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Immunity of strains of Agaricus bitorquis to mushroom virus disease

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Abstract

Agaricus bitorquis, a species originally found in nature, produces snowwhite, tasty and well storable fruiting bodies on the common mushroom substrate. Several strains of this species were tested for susceptibility to mushroom virus, viz. the original wild isolates *A. bitorquis* Nos 1, 2, 4 and 6, and the selection and breeding products 'Somycel 2.017', Horst B30, Horst B6, Horst K26 and Horst K32. Inoculum consisted of either mycelium or spores from virus-infected mushrooms (*A. bisporus*), or both. Trays with *A. bisporus* were always inoculated simultaneously to check the infectivity of the inoculum. Unlike *A. bisporus*, the strains of *A. bitorquis* tested did not show symptoms of virus disease after inoculation. Their fruiting bodies did not present mushroom virus particles, either in extracted cell-free preparations, or in ultrathin sections of the tissue. Yields of *A. bitorquis* were not decreased by inoculation. The efficacy of the inoculation technique is discussed. The recent commercial availability of immune mushroom strains was a relief to Dutch mushroom growers, who found the disease difficult to eradicate by sanitary measures only.

Introduction

In the Netherlands, virus disease is a considerable threat to cultivated mushroom, *Agaricus bisporus* (Lange) Imbach (Fig. 1). The disease is caused by three types of virus particle, often in combination: isometric particles 25 nm in diameter, bacilliform particles 19 nm in diameter and 50 nm long (Hollings, 1962), and isometric particles 34 nm in diameter (Dieleman-van Zaayen and Temmink, 1968). Spread of the disease is by viable infected mycelium (Gandy, 1960) and by spores from infected mushrooms (Schisler et al., 1967; Dieleman-van Zaayen, 1972a) through anastomosis with healthy mycelium. Sanitary measures that prevent the spread of infected mycelium and spores can prevent or control the disease. General implementation of a list of measures, requiring continuous effort of each grower, considerably reduced crop loss in the Netherlands over some years (Dieleman-van Zaayen, 1972b). Growing virus-resistant mushroom strains would save much labour and diminish risks. Therefore attempts were made to obtain such strains.

The white species *Agaricus bitorquis* (Quél.) Sacc. was gathered from nature in 1968. It colonized the common mushroom substrate, i.e. horse manure compost, provided the growing temperatures exceeded those required by *A. bisporus* (Hasselbach and Mutsers, 1971). In preliminary trials in 1969 a remarkably high tolerance to virus disease was noticed (Dieleman-van Zaayen, 1972b). Breeding with *A. bitorquis* is described by Fritsche (1976).

The present study details the immunity of several strains of *A. bitorquis* to mush-

Fig. 1. Virus-infected crop of *A. bisporus*. Photograph: Th. G. M. Pompen, Horst.

Fig. 1. Viruszieke teelt van A. bisporus. Foto: Th. G. M. Pompen, Horst.

room virus disease. Fruiting bodies of this species were grown in inoculated trays and tested for the presence of virus. Inoculum consisted of mycelium and/or spores from virus-infected mushrooms (*A. bisporus*). Anastomoses between hyphal tips of *A. bisporus* and *A. bitorquis* were frequently observed (Hasselbach and Derks, personal communication).

Materials and methods

Experiments in vitro

Transmission of the viral agent in vitro was tested on 2% Biomals agar (a malt product) in Petri dishes at 25 °C. Plates were inoculated with:

a. a disc of agar with healthy mycelium of *A. bisporus* (white strain 'Somycel 32') and a disc with a virus-infected mycelial culture of *A. bisporus* (No. 92) showing slow growth, at a distance of ca 3 cm;
b. a disc with mycelium of *A. bitorquis* and a disc with virus-infected No. 92, at a distance of ca 3 cm;
c. a disc with healthy mycelium of *A. bisporus* ('Somycel 32');
d. a disc with mycelium of *A. bitorquis*.

The experiments were in duplicate. Strains 1 and 2 of *A. bitorquis* were used (Table 1). After adjacent cultures of a) and b) had grown together for some days, all cultures were transferred to a fresh nutrient medium and ca 20 days later growth rate was mutually compared.

Table 1. Strains of *A. bitorquis* tested for susceptibility to mushroom virus.

Strain number or code	Origin	Derived from
No. 1	nature	
No. 2	nature	mycelium isolated from
No. 4	nature	fruiting bodies
No. 6	nature	
Horst B30	No. 2	selection (multi spore culture)
Horst B6	No. 1 × 2	single spore cultures
Horst K26	No. 2 × 4	single spore cultures
Horst K32	No. 2 × 6	single spore cultures
'Somycel 2.017'	Poppe (1972)	commercial spawn

Tabel 1. Rassen van A. bitorquis die getoetst zijn op hun vatbaarheid voor champignonvirus.

Trials in mushroom growing-rooms

Strains of A. bitorquis. The strains of *A. bitorquis* tested for susceptibility to virus, and their origin, are listed in Table 1.

Preparation of spawn. To 10 kg sorghum grains, boiled in ca 12 l water for 20–25 minutes, 200 g gypsum ($CaSO_4$) and 50 g chalk ($CaCO_3$) were added after cooling down (Stoller, 1962). The mixture was thoroughly stirred and divided among 1 l bottles, which were closed with cottonwool and paper plugs, and sterilized for 2 h at 121 °C. After cooling, the bottles were vigorously shaken and a piece of agar with mycelium of *A. bitorquis* was tranferred to each of them. Spawn running was at 28 °C for about 3 weeks; twice during this period the bottles were shaken. On the day of spawning the desired amount of spawn was weighed out (5 kg/1000 kg compost). 'Somycel 2.017' was obtained commercially.

Mushroom growing. Mushrooms were grown by the single zone system. Standard mushroom trays (0.27 m^2) were filled with 21.6 kg (ca 80 kg/m^2) compost. The compost was peak-heated for 10 days. A maximum air temperature of 57 °C was recorded on the first day. Subsequently, the compost was spawned with laboratory-prepared or commercial spawn of *A. bitorquis* or with commercial spawn of *A. bisporus* (white strain, Sinden A_1). During mycelial growth, the compost temperature was kept at 30 °C.

Twelve days after spawning, the compost was fully colonized by mushroom mycelium. It was then cased with a 4 cm layer of a mixture of black peat (ca 60%), sphagnum peat (ca 34%), sand (ca 3%) and marl (ca 3%). Horst B6 and Horst B30 demanded compressing of the casing layer.

Mushrooms appeared 3 to 4 weeks after casing, depending on the strain. The compost temperature during cropping was 25 °C. For the next 3 to 6 weeks mushrooms were harvested every 2 to 3 days and weighed in each of usually eight replicates in randomized blocks. The yields were processed by analysis of variance (Exp. 228–478). All growing experiments with virus-infected material were in an isolated growing-room. To prevent other growing-rooms from becoming infected, precautions were taken according to Dieleman-van Zaayen (1972b).

Inoculation with virus-infected material: a) Mycelium. Virus-infected mycelial cultures of *A. bisporus* were obtained and maintained as described elsewhere (Dieleman-van Zaayen, 1972b). Inoculum was prepared of some of the most infectious cultures as described above for spawn, except for a spawn running temperature of 25°C instead of 28°C.
b) Basidiospores. Spores were collected by placing virus-infected mushrooms (*A. bisporus*) on filter paper under a glass beaker. After some days at room temperature the mushrooms dropped their spores, and a brown 'spore print' appeared on the filter paper. The prints were stored in Petri dishes in plastic bags at 4 °C until use.

Inoculation. Five g of infected mycelium on sorghum grains was put in the centre of a tray in the isolated growing-room, at a depth of 5 cm in the compost. The spore prints were cut into equal shreds. Usually several shreds derived from different mushrooms were placed together at a depth of 5 cm in the compost in the centre of a tray to be inoculated. Inoculation was immediately after spawning. Uninoculated trays spawned with strains of *A. bitorquis* acted as control.

To verify infectivity of the infected mycelium and the 'diseased' spores, trays spawned with *A. bisporus* were inoculated simultaneously and kept in the same growing-room.

In experiment V15 plots with *A. bitorquis* were inoculated with spores collected from fruiting bodies of *A. bitorquis* of a previous infection experiment.

Electron microscopy

Cell-free preparations. Fruiting bodies of *A. bitorquis* and *A. bisporus* from inoculated and control trays were tested for virus by a method developed by Dieleman-van Zaayen and Temmink (1968), slightly amended by Dieleman-van Zaayen (1972b). This method includes ultrasonic treatment of the ground fruiting bodies (Hollings et al., 1965), followed by a procedure based on an organic solvent phase system (Kitano et al., 1961). As for *A. bitorquis*, samples were collected mainly from suspected trays that yielded lower than the control trays.

Ultrathin sections. Fruiting bodies of *A. bitorquis* from inoculated and control trays were also subjected to fixation, embedding, sectioning and staining according to Dieleman-van Zaayen and Igesz (1969) for the detection of virus particles in situ.

Results

Experiments in vitro

After anastomosis with virus-infected culture No. 92, mycelium of *A. bisporus* grew more slowly than control mycelium, presumably because of transmission of virus. Infected mushroom mycelium often grows slowly and degenerates on a nutrient medium (Gandy, 1960). After anastomosis with No. 92 mycelial growth of strains of *A. bitorquis* did not differ from that of the control (Fig. 2). Detection of virus particles in mycelium by direct observation of sonicated mycelium in the electron microscope (Hollings et al., 1965) is, even with mycelium of *A. bisporus*, in our experience unsatisfactory and was not applied.

Fig. 2. Similar growth rates of mycelium of *A. bitorquis* (below) on 2% Biomals agar without (C) and after (V) anastomosis with virus-infected culture *A. bisporus* No. 92, as compared with growth decrease of mycelium of *A. bisporus* ('Somycel 32', above), after (V) anastomosis with No. 92. The healthy control ('Som. 32', C) shows a normal growth rate. All mycelial cultures were transferred on the same day. Infected culture No. 92 acts as a standard on all plates. Photograph: IPO, Wageningen.

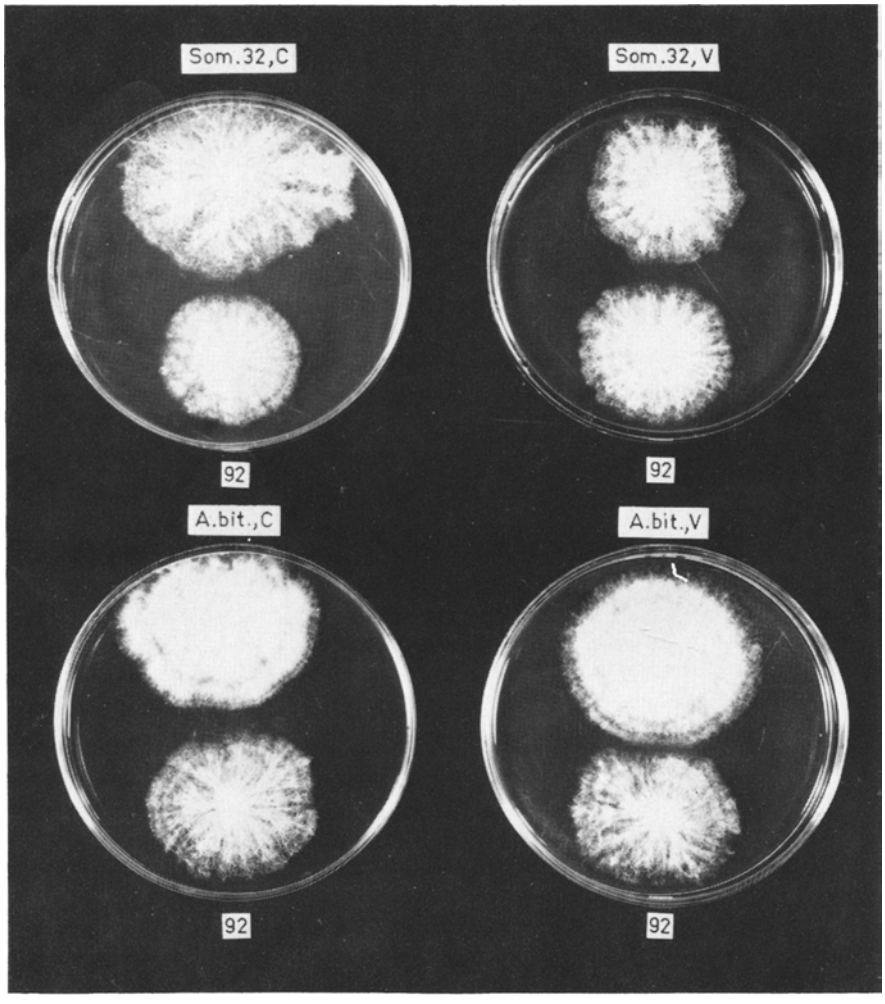

Fig. 2. Gelijke groeisnelheid van mycelium van A. bitorquis (onder) op 2% Biomals agar na (V) en zonder (C) anastomose met virusziek mycelium nr 92 van A. bisporus, vergeleken met groeiremming in mycelium van A. bisporus ('Som. 32', boven) na anastomose (V) met nr 92. De gezonde controle ('Som. 32', C) vertoont een normale groei. Alle mycelia werden op dezelfde dag overgeënt. De virusziekе culture nr 92 dient als standaard op alle schalen. Foto: IPO, Wageningen.

Table 2. Average[1] yields[2] of strains of *A. bitorquis* inoculated with mycelium and spores from virus-infected mushrooms *(A. bisporus)*. Symptoms and virus particles were not observed in any.

Trial No.	Strain of *A. bitorquis*	Yields (kg/m^2)		
		untreated	inoculated with infected mycelium	'diseased' spores
V9[3]	No. 1	13.16 (2)[4]	13.48 (2)[4]	14.16 (1)[4]
V11[3]	No. 1	12.25 (1)	12.38 (8)	12.41 (4)
	No. 2	8.02 (1)	8.85 (8)	8.42 (4)
V15[3]	No. 1	5.44 (1)	–	5.34 (2)
				6.36 (1)[5]
228	Horst B30	16.20 (8)	15.21 (7 × 8)	15.62 (8)
333	Horst B30	14.76 (8)	14.93 (2 × 8)	15.50 (8)
	'Somycel 2.017'	12.65 (8)	13.01 (2 × 8)	13.69 (8)
358	Horst B30	10.84 (8)	9.88 (7)	9.67 (8)
	'Somycel 2.017'	11.44 (8)	10.89 (7)	11.70 (8)
383	No. 4	15.63 (8)	16.21 (8)	16.09 (8)
	No. 6	13.04 (8)	13.39 (8)	12.61 (8)
	Horst B6	13.63 (8)	14.43 (8)	14.72 (8)
463	Horst K26	20.41 (9)	19.10[6] (9)	
478	Horst K32	14.24 (9)	13.79[6] (9)	

[1] Averages of 1–9 replicates.
[2] Yields in cut mushrooms are in 3.5 week of picking in trial V15, 5.5 week of picking in trial 228, 6 weeks of picking in trial 333 and ca 4 weeks of picking in the other trials. Yields were adjusted for 100 kg compost/m^2.
[3] Experiments in plots in an isolated growing-room of the former building of the Mushroom Experimental Station. Yields were not processed by analysis of variance.
[4] Figures in parentheses represent number of replicates.
[5] Inoculated with spores and germinated spores from fruiting bodies of *A. bitorquis* from an inoculated plot in trial V11.
[6] Infected mycelium and 'diseased' spores were inoculated together.

Tabel 2. Gemiddelde opbrengsten van rassen van A. bitorquis, die geïnoculeerd werden met mycelium en sporen van viruszieke champignons (A. bisporus). Er werden geen symptomen en geen virusdeeltjes waargenomen.

Trials in mushroom growing-rooms

Results of inoculation experiments with *A. bitorquis*-strains are given in Table 2. Yields varied due among others to various harvesting periods, and to our initial ignorance of conditions required by *A. bitorquis*. Yields in the isolated growing-rooms usually were not optimal, since the aim was primarily to establish infection. Decreases or increases in yield following inoculation with virus-infected material were not significant in any case. Strains of *A. bitorquis* never showed symptoms of virus disease after inoculation (Fig. 3). Virus particles could never be isolated from fruiting bodies of *A. bitorquis*.

The infected mycelium and 'diseased' spores used as inoculum were in each case infectious as shown by decreased yields, symptoms of the disease, and many virus particles (25 nm, 34 nm and 19 × 50 nm, Fig. 4) extracted from mushrooms on inoculated trays with *A. bisporus*. Because yields of inoculated trays with *A. bisporus*

Fig. 3. Crop of *A. bitorquis*, 5 weeks after inoculation with infected mycelium, in an isolated growing-room (trial V9). Photograph: IPO, Wageningen.

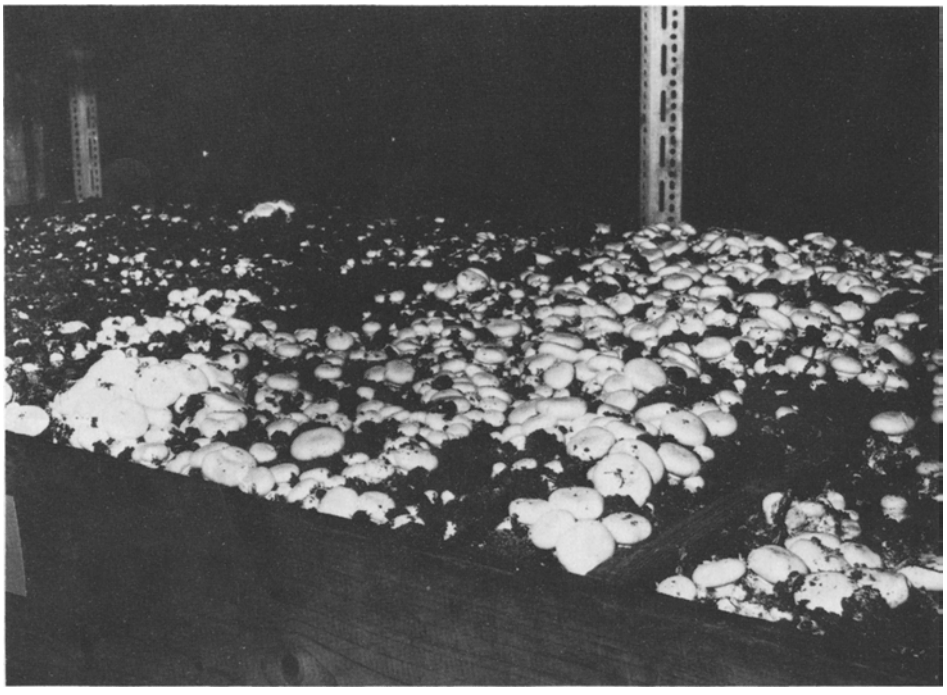

Fig. 3. Champignons van A. bitorquis, 5 weken na inoculatie met ziek mycelium, in een geïsoleerde teeltruimte (proef V9). Foto: IPO, Wageningen.

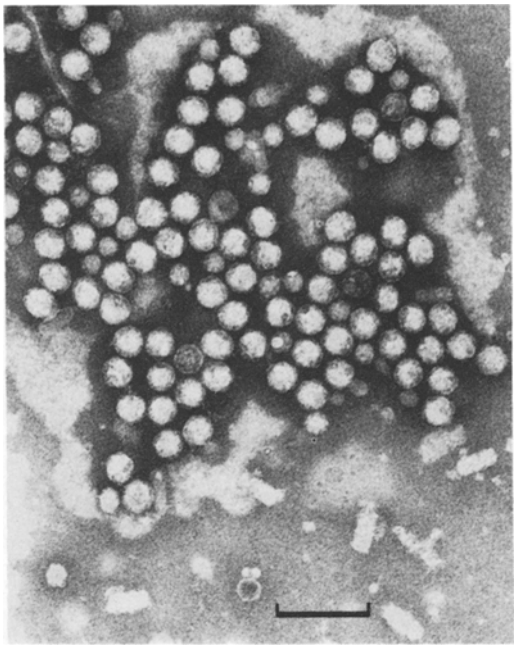

Fig. 4. Cell-free preparation from diseased mushrooms *(A. bisporus)* with isometric virus particles 25 and 34 nm in diameter, and bacilliform virus particles 19 nm in diameter and 50 nm long. The bar represents 100 nm.

Fig. 4. Celvrij preparaat van zieke champignons (A. bisporus), met isometrische virusdeeltjes met een diameter van 25 en 34 nm en bacilliforme virusdeeltjes met een diameter van 19 nm en 50 nm lang. De maatstreep geeft 100 nm aan.

were very low, not only by virus disease but also by the high temperatures required by *A. bitorquis*, they have been omitted from Table 2.

Electron microscopy of tissue

In ultrathin sections of fruiting bodies of *A. bitorquis* from inoculated and control trays no viruslike particles were detected. Two, possibly three types of mushroom virus particles had earlier been observed in ultrathin sections of infected fruiting bodies of *A. bisporus* (Dieleman-van Zaayen, 1972a).

Discussion

Until recently, mushroom virus disease could only be prevented or controlled by hygienic measures preventing spread of infected mycelium and spores (Dieleman-van Zaayen, 1972b). At the outset, general implementation of the measures in the Netherlands considerably reduced crop loss: from 4.5% of the annual production of 17.5 million kg mushrooms in 1967, to 1.3% of 23 million kg in 1969 and 1.3% of 30 million kg in 1970 (Dieleman-van Zaayen, 1972b). In 1973, however, the loss by virus disease rose to 3.5% of a production of 41 million kg or some 1.5 million kg mushrooms. This increase could inter alia be attributed to loosely applying of the sanitary measures and picking of big, open, spore-dispersing mushrooms as a result of high labour costs.

Better results were to be expected from tolerant or resistant mushroom varieties. Kneebone et al. (1962) reported that cross-inoculation between white and cream varieties of *A. bisporus* using diseased mycelium was unsuccessful, and growers in the USA were advised to shift to other varieties of *A. bisporus* to avoid the disease. Schisler et al. (1967) however, succeeded in transmitting the disease by cross-inoculations using spores from diseased and healthy mushrooms of white and cream varieties of *A. bisporus*. This was confirmed by infection trials in our isolated growing-rooms in which all other available strains (cream, brown) of *A. bisporus* tested, after various lengths of time, became equally diseased as snowwhite strains. Virus particles of 25, 34 and 19 × 50 nm could be extracted from the mushrooms. On contaminated Dutch mushroom farms the disease could not be eradicated by shifting to other varieties of *A. bisporus*.

Strains of *A. bitorquis* have now been shown immune to mushroom virus disease. Virus particles could not be extracted from fruiting bodies after inoculation, nor could they be detected in mushroom tissue. Trays or plots with *A. bitorquis* were inoculated with mycelial cultures or (and) spores of virus-infected *A. bisporus*. Injection of a cell-free mushroom virus preparation into fruiting bodies of *A. bitorquis* was omitted, since this technique (Gandy and Hollings, 1962; Dieleman-van Zaayen and Temmink, 1968) very seldom led to infection of *A. bisporus*. This species can get infected very easily with diseased mycelium or spores.

Anastomoses between hyphal tips of *A. bisporus* and the closely related species *A. bitorquis* were frequently observed (Hasselbach and Derks, personal communication). Anastomoses between germ tubes of one species and growing hyphal tips of the other, as occurs between different varieties of *A. bisporus*, may even be more frequent. In this way transmission of virus from *A. bisporus* to *A. bitorquis* could be possible,

Fig. 5. *Agaricus bitorquis*, highly productive strain Horst K26, immune to mushroom virus disease. Photograph: Th. G. M. Pompen, Horst.

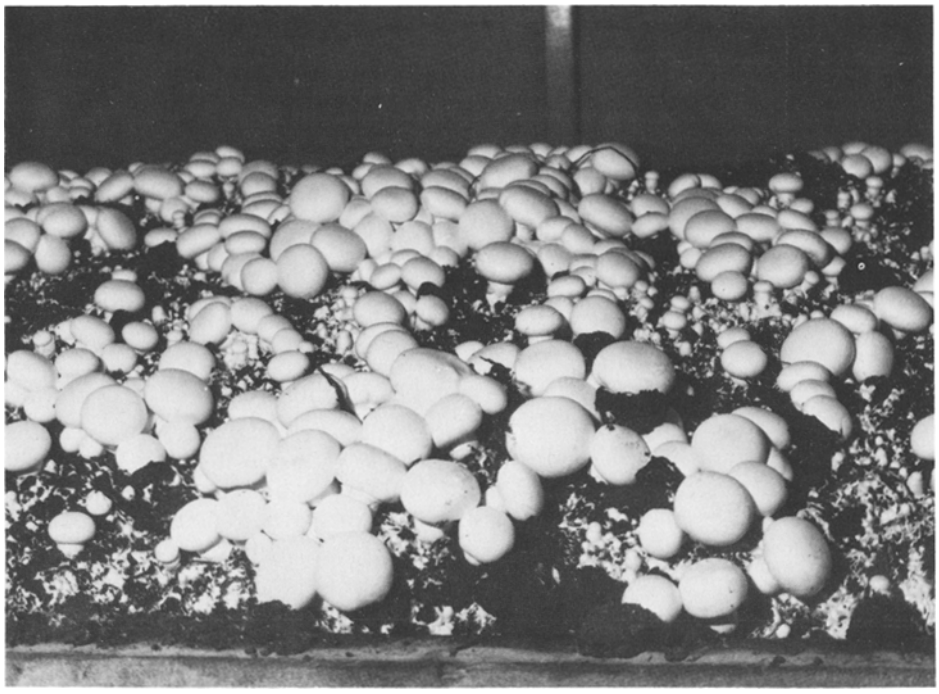

Fig. 5. Agaricus bitorquis, het zeer produktieve ras Horst K26, dat onvatbaar is voor champignonvirus. Foto: Th. G. M. Pompen, Horst.

but no virus particles were detected in the fruiting bodies of *A. bitorquis*. Apparently, virus particles did not penetrate into tissue of *A. bitorquis* or were unable to multiply after entering a cell. The inoculation technique may have failed. However, viral transmission is achieved by fungi through heterokaryosis (Lemke and Nash, 1974). Interspecific heterokaryons between *A. bisporus* and *A. bitorquis* (syn. *A. edulis*) were produced in vitro (Raper and Raper, 1972).

Even when inoculation was with spores of *A. bitorquis* from an inoculated tray (V15), no virus particles could be extracted from the symptomless fruiting bodies. Therefore it is out of the question that *A. bitorquis* would contain virus in a concentration below the detection threshold.

The higher growing temperatures required for *A. bitorquis* cannot have been injurious to the mushroom virus particles, since fruiting bodies of *A. bisporus* yielded many virus particles at high temperatures unfavourable to this species. In 1973, two seriously contaminated mushroom farms were supplied with spawn of Horst B30. No symptoms of virus disease and no virus particles could be detected in the *A. bitorquis*-crop and the yield was satisfactory. In 1974 Horst B30 was commercially available, as was the French strain 'Somycel 2.017', which was found to be immune in the above experiments. Introduction of both strains considerably decreased crop loss by mushroom virus disease in the Netherlands. Admittedly, they are not easy to grow and their yields are not impressive. However, *A. bitorquis* produces tasty snow-

white fruiting bodies with good keeping quality. Of the new commercially available strains Horst K26 and Horst K32 (Fritsche and Pompen, 1975), the first in particular produces high yields (Fritsche, 1976; Table 2) and mushrooms of good shape (Fig. 5) and high quality.

Acknowledgment

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Samenvatting

Onvatbaarheid van rassen van Agaricus bitorquis voor virusziekte van champignons

De oorspronkelijk wilde soort *Agaricus bitorquis* produceert helderwitte, smakelijke en lang houdbare vruchtlichamen op het normale champignonsubstraat. Verschillende rassen van deze soort werden getoetst op vatbaarheid voor champignonvirus, namelijk de oorspronkelijk wilde isolaties *A. bitorquis* nr 1, 2, 4 en 6 en de selectie- en veredelingsprodukten 'Somycel 2.017', Horst B30, Horst B6, Horst K26 en Horst K32 (Tabel 1). Het inoculum bestond uit mycelium of sporen van viruszieke champignons *(A. bisporus)*, of uit beide. De proeven omvatten steeds objecten waarin kisten met *A. bisporus* werden geïnoculeerd met identiek materiaal als toets op de infectiositeit van het inoculum. In tegenstelling tot *A. bisporus*, vertoonden de getoetste rassen van *A. bitorquis* na inoculatie geen verschijnselen van virusziekte (afstervingsziekte) (Fig. 3). Champignonvirusdeeltjes (Fig. 4) konden niet worden geextraheerd uit de vruchtlichamen van *A. bitorquis*, noch worden aangetoond in ultradunne coupes hiervan. Er trad geen oogstderving op na inoculatie (Tabel 2). De doelmatigheid van de gebruikte inoculatiemethodiek wordt besproken. Aangezien de virusziekte van champignons nog steeds een ernstige ziekte is (Fig. 1) die nooit geheel uitgeroeid zal kunnen worden door het toepassen van hygiënische maatregelen, is het voor Nederlandse champignontelers een verademing dat er thans onvatbare champignonrassen in de handel zijn (Fig. 5).

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