-diseased mushrooms were exposed to formaldehyde vapour in the fumigation chamber. Each isolate, growing on 2 % Biomals agar in test tubes, was treated in duplicate. Cottonwool plugs were replaced by sterilized filter paper as described before. After formaldehyde fumigation, pieces of agar with mycelium were transferred to tubes with fresh agar. Growth of the mycelium was determined after 24 days. For each treatment one spore print was fumigated in a Petri dish, the lid of which had been replaced by a cover of filter paper. After fumigation some spores were transferred to 5 Petri dishes with agar per treatment. Fumigation took place for 1, 2, 3 and 24 h. Formaldehyde vapour was produced inside the fumigation chamber by mixing 52.5 g potassium permanganate with 105 ml

METHYL BROMIDE FUMIGATION AND MUSHROOM VIRUS DISEASE

Table 8. Influence of formaldehyde fumigation on virus-diseased and healthy mycelium and on germination of spores from virus-diseased mushrooms (Exp. IV).

Treatment; exposure time (h)	Isolate No										Spores (5 repetitions per treatment)
	40		52		92		112		Sinden (healthy)		
	I	II	I	II	I	II	I	II	I	II	
— (control)	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	−	−
2	+	+	+	+	+	+	+	+	+	+	−
3	+	+	+	+	+	−	+	+	+	−	−
24	−	−	−	−	−	−	−	−	−	−	−

+ Growth of mycelium; germination; — No growth; no germination.

commercial formaldehyde 36 %, and immediately afterwards the chamber door was closed. During treatments the inside temperature was kept at 25°C. After each treatment the chamber was thoroughly ventilated for at least 30 min. During this experiment the gassing method made it impossible to reduce the pressure to 70 cm of mercury, as in the methyl bromide experiments II and III.

Table 8 shows the results. This time the growth of the mycelium was not measured because treated mycelia, which started to grow, developed quite normally compared with untreated.

The sensitivity of spores was again demonstrated. Spores were already destroyed after one hour in formaldehyde vapour, whereas most mycelia still grew after a treatment for three hours.

However 24 h of formaldehyde vapour in the indicated concentration was sufficient to kill both spores and mycelium. Even a shorter period may do, but this was not investigated.

Discussion

The results prove that methyl bromide is an inadequate after-crop sterilant to control the virus disease of cultivated mushrooms, except for destruction of mushroom spores. The results with spores correspond to those obtained by Hayes (1969). Mushroom spores, however, can be destroyed in a much easier and much less dangerous way, either by heat treatment for one hour at 70°C or with a certain dosage of formaldehyde vapour for one hour (Experiment IV). Spores from virus-diseased mushrooms seemed more sensitive to methyl bromide fumigation than spores from healthy mushrooms. This might be due to a reduction of spore wall thickness by infection, as already supposed by Schisler et al. (1967).

The results of fumigating mycelium and spawn were variable. Grain spawn makes the mycelia even more resistant. Hayes (1969) also reported variable results, which he attributed to an ineffective concentration of methyl bromide or too low temperature during fumigation. Hayes's trials, restricted to healthy mushroom tissue, were done in polythene chambers and in growing houses of several commercial mushroom farms, whereas our experiments were carried out in a gastight experimental fumigation chamber with adjustable equipment. In Hayes's opinion, a minimum air temperature of 67°F (= 19.5°C) should be maintained during fumigation. This was so in all our

experiments. Nevertheless, fumigation did not kill all mycelia at about 600 CTP (Tables 4 and 5) and did not completely destroy spawn at about 800 CTP (Tables 6 and 7). This implies that fumigation of mushroom houses would demand extremely high CTP's of methyl bromide to kill all virus-diseased mycelium.

Our experiments, partly practical (trays and compost), partly fundamental (mycelium, spawn, spores in tubes or Petri dishes), were perhaps more sensitive than Hayes's trials, since ours concerned virus-diseased mycelium. For instance, with trays destruction of healthy mycelium is difficult to demonstrate, but if virus-diseased mycelium grown into the wood of a tray has not been destroyed during fumigation, it will anastomose with healthy mycelium of the next crop and thus transmit the virus. If virus is demonstrated in mushrooms of this new crop, the tray obviously was not effectively disinfected.

Since no other reliable test for virus is available so far, the virus-diseased nature of a mushroom crop can only be established with an electron microscope: see for instance Fig. 2 and 3 (fumigated compost, 800 CTP). In seemingly healthy crops the electron microscope revealed virus particles (Table 2A).

Some reports on application of methyl bromide in England are promising. There, however, a good deal of the mushrooms are picked as 'open' mushrooms, so that the amount of spores in the mushroom growing areas must be tremendous. For example, a mushroom with a cap 8 cm diameter can produce 1,300 million spores (Sarazin, 1955).

Any treatment affecting spores will cause a reduction of virus disease. A less dangerous and equally effective treatment could have been considered, however. In November 1968 hygienic measures to prevent and control mushroom virus disease, based on our research, were advocated among mushroom growers in the Netherlands. An official inquiry over 1969 showed that the overall application of these measures considerably reduced the total crop loss in this country from 4.5 % of the total production in 1967 to 1.3 % in 1969 (Dieleman-van Zaayen and Van Tilburg, 1970).

Our experiments explicitly indicate that we cannot agree with Hayes's statement: 'Traditional cooking out with steam can, with advantage, be replaced by methyl bromide fumigation'. Moreover, the application of methyl bromide is dangerous. The danger is further increased since this poisonous gas is odorless in contrast to formaldehyde. Special equipment and continual control of the remaining gas after fumigation are very costly. The fumigation also cannot be carried out by the growers themselves. In the Netherlands it is impossible to apply the gas in mushroom farms, since most farm buildings are attached to the growers' private houses.

Crop disinfection with live steam (12 hours at 70°C, as we tested thoroughly) or, if no steam installation is available, with formaldehyde vapour (24 h; 7 l commercial formaldehyde 36 % and 3.5 kg KMnO4 per 200 m³; temperature 25°C) must be preferred, though the disadvantages of steam (decline of cropping structures by heat stress) and of formaldehyde (poor penetration) are known. Consequently, the wood of trays and shelves has to be treated with the wood preservative sodium pentachlorophenate. This should also be applied after fumigation with methyl bromide.

For a (central) disinfection of picking boxes methyl bromide is unsuited.

Acknowledgments

Spread, prevention and control of mushroom virus disease

SUMMARY

In an isolated growing-room, mushroom virus disease was spread by viable my-
celium and by spores from diseased mushrooms, as reported earlier by several
authors. The trials demonstrated the importance of time of infection and the
relative unimportance of the amount of inoculum. Intensive research into this
disease resulted in a list of measures to prevent or control it, applicable
to Dutch mushroom farms but, with slight adaptation, also suited for more-
zone systems. General implementation of the measures in the Netherlands re-
duced the total crop loss considerably.
    Trials to obtain virus-free material by various techniques (heat treat-
ment at 33°C, repetitive transfer from the outermost periphery of mycelial
cultures) failed. Better results may be expected from a wild mushroom with
a high tolerance, or even resistance, to virus disease.

INTRODUCTION

In recent years the Dutch mushroom farming has suffered severe losses from a
virus disease of the cultivated mushroom, *Agaricus bisporus* (Lange) Sing..
Hollings (1962) detected virus particles in association with a similar dis-
ease in England.

Several authors (Gandy, 1962; Schisler et al., 1967; Dieleman-van
Zaayen, 1969) have reviewed the various types of symptoms. Frequent symptoms
are bare zones where the mycelium grows only slightly, if at all, into the
casing soil, and mushrooms of poor quality standing loose in the casing soil,
often with a long stipe and an off-white cap that matures too early. Usually
crop yield is seriously reduced.

Since Dutch research into the disease started in 1966, usually three
types of virus particles, often in combination, have been observed in cell-
free preparations from diseased mushrooms: isometric particles with diame-
ters of 25 and 34 nm, and elongated particles with rounded ends, 19 x 50 nm
(Dieleman-van Zaayen and Temmink, 1968). We have isolated these particle
types also from samples of diseased mushrooms from Belgium, Bulgaria, Germa-
ny, Italy, Switzerland and the United States. When a cell-free virus prepa-
ration was injected into the stipes of young mushrooms on a growing tray,

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symptoms developed a few weeks later in mushrooms of a subsequent flush; from these fruiting bodies, the three types of virus particles could be re-isolated, predominantly the 34-nm particles, which also prevailed in the inoculum (Dieleman-van Zaayen and Temmink, 1968). This provided the final proof that the disease was indeed caused by virus particles isolated from diseased mushrooms. In 1968 Hollings also observed the 34-nm virus particles, so far overlooked most probably because of their instability.

A survey among the more than 1 000 Dutch growers showed that in 1967 and the first half of 1968, one out of three mushroom farms was contaminated; on these farms average yield loss was 15%. Thus in 1967 in the Netherlands, 4.5% or about 790 000 kg of mushrooms were lost, total yield being 17.5 million kg.

The disease is spread by viable mycelium (Gandy, 1960a) and by spores from diseased mushrooms (Schisler et al., 1967). Spread with spores was confirmed by infection experiments (Dieleman-van Zaayen, 1970) and by the detection of virus particles in ultrathin sections of spores from infected mushrooms (Dieleman-van Zaayen, 1972).

Intensive research has been done to control the disease. Two aspects of control are dealt with here:
a. of interest to mushroom growers, the drawing up from research data of a list of control measures;
b. of interest to spawnmakers, preparation of virus-free material, for instance by heat treatment.

MATERIAL AND METHODS

Research in an isolated growing-room

Most of the virus growing trials were in a growing-room of the former building of the Mushroom Experimental Station at Horst (L.). To prevent the other growing-rooms from becoming infected, certain precautions and rules were kept. These included: a closed chamber of hardboard in front of the growing-room so that one could not proceed directly from the growing-room into the working-corridor. In this chamber, where clothing and footwear could be changed, facilities were available for disinfecting hands, footwear and tools, and for weighing the mushrooms picked in the growing-room. The mushrooms were afterwards packed for destruction. The growing-room was tended by one person, who never entered the other growing-rooms of the Experimental

Station. Generally visitors were not admitted to the virus-contaminated growing-room. The mushrooms were harvested as closed mushrooms and spore filters were placed in all ventilation openings. Strict hygiene was observed. Picking was restricted to a maximum of three flushes.

The growing-room contained on either side of the centre path four shelves, one above the other. Each shelf was divided into four plots, each of 2 m^2. There were thus 32 plots in all and the total growing-area was 64 m^2. Trays could be placed in the room for extra trials.

Mushrooms were grown by the single-zone system. Shelves and trays were always filled with compost, about 100 kg/m^2, from the Co-operative Composting Enterprise at Ottersum. Compost was pasteurized (up to 60°C) for about 10 days. The spawning rate was 375 g/m^2 of commercial spawn (snowwhite strains) for the plots and about 40 g per tray, unless otherwise stated.

Inoculation with virus-infected material

Isolation of mycelium; the Virus Collection
Various virus-infected and some uninfected mycelial cultures were obtained by isolating tissue fragments from samples of fruiting bodies sent in by growers to be tested for virus (Section: Detection of virus particles, page 99). The fragments were plated on 2% Biomals agar (a malt product) at 25°C. After transfer to tubes, the mycelial cultures were stored at 10-12°C, and transferred to a fresh nutrient medium every four months.

In that way a collection was established of virus-infected and some uninfected mycelia, about 70 cultures in all. About 50 were used frequently in several experiments. The mycelia were numbered serially by date of arrival of the mushroom samples to be tested; from the corresponding mushroom samples, the number of virus particles was known. The collection of these mycelial cultures is referred to here as 'Virus Collection'.

Preparation of inoculum
Preliminary trials are described elsewhere (Dieleman-van Zaayen, 1970). They used compost with virus-infected mycelium from a contaminated mushroom farm as inoculum. This dubious procedure was gradually improved. At first several virus-infected mycelial cultures from the Virus Collection were transferred to and multiplied on peak-heated, ground and sterilized compost in Petri dishes. Mushroom mycelium grew well on this medium. On the day of inoculation, mycelium was carefully scraped from the compost medium; inevitably the

inoculum contained some nutrient medium.

Soon this inoculum was replaced by 'infected spawn' (Schisler et al., 1967). The material was easy to handle at inoculation. It was prepared in the following way:

to 1 kg sorghum grains, boiled in 1 litre water for 5-10 min, 5.3 g chalk ($CaCO_3$) and 21.2 g gypsum ($CaSO_4$) were added (Stoller, 1962). The mixture was thoroughly stirred and divided among small flasks. These were closed with cotton-wool plugs and sterilized for 2 h at 120^OC. After cooling, the flasks were vigorously shaken and a piece of agar with infected mycelium was transferred to the grains. Spawn running was at 25^OC for about 3 weeks; twice during this period the flasks were vigorously shaken. The 'infected spawn' was then ready for use.

Inoculation

On the day of inoculation the desired amount of inoculum (ground compost with mycelium, or grains with mycelium) was weighed out and put in the centre of a plot or tray in the isolated growing-room, at a depth of 5 cm in the compost. Inoculation was always done before casing.

Although inoculation with spores from diseased mushrooms is certainly possible (Schisler et al., 1967; Dieleman-van Zaayen, 1970), it was usually done with infected mycelium, which was easier to handle and gave more reliable results.

Detection of virus particles

Fruiting bodies were diagnosed for virus particles by a method developed by Dieleman-van Zaayen and Temmink (1968). Mushrooms, stored at 4^OC for 3-10 days, were rinsed under running tap-water and blotted with filter paper. After removal of the lower parts of the stipes, the fruiting bodies were ground for 2 min in a Waring Blendor in 30 ml 0.033 M phosphate buffer containing 0.1% thioglycollic acid, final pH 6.8, per 10 g tissue. Homogenate in 50-ml portions was subjected to ultrasonic treatment (Hollings et al., 1965) with a Kerry Vibrason cell disrupter (probe diam. 0.9 cm, output 50 W) for 10 min. During treatment, the thick-walled glass tube (internal diam. 3.4 cm), which contained the homogenate, was kept at 4^OC. A procedure modified from Kitano et al. (1961) was used on the sonicated homogenate. To one volume of homogenate, an equal volume of 2.5 M potassium phosphate buffer pH 6.8 and 0.8 volume of a mixture (1:2) of 2-butoxyethanol and 2-ethoxy-

ethanol, were added. The phosphate buffer was prepared by mixing hot solu-
tions of 2.5 M KH_2PO_4 and 2.5 M K_2HPO_4 until pH reached 6.8. The components
were mixed gently by hand. Centrifugation at 1000 x g for 5 min resulted in
partition of an aqueous bottom phase, an organic top phase and a gelatinous
interphase. After decantation of the liquids the interphase was resuspended
in 25-30 ml 0.033 M phosphate buffer, pH 6.8, per 100 ml sonicated homoge-
nate. Centrifugation at 5000 x g for 10 min gave a clear supernatant, which
was ultracentrifuged at 105 000 x g for 60 min. As much as possible of the
procedure was at 4^oC.

The pellet resulting from ultracentrifugation was resuspended in 0.5 ml
0.033 M phosphate buffer pH 6.8 per 100 ml crude homogenate. The suspension
was kept for some hours at 4^oC and sometimes finally centrifuged at 5000 x g
for 5 min but not in later trials because of undesired precipitation of vi-
rus.

After negative staining with 2% phosphotungstic acid pH 6.0, the virus
particles were observed in a Siemens Elmiskop 1 or a Philips EM-300 electron
microscope.

Material and methods will be detailed under the individual trials.

EXPERIMENTS AND RESULTS

The isolation of the virus growing-room

During trials lasting about $3\frac{1}{2}$ years in the isolated growing-room of the for-
mer building of the Experimental Station, not one of the other growing-rooms
became infected with the disease. Although such a well isolated growing-room,
tended by only one person, cannot be compared with a mushroom farm, it still
indicates what control measures are possible.

Infection experiments

Earlier trials on infection in the isolated growing-room had already confirm-
ed that the disease is very easily spread by viable mycelium or by spores
from diseased mushrooms (Dieleman-van Zaayen, 1970). Virus-infected mycelium,
left behind on or in wood after inadequate disinfection at the end of a crop,
could anastomose with healthy mycelium in a following crop and thus transmit
the virus.

Various authors, for instance Rasmussen et al. (1969), stressed the

danger of infection between spawning and casing. Below are described two of the trials to find the most dangerous time of infection and the influence of amount of inoculum.
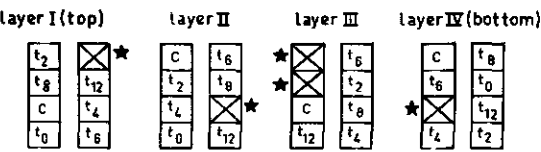
Trial 1: time of infection
A constant amount of inoculum was introduced in seven plots of one layer (a layer consists of two shelves next to one another) on the day of spawning (t_0), every 2 days after spawning(t_2, t_4, t_6, t_8) or 12 days after spawning (t_{12}). A control (C) was not inoculated.

The remaining plots were used for other tests. This trial was carried out in layers since there were rather great differences in climate between the layers at the former building of the Experimental Station. Because of this, each plot of one layer was treated differently, so that each treatment occurred once in each layer. The various treatments were divided randomly over the plots of a layer. The layout is shown in Fig. 1. The plots were inoculated in the centre with 2.5 g 'infected spawn' consisting of a mixture of Virus Collection No 92 and 94.

All inoculated plots showed symptoms of the disease, and virus particles, of 25 and 34 nm mainly, could be isolated from the mushrooms. The controls remained healthy.

Uncut mushrooms from each plot were weighed separately. After 25 days of harvesting (three flushes), the growing-room was cooked out, as is common procedure in virus experiments. The test was in quadruplicate so that the results could be processed by variance analysis, but this proved completely superfluous: the differences were striking. The yields of four plots that had received a treatment at one time were now added, and yields/m^2 were adjusted (-20%) as if the mushrooms had been cut. For the missing treatment t_0 in layer III, which plot was used for other trials, the expected yield was extrapolated from all other yields by the missing-plot technique.

Results are given in Table 1 and plotted in Fig. 2. Note that the con-

★ These plots were used for other experiments.
For the same reason, from layer III, t_0 had to be omitted.

Fig. 1. Layout of Trial 1 on time of infection.

0.5% solution of commercial formaldehyde until casing. This percentage is sufficient to kill mushroom spores, but the mycelium growing in the compost underneath is not harmed, as tests indicated;

4. strict hygiene.

It is not sufficient to implement one or two control measures only; the whole list of measures must be implemented to free a farm from virus.

For more-zone systems, only slight adaptation of these measures would be needed.

Reduction of crop loss

In the Netherlands, an official enquiry for 1969 in which 97% of the growers participated, showed that implementation of all the measures considerably reduced crop loss: from 4.5% of the total production in 1967 to 1.3% in 1969 (Table 4). Although the enquiry over 1970 is only partly processed (up to 80%), preliminary results indicate exactly the same figure, 1.3%, for total crop loss; the annual production amounted to 30 million kg cut mushrooms. The total crop loss due to virus disease has already decreased from 4.5% to 1.3%, but in future this figure might fall even lower when the new returnable picking boxes are centrally disinfected. Picking boxes used a number of times by different growers and redistributed to others by regional auctions have earlier been shown to contribute to spread of the disease throughout the country.

The *conclusion* is: prevention and control of virus disease is certainly possible. The measures have proved to be effective. It should be realized, however, that this requires continuous effort from every grower.

Table 4. Commercial losses from mushroom virus disease in the Netherlands. Data from official enquiries and a random test among more than 1 000 Dutch growers.

Year	Contaminated farms (%)	Avg crop loss per contaminated farm (%)	Total crop loss (%)	Annual production (kg)	Crop loss (kg)
1967	>30	15.0	4.5	17.5 million	787,500
1968	25.1	16.7	4.2	20 million	840,000
Control measures (November 1968)					
1969	11.2	11.4	1.3	23 million	299,000

Trials to obtain virus-free material

Heat treatment of mycelium

Heat treatment   Virus-infected mushroom mycelium often grows slowly and de-
generates on a nutrient medium but Gandy (1960b) reported that treatment at
33°C resulted in most cases in better and faster growth. Presumably this
treatment, which lasted two weeks (Gandy and Hollings, 1962), freed the fun-
gus from virus. However heat-treated mycelium was never tested for virus
particles.

To verify this assumption the Virus Collection, including some healthy
mycelia, was kept in an incubator:
a. for 3 weeks at 33°C: 36 different mycelia including 10 uninfected cultures;
b. for 3 weeks at 33°C: other material from the same 36 mycelial cultures;
c. for 2 weeks at 34°C: the same 36 cultures plus 22 virus-infected and 8
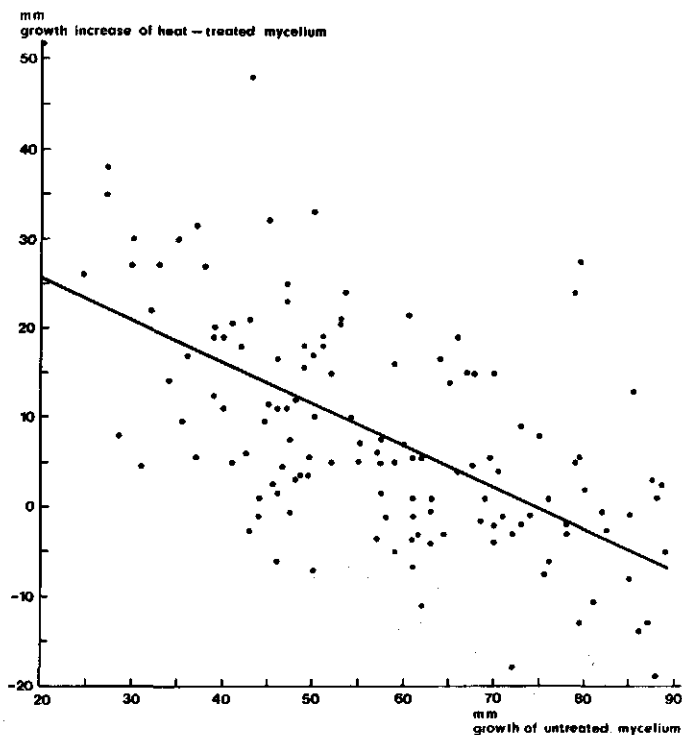uninfected mycelia (including 2 wild strains).

Fig. 5. Differences in growth (mm) between heat-treated and untreated mycelial cultures against growth rate of the untreated cultures. Heat treatment was for 3 weeks at 33°C or for 2 weeks at 34°C. Regression equation: $y = -0.46x + 34.6$, with $r = -0.61$ (significant at 1% level). Means: growth increase = 8.2 mm; growth rate of untreated culture = 57.7 mm.
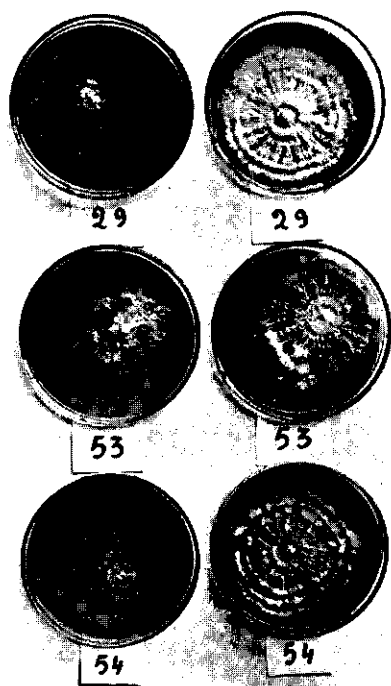
Fig. 6. Heat treatment (2 weeks at 30°C) of virus-infected mycelial cultures No 29, 53 and 54 on 2% Biomals agar. Left untreated cultures; right the same cultures after treatment. All transfers were made on the same day.

For this purpose transfers of the mycelial cultures were made to 4 test tubes with 2% Biomals agar per isolate; the tubes were kept at 25°C. Five days later 2 tubes were incubated at 33°C or 34°C for three or two weeks. The other 2 tubes remained at 25°C as controls. After incubation, all tubes were again kept at 25°C, and about 5 days later all cultures were transferred to a fresh nutrient medium in tubes. Then the growth in length (mm) of the colonies was measured after 20 days. The two replicates were averaged and growth increase or decrease in relation to control was calculated.

The results are shown in Fig. 5. The negative relationship between growth increase of heat-treated mycelia and their growth rate when untreated, can be expressed by the regression equation $y = -0.46x + 34.6$ ($r = -0.61$; significant at 1% level), represented by the regression line in the graph. The equation was obtained by averaging the regression equations of the individual treatments. Although a certain relationship could be demonstrated, it is clear that the results had a wide spread. Replicates often differed widely. In general, slowly growing cultures displayed a much better and faster growth after heat treatment (Fig. 6), whereas mycelia with a good or excellent growth did not react or deteriorated with the treatment. This

Table 5. Difference in growth of mycelial cultures, after various treatments
(d), from controls (25°C, last column). Averages of two colonies on 2%
Biomals agar in test tubes.

Culture No	Growth increase or decrease (mm)							Growth of control (mm)
	2 wk[1] -20°C	2 wk 28°C	2 wk 30°C	2 wk 33°C	3 wk 33°C	2 wk 34°C	2 wk 35°C	
Virus-infected								
1	-10	- 4	- 2	+10	+10	- 3	0	60
7	†[2]	+ 1	+15	+17	+20	+ 7	†	49
92	-23	- 2	+16	+24	+19	+39	+ 3	43
123	- 1	-11	+ 2	+ 2	+22	+16	+ 7	39
135	- 3	+ 5	+14	+24	+14	+12	+ 5	47
154	†	- 7	+18	+18	+31	+27	+ 9	32
Healthy								
37	+ 2	- 8	-11	+19	+ 8	-18	†	63
43	†	0	+11	+15	+ 8	-	†	80
130	+ 4	+ 3	- 4	+ 7	+ 1	+ 8	-	58
152	-13	+ 1	0	+ 3	+14	- 9	-22	44

1. wk= weeks
2. †= culture did not survive

applies to uninfected and infected cultures, and to wild strains.

Further treatments:

d. Material from 6 infected and 4 uninfected mycelial cultures was subjected
to one of the following treatments in duplicate: for 2 weeks at -20°C; 2
weeks at 28°C; 2 weeks at 30°C; 2 weeks at 33°C; 3 weeks at 33°C; 2 weeks at
34°C; and 2 weeks at 35°C. Controls were kept at 25°C.

After the treatments, the cultures were again kept at 25°C and 5 days
later they were transferred to a fresh medium. Growth rates were measured
on agar in test tubes 20 days after transfer. Averages were calculated; the
growth increases or decreases of the treated mycelia in relation to the con-
trols are given in Table 5. Treatments at -20°C and 28°C did not influence
the mycelial growth rate; -20°C even killed some cultures, as did 35°C.
Some isolates (No 7, No 43) seemed to be more sensitive than others. Incu-
bation at 30-34°C, however, often resulted in growth increase although with
great irregularity. Note that some of the uninfected mycelia showed a simi-
lar trend.

Tests for virus particles   To test for virus particles in mycelium,
Hollings et al. (1965) suggested direct observation of sonicated mycelium
with the electron microscope. In our experience, this method is unsatisfac-

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in 3rd flush

1. Inoculum consisted of laboratory-prepared spawn.
2. 80% of true yield

transfer from centre or periphery of a virus-infected mycelial culture (No 112). Although a certain growth increase after transfer from the centre can also be noticed, the increase is more pronounced with peripheral transfer. The other cultures reacted similarly; however the results were variable as with heat treatment, although most replicates varied only slightly.

A number of resulting cultures from the last transfer of the seven different mycelia under treatment were incubated for two weeks at 34°C. Controls remained at 25°C. Twenty days after transfer to fresh medium, growth was measured (Table 9). Tests were on 2% Biomals agar in test tubes in duplicate. Expectedly, the results were variable but followed the general trend of heat treatment: growth increase of the slowly growing cultures, and no or negative reaction of the faster growing mycelia.

Tests for virus particles   Spawn inoculum was prepared from No 52, CCCC and CCCC heat-treated (HT), 52 PPPP and PPPP-HT. Small growing trays were inoculated with 5 g laboratory-prepared spawn each. The trial was in duplicate. The results indicated that No 52 PPPP, PPPP-HT and CCCC-HT were not freed from virus particles: two or three particle types could be isolated from mushrooms on the trays. Earlier similar tests with No 92 failed because of

Table 9. Growth increase or decrease (mm) of mycelial cultures, earlier subjected to repetitive transfer from centre or periphery, after heat treatment (2 weeks at 34°C). Each value represents an average of two cultures in test tubes. C= growth of untreated control (25°C); GI= difference in growth from control.

Culture	No 1		No 28		No 37[1]		No 52		No 92		No 94		No 112	
	C	GI	C	GI	C	GI	C	GI	C	GI	C	GI	C	GI
CCCC	58	+15			69	+2	68	-2	45	+20			55	- 5
CPCC			50	- 2										
CCPP	55	+20			67	0	58	+4	56	+ 3	63	- 3	70	- 4
CPPP	67	+16	50	- 1	76	-3	68	0	30	+25			71	+ 5
PCCC	67	+21	55	+ 2	76	-7	63	+1	32	+20				
PCPC													61	+10
PPCC	67	+12	55	+ 6			62	+3					76	*
PCPP					66	+5								
PPPP	70	-13	51	+12	72	*	72	-7	56	+13	62	+13	84	- 7

*. Contaminated

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Table 10. Yields of, and presence of virus particles in, mushrooms grown in plots inoculated (Trial I) or spawned (Trial II) with spawn from virus-infected No 112. This mycelial culture was subjected to repetitive transfer from centre (C) or periphery (P), and sometimes heat-treated (HT, 2 weeks at 34°C). Averages of two plots.

Treatment	Inoculum (g/plot)	Adjusted[1] yield[2] (kg/m^2)	Virus particles[3] (nm)
Trial I			
CCCC	2.5	0.26	abundant 25 and *34*
CCCC-HT	2.5	10.1	few *25*, 34, 19x50
PPPP	2.5	10.6	few 25, and probably 34
PPPP-HT	2.5	9.7	few 25 and 34
Trial II			
No 112 (untreated)	450	7.4	many 25, *34*, 19x50
PPPP	750	19.3	common *25* and 34
PPPP-HT	750	17.6	common *25*, 19x50 and probably 34

1. 80% of true yield.
2. Yields are for 22 and 31 days of picking in Trials I and II, respectively.
3. The figures in italics indicate the particle type(s) prevailing in that virus preparation.

several unfavourable circumstances.

The results of two trials in duplicate with No 112 in plots are shown in Table 10. In the first trial (Trial I), plots were inoculated at spawning with 2.5 g spawn each, prepared from No 112 CCCC, CCCC-HT, PPPP or PPPP-HT. In Trial II, plots were spawned with inoculum (grain spawn) prepared from No 112 PPPP (375 g/m^2), PPPP-HT (375 g/m^2) and No 112 (450 g 'infected spawn' and 300 g commercial spawn per plot). Virus Collection No 112 is similar to No 112 CCCC. Yields were estimated in 22 days (Trial I) and 31 days (Trial II) of harvesting. Control plots yielded 10.5 to 11 kg/m^2 (Trial I) and 18 kg/m^2 (Trial II) cut mushrooms. Table 10 shows that repetitive transfer from the outer periphery of the colony increased yields as does heat treatment; combination of both, however, seemed slightly less favourable. Although the yields of the treated cultures were quite as good as controls, the mycelia certainly were not virus-free. Whether the virus, still present after treatment, may be an attenuated strain, or may later cause a new outbreak, has not yet been investigated; hence treated mycelium should be handled cautiously.

There is no distinct difference between heat treatment and repetitive transfer from the outer periphery; the effect observed after heat treatment, better growth of the mycelium, may even have been due to transfer, after

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incubation, from the periphery (Gandy and Hollings, 1962), which generally consists of vigorously growing mycelium.

DISCUSSION

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4.5 Additional information and discussion

Mushroom virus disease is spread by viable infected mycelium and by virus-
infected spores, as has been reported by different authors and is confirmed
by the trials described in this Chapter. Spread by mycelium can be complete-
ly prevented, chiefly by applying the wood-preservative sodium pentachloro-
phenate. Spread by spores, however, is harder to control. This means of
spread is very effective: infected mushrooms usually mature too early (Sec-
tion 2.1) so that growers cannot pick them all before they open and before
spores are released; virus-infected spores germinate better and earlier
than uninfected spores (Section 4.1) and may contain many virus particles
(Section 3.1). Moreover, single spores are invisible and growers can only
suspect their presence; spores are light in weight and can easily be carried
by air-currents or wind. Schisler et al. (1967) mention a longevity of more
than 30 years for healthy mushroom spores, under undefined conditions. Our
data indicate that virus-infected spores still germinated after storage for
$3\frac{1}{2}$ years (the storage period did not yet exceed $3\frac{1}{2}$ years) on filter paper
in a refrigerator at 4°C. In less favourable conditions as occur in nature
(moister; higher temperatures), their life may be shortened. The danger of
spores will be obvious, especially in areas where farms are built close
together as in some parts of the Netherlands. But within one contaminated
mushroom farm, spores can already cause much trouble.

Spawn has not proved an important means of spread, at least in this
country.

Spread of mushroom virus disease by an insect vector is highly improb-
able: phorid flies, for instance, only seem to lick mushrooms since their
mouth-parts are suctorial; the larvae feed on the mycelium and do not tunnel
in the stems and caps of fruiting bodies (Hussey, 1959); moreover, no Diptera
with suctorial mouth-parts are known that are vectors of viruses. An insect
vector is also not necessary, since the rapid spread of mushroom virus dis-
ease can be explained by spores. But British workers evidently did not rate
spread by spores at its true value and explained the rapid spread of the
disease, even to control trays of their trials, from virus transmission by
the phorid fly *Megaselia halterata* Wood (Hollings, 1967). The mushroom build-
ing where these trials were done had no spore filters (Hollings et al., 1971).
Experiments on virus transmission by these flies had no controls and are

only in the destruction of spores; but spores can be killed in easier and safer ways, e.g. by brief heat treatment or brief exposure to formaldehyde vapour. Mycelium and especially spawn were found to be moderately resistant to the fumigant, and various uninfected and infected mycelial cultures differed in sensitivity to methyl bromide.

This variability was also noticed in attempts to free mycelium from virus. Heat treatment and repetitive transfer from the outermost hyphal tips did not result in virus-free cultures. Yet growth of mycelium often improved, but with wide variation, and mushroom yields usually increased after treatment. Nothing is yet known about what happens during treatment, nor about the effects on multiplication of virus in the mycelium.

Because of the difficulties encountered during purification, I have not yet succeeded in preparing specific antisera against the three different types of virus particle. Although serology may be an important tool in establishing relationships between the particle types, satisfactory antisera can only be prepared after purification and complete isolation of each kind of particle. It will probably never provide a sensitive test method because of the extremely low virus concentrations sometimes observed in cell-free preparations from obviously diseased mushrooms (low, as compared to virus concentrations in many flowering plants).

The diversity of virus concentrations in mushrooms, the virus multiplication and the build-up of virus concentrations in mushroom cultures are interesting problems. A simple infectivity test would facilitate further research on purification. Improved purification and separation techniques are indispensable for elucidation of any mutual relationship between the three types of virus particle, their properties and the role of each type in causing the disease and in symptoms.

Since properties and relationships of the mushroom virus particles are not yet known, it seems premature to name or number the different particle types as British workers have done (Hollings & Stone, 1971).

A mutual relationship between the three types cannot be excluded, since they are usually found together. From *Penicillium stoloniferum*, three types of virus-like particles were isolated, two of which were electrophoretically and serologically distinct (Buck & Kempson-Jones, 1970; Bozarth et al., 1971). From *Aspergillus foetidus*, two types of virus-like particles were recently isolated, similar in diameter but different in double-stranded RNA components. They were probably serologically unrelated. Ratti & Buck (1972) have suggested a multicomponent system of these virus-like particles.

A similar system could exist for mushroom virus particles. The possibility of satellite viruses, or mutual helper viruses cannot be excluded. But the particle types may alternatively represent distinct viruses. Comparisons with the double-stranded RNA-containing virus-like particles in *Penicillium* and *Aspergillus* spp. should be considered carefully, since those particles have not yet been proved to be pathogens or infectious agents; the work of Lhoas (1971) is not conclusive.

The mushroom virus particles will have to be purified and isolated. It would then be possible to investigate such questions as the nature of the nucleic acid, and perhaps to make more comparisons with viruses of higher plants.

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Summary

Diseased cultivated mushrooms, *Agaricus bisporus* (Lange) Sing., usually yielded three types of virus particle, often in combination: isometric particles 25 and 34 nm in diameter, and bacilliform particles 19 nm in diameter and 50 nm long. Injection of a cell-free virus preparation into young mushrooms on a growing-tray, and subsequent reisolation of the three types of virus particles, in a later flush, from fruiting bodies showing symptoms, proved that the particles caused the disease (Section 2.1).

Symptoms were highly variable (Section 2.1). However isolation of virus particles from suspect mushrooms is decisive (Section 2.2).

In ultrathin sections of vegetative mycelium, dense aggregates of virus particles were detected often near a septum or close to a nucleus. Prestaining en bloc with aqueous uranyl acetate proved essential to demonstrate the mushroom virus particles (Section 3.1). Later studies revealed the particles in mycelium to be 34 nm in diameter. In ultrathin sections of fruiting bodies, they were often abundant in the cytoplasm, in loose aggregates or dispersed, and sometimes in vacuoles. They were observed in dolipores; hence 34-nm particles seem to pass from cell to cell. Virus particles 19 nm x 50 nm were seldom found in sections of the stipe; they were difficult to detect. Isometric particles 25 nm in diameter, resembling ribosomes, occurred in deranged cells of the cap of mushrooms from a sample, known to contain many 25-nm virus particles. These isometric particles were often clumped together and membrane-bound. In ultrathin sections of basidiospores from diseased mushrooms, virus particles 34 nm in diameter were grouped together in small vacuoles and occasionally in spore cytoplasm, up to some hundreds of particles per section of one spore. No virus-like particles were found in tissue or spores from healthy mushrooms (Section 3.1).

The 25-nm mushroom virus particles turned out to be identical in appearance to turnip yellow mosaic virus, i.e. a $T = 3$ structure with hexamer-pentamer clustering of subunits (Section 3.2). A parallel resemblance was observed between rod-shaped virus-like particles, frequently isolated from apothecia of *Peziza ostracoderma* Korf, and tobacco mosaic virus. *P. ostra-*

coderma (syn. *Plicaria fulva* R. Schneider) often occurs as a contaminant in mushroom farms. Negatively stained virus-like particles were on average 17 mm in diameter x 350 mm long and had a clear axial canal. Occasionally such particles were observed in cell-free preparations from diseased cultivated mushrooms (Sections 3.2 and 3.3). Apothecia of *P. ostracoderma* that contained particles did not differ macroscopically from those without particles (Section 3.3). Ultrathin sections of apothecia, from samples known to contain the virus-like particles, revealed the presence of crystal-like aggregates of the particles in cells just below the asci. The aggregates were often found in vacuoles and consisted of rods arranged in angled layers. However similarities as in aggregation, to some strains of tobacco mosaic virus, were accompanied by distinct differences, for instance in structure (Section 3.3). So far the significance of the rods in *A. bisporus* is unknown.

In Section 4.1, practical aspects were tackled. Trials in an isolated growing-room of the Mushroom Experimental Station confirmed that the disease spreads with viable mycelium and with spores from diseased mushrooms, as reported earlier by different authors, and demonstrated the importance of time of infection: early infection considerably reduced crop yield, whereas later infection did not. Returnable picking boxes contributed to the spread of the disease all over the country. Intensive research resulted in a list of measures to prevent or control the disease. It was implemented among Dutch growers in November 1968. The measures are based mainly on strict hygiene and are meant to prevent the spread of infected mycelium and spores.

Extensive investigations on spawn, in which more than 100 growers participated for over a year, were not decisive, partly through lack of a reliable test for virus in spawn. Virus may spread with spawn, but other means are considered more important (Section 4.2).

, Methyl bromide fumigant does not control mushroom virus disease, except partially by destroying mushroom spores. Spores, however, can be destroyed easier and less dangerously. The results of fumigating mushroom mycelium were variable, and of spawn disappointing (Section 4.3). The use of this chemical should be discouraged.

Section 4.4 describes trials in the isolated growing-room on, inter alia, the importance of time of infection and the inconsequence of amount of inoculum. It gives the definitive version of the list of measures to prevent or control disease. General implementation of the measures in the Netherlands considerably reduced crop loss, as appeared from annual inquiries among all mushroom growers. Attempts to free mycelium from virus by various

techniques (heat treatment at 33^OC, repetitive transfer from the outermost periphery of hyphae) failed. For a virus-free start, and for control, better results may be expected by breeding from a mushroom strain that seems re-sistant to virus disease.