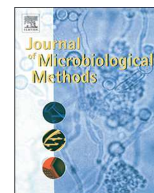
Journal of Microbiological Methods 173 (2020) 105913

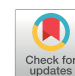
Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth



FISHing in fungi: Visualisation of mushroom virus X in the mycelium of *Agaricus bisporus* by fluorescence *in situ* hybridisation



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ARTICLE INFO

Keywords:
Agaricus bisporus
Fluorescence *in situ* hybridisation
FISH
Mushroom virus X
MVX
Mycovirology
Mycovirus

ABSTRACT

Agaricus bisporus is a commercial mushroom crop susceptible to a disease caused by a complex of viruses known collectively as mushroom virus X (MVX). Symptoms of MVX include bare patches and mushroom cap discolouration (browning) in the fruiting bodies, phenotypes associated with the viruses AbV6 and AbV16, respectively. Limited understanding exists of the localisation and mobilisation of these viruses within the mycelium of *A. bisporus*. To this end, a non-destructive fluorescence *in situ* hybridisation (FISH) method was developed for *in situ* targeting of AbV6 and AbV16 in *A. bisporus* mycelium. An MVX strain associated with the bare patch disease phenotype revealed predominantly high signal towards the growing edges of cultures when probed for AbV6, with a 'halo-effect' of high signal intensity around putative vacuoles. An MVX strain associated with the browning disease phenotype showed high signal intensities within reticulating networks of hyphae in a highly compartmentalised manner when probed for AbV16. Localisation of the two viruses in MVX-infected cultures appears independent, as both viruses were found in completely discrete areas of the mycelium in differential patterns. FISH detected low level presence of the two viruses, AbV6 and AbV16 in a number of cultures which had tested negative for the viruses by RT-PCR. This suggests that FISH may be more sensitive at detecting viruses at low levels than molecular methods. This study demonstrates that FISH is a powerful tool in the field of mycovirology.

1. Introduction

Fluorescence *in situ* hybridisation (FISH) is a method for the hybridisation of targeted nucleic acid sequences using bespoke fluorophore-conjugated nucleic acid probes. A technique developed in the 1980's, the initial intricacies of the technique led to its use being considered challenging and difficult. Innovations in subsequent years have resulted in FISH becoming a more accessible method, with a wide breadth of applications (Huber et al., 2018), with cytogenics arguably reaping the greatest degree in advances (Jiang, 2019).

FISH is a powerful tool in microbiology. For example, it has been used in the detection of community structures in biofilms (Almeida et al., 2011; Aoi, 2002), aquatic microbiome sampling (Dawson et al., 2012; Kurisu et al., 2015; Medlin and Orozco, 2017), cultural heritage conservation (La Cono and Urzi, 2003; Urzi and De Leo, 2001), in clinical samples of blood sera (Da Silva et al., 2015) and many other uses. FISH has been used for the localisation of bacteria, fungi and viruses within respective hosts (reviewed in (Kliot and Ghanim, 2016)). The first use of FISH targeting fungi was in the yeast-like fungus *Aureobasidium pullulans* (Li et al., 1997). The greatest hurdle in applications of FISH in localisation studies is commonly the issue of probe penetration (Kliot and Ghanim, 2016). This is particularly relevant to fungi, as the fungal cell wall is a complex structure of hydrophobic scaffolds of α-1,3-glucan and chitin encased in layers of convoluted linkages of β-glucans, glycoproteins and α-1,3-glucan (Kang et al., 2018). The lack of or complete absence of permeability of the fungal cell wall, can act as a

Abbreviations: FISH, Fluorescence *in situ* hybridisation; AbV(*N*), *Agaricus bisporus* virus (*number*); MVX, Mushroom virus X; DAPI, 4′,6-diamidino-2-phenylindole; FITC, Fluorescein isothiocyanate; CYM, Complete yeast media; PBS, Phosphate-buffered saline; BSA, Bovine serum albumin; HCl, Hydrochloric acid; SDS, Sodium dodecyl sulphate; PNA, Peptide nucleic acid

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https://doi.org/10.1016/j.mimet.2020.105913
Received 21 November 2019; Received in revised form 1 April 2020; Accepted 1 April 2020
Available online 08 April 2020
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barrier to probe penetration (Brul et al., 1997; Teertstra et al., 2004). Due to the hydrophobicity and varying degrees of negative charge observed in the fungal cell walls (Free, 2013), non-charged semi-synthetic hybridisation probe alternates may be used, known as peptide nucleic acid (PNA) probes (Nielsen and Egholm, 1999). PNA probes have seen many uses of FISH in fungi (Da Silva et al., 2015; Ferreira et al., 2017; Nakada et al., 2013; Reller et al., 2007; Teertstra et al., 2004). Although, PNA probes are expensive and their use can be highly cost prohibitive. The process or permeabilising the cells of fixed fungal mycelium, without destructive proteases and chitinases, is an effective way of allowing access of DNA probes to their targets and minimizing the dependency on use of PNA technology (Villa et al., 2009).

To date, mycovirology has yet to fully harness the application of FISH. A single study probed a DNA mycovirus in the mycelium of *Sclerotinia sclerotiorum*, but this was achieved using methods incorporating lytic enzymes (which were avoided in this study) and the resolution of nucleus fluorescence and probed virus fluorescence was limited (Yu et al., 2013).

In this study, the technique of FISH was applied for the investigation of two viruses in the mushroom virus X (MVX) complex. The application of FISH was used to assess whether both viruses could be probed for their detection and for the understanding of their spatial distributions within the mycelium of *A. bisporus*. The targets were AbV6 and AbV16, which are multipartite and bipartite viruses, respectively (Deakin et al., 2017). As such, the probes designed specifically targeted AbV16 RNA 1(^{MVX} 1.8 kbp) and AbV6 RNA 2 (^{MVX} 3.6 kbp) (Grogan et al., 2003). AbV16 RNA 1 is causal for the brown disease phenotype, and present particularly in MVX-infected crops in Ireland (Eastwood et al., 2015; Fleming-Archibald et al., 2015; Grogan et al., 2003). AbV6 was associated with the bare patch disease phenotype, prevalent in diseased crops in the UK in some of the earliest reports of MVX (Grogan et al., 2003) although a definitive correlation with symptoms has not been established. It has been detected in high abundance in AbV16 infected crops with no bare patch symptoms (unpublished data). Low levels were also reported in a 'non-diseased commercial culture' (Deakin et al., 2017). The work reported here involves an adapted method of FISH, whereby *A. bisporus* cultures (with and without MVX viruses) are grown, permeabilised, hybridised and visualised *in situ*, so as to negate any disturbance to the mycelium. This technique uses non-destructive permeabilization methods (Villa et al., 2009) which allow the use of DNA probes, making the method cost-effective and reproducible. This is the first robust application of FISH on mycoviruses within the mycelium of a fungal host.

2. Methods

2.1. Strains and culture conditions

MVX-infected *A. bisporus* cultures MVX-1283, MVX-2735, and MVX-1153 were derived from mushroom samples that were taken from symptomatic crops in the UK and Ireland between 2000- and 2016 (Table 1). Presence of AbV16 RNA 1 was confirmed by RT-PCR according to (Fleming-Archibald et al., 2015). Presence of AbV6 RNA 2 was confirmed using the same methodology, and PCR primers designed specifically for AbV6 RNA 2 (F: GGCAGGAGCAGATGAACATT R: ACC TGGAACAGCAGCAAAAC; product size 305 bp; Fleming-Archibald, unpublished) based on the published sequence (Genbank KY357490). MVX cultures were retrieved from liquid nitrogen storage and grown on complete yeast medium (CYM) containing 2 g proteose peptone, 2 g yeast extract, 20 g glucose, 0.5 g $MgSO_4$, 0.46 g KH_2PO_4, 1 g K_2HPO_4, 10 g agar in 500 ml dH_20. Cultures were incubated at 25 °C for two weeks, in the dark prior to use. A commercial strain of *A. bisporus* (Sylvan A15; www.sylvaninc.com) was used as a non-MVX control strain in this study. A culture was derived from a spawn grain, taken from a new bag of commercial spawn and grown on CYM and incubated at 25 °C for two weeks, in the dark prior to use.

2.2. Slide culture preparations

Microscope slides (75 mm × 25 mm) were sterilised by autoclaving. To obtain a thin solid layer of medium, 600 µl of molten CYM was carefully pipetted to the centre of a microscope slide and immediately covered by another microscope slide. The medium was left to solidify for 1 min and the slide placed on top was carefully separated and removed with sterile forceps. The slide with the adhered CYM layer was added to a Petri dish containing a sterile, dH_2O-moistened filter paper to act as a humidity chamber (Fig. 1A). Agar plugs of *A. bisporus* cultures were placed on top of the thin, flat layer of solid media and incubated at 25 °C for one week, in the dark. CYM was considered a suitable medium to use in this study as it is commonly used to cultivate *A. bisporus* mycelium *in vitro* (De La Bastide et al., 1997; Kaur et al., 2011; Li et al., 1994; Masoumi et al., 2015). Control steps were taken to ensure CYM did not harbour problematic levels of nascent auto-fluorescence that would impede fluorescence observations (data not shown).

2.3. Fixation, permeabilisation and hybridisation of DNA probes

Slide cultures of *A. bisporus* were fixed for 1 h on ice with freshly prepared 4% formaldehyde in PBS, pH 7.4. The agar plugs were gently removed with sterile forceps, leaving a flat layer of hyphae on the medium. Cultures were washed twice in PBS and then dehydrated by submerging in 50% ethanol (v/v) and stored overnight at −20 °C. Cultures were rinsed once and submerged in PBS for 20 min then washed in pre-hybridisation wash buffer (0.5% Tween-20, 0.2% BSA in PBS) for 1 h 30 min followed by thorough rinsing in dH_2O. Permeabilisation of hyphae was carried out by soaking in permeabilisation buffer (1% Triton X-100 in 0.05 M Tris-HCl, 0.04 M EDTA, 0.1 M β-mercaptoethanol) at 30 °C for 30 min and at 40 °C for an additional 15 min (Villa et al., 2009). Osmotic pressure was applied through submerging cultures in glycerol solution (31.75 ml glycerol (Sigma-Aldrich) and 68.25 ml dH_2O) on ice (Fig. 1B) for 30 min (Villa et al., 2009). Dry filter paper was dabbed near the edges of cultures to facilitate the removal of permeabilising agents and allowed to air-dry, briefly. Hybridisation buffer (0.9 M NaCl, 0.02 M tris-HCl pH 7.2, 0.01% w/v SDS, 20% v/v deionised formamide, 50 ng/µl DNA probe

Table 1
Information on the *A. bisporus* strains used for FISH.

A. bisporus Strain	Disease phenotype	MVX in original culture	Year collected	Country of origin	RT-PCR 2017 AbV6	RT-PCR 2017 AbV16
MVX-1283	Bare patches on crop bed	AbV6	2000	UK	+	−
MVX-2735[a]	Brown caps	AbV16	2002	Ireland	−	−
MVX-1153	Brown caps	AbV16	2016	Ireland	−	+
Non-MVX (A15)	None (control)	None	2017	France	−	−

MVX cultures were derived from mushrooms collected at different locations from crops displaying MVX symptoms and which were shown to contain AbV6 or AbV16 at the time of collection. The results of RT-PCR tests (+ = positive; − = negative) on the cultures used in these studies in 2017 are also shown.

[a] MVX 2735 had originally contained AbV16 and had originally tested positive by RT-PCR but in recent years the culture is frequently negative by RT-PCR for AbV16.

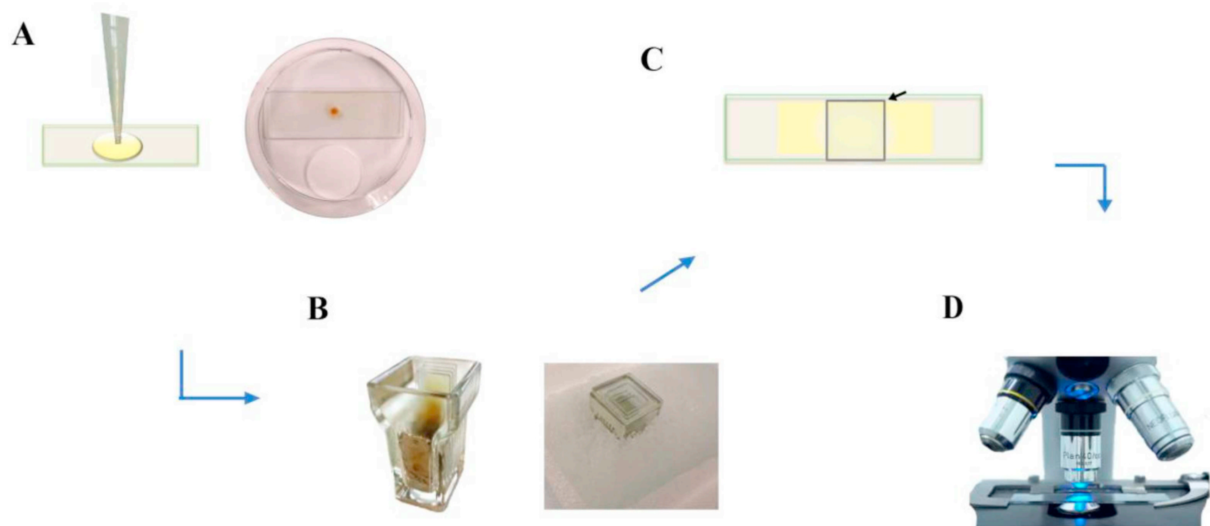

Fig. 1. Schematic of the MVX-FISH workflow. (A-D) Represents the order of the main aspects of the method. A: Slide cultures. A slide is coated in CYM by pipetting and then added to a petri dish containing a moist filter paper to provide a humid environment. An agar plug colonised with *A. bisporus* hyphae is added to the slide. B: Pre-treatments. Coplin jars are used to submerge cultures in solutions for fixation, washing and permeabilisation. Jars can be imbedded in ice, heated and agitated when necessary. C: Hybridisation. For hybridisation, a cover slip (black arrow) is glued over the preparation with a latex glue. D: Visualisation. A fluorescence microscope is used to visualise cultures on the same slide used from A.

and 1 mg/ml 1× RNase-free BSA) was pre-warmed and 20 µl slowly added to the surface of hyphae. A HybriSlip™ (22 mm × 22 mm; Sigma-Aldrich) was placed over the hybridisation buffer carefully to avoid bubbles forming in the culture/cover slip interface. Latex glue was applied around the entire circumference of the HybriSlip to seal in the hybridisation buffer (Fig. 1C). Cultures were added to a hybridisation chamber (high humidity) and incubated at 52.8 °C for 14 h in the dark. The hybridisation chamber was allowed to cool to room temperature over 5 min before proceeding further. Latex glue and cover slip were carefully removed and cultures were rinsed with dH_2O. Cultures were washed by submerging slides in post-hybridisation wash buffer (0.2 M NaCl, 0.02 M tris-HCl pH 7.2, 0.01% w/v SDS) at 47 °C for 20 min on a rocking platform. Cultures were further rinsed in dH_2O and air-dried for 5 min. 15 µl of Vectashield Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) stain (Vector Labs, Burlingame, CA, USA) was added to cultures and incubated at 4 °C for 30 min, in the dark. ssRNA target specificity of hybridisation was assessed by flushing cultures with and without the presence of RNase cocktails, as detailed in previous work (Teertstra et al., 2004). RNase-treated slides functioned as negative controls.

2.4. MVX FISH probes

FISH probes were made by designing oligonucleotide DNA probes (Eurofins Genomics, Ebersberg, Germany) complementary to the sequences of AbV16 RNA 1 and AbV6 RNA 2 (Deakin et al., 2017). The two FISH probes (5′- TGTTATGTGGGTGTAGTTAG-3′ for AbV16 RNA 1, designed from GenBank accession: KY357502.1 and 5′-TGTCAGGTGT TCAACCAGC-3′ for AbV6 RNA 2, designed from GenBank accession: KY357490.1) were 5′-end fluorescein isothiocyanate (FITC) labelled (Eurofins Genomics, Ebersberg, Germany). At the concentrations used, the DNA probes for AbV16 RNA 1 and AbV6 RNA 2 had a Tm of 52.4 °C and 62.5 °C, respectively.

2.5. Fluorescence microscopy

Fluorescein and UV fluorescence were monitored using a FITC and DAPI filter set, respectively. Fluorescence light microscopy was conducted with an Olympus BX43 system microscope coupled with a CoolLED pE-300 Illuminator. Images were capture by an Olympus SZX16 camera mounted on the microscope, connected to a computer.

3. Results

3.1. Permeabilisation of A. bisporus hyphae and FISH controls

Permeabilisation was necessary to facilitate FISH probe penetration of fungal cell walls. Porousness of cells was achieved using the nuclear stain DAPI (Fig. 2). DAPI was added to every culture preparation as quality control to reduce the likelihood of false negatives *i.e.* where no fluorescent signal is a result of inefficient cell permeabilisation and not the absence of the target virus. Additionally, RNase-cocktails were used to introduce negative controls. Specificity of probes to bind to target RNA was evaluated by flushing permeabilised hyphae with RNase, which can be confirmed by a loss in signal due to the destruction of virus RNA (Fig. S1). Non-specific binding of DNA probes and nuclei can occur by affinities of probe nucleotide sequences to chromosomal regions. To ensure DNA probes were not non-specifically binding to nuclei within cells, DAPI stain fluorescence was compared to FITC fluorescence to ensure no superimposable signal intensity was captured (Fig. S2). These measures provide confidence that the observed fluorescence is as a result of the hybridisation of the probes to viruses within the mycelium.

3.2. FISH detection of AbV6 in A. bisporus strains

Each strain was hybridised with AbV6 probes. MVX-1283 was positive for AbV6 infection upon original testing and also when tested by RT-PCR in 2017 (Table 1) and was the only strain that showed high levels of fluorescence when probed for AbV6 (Fig. 3). Localisation of AbV6 was observed primarily at the margins of the colony. The intensity of fluorescence was high, occurring throughout the length of the hyphae. The fluorescence was strong enough in certain hyphae that a particular pattern was observed, whereby negative staining zones (possibly vacuoles) were surrounded by very high fluorescence (Fig. 3B, D), giving an apparent ‘halo-effect’. Although the non-MVX control strain had tested negative by RT-PCR for AbV6 (Table 1), some fluorescence was recorded in all replicate preparations (n = 25) when AbV6 was the target. The extent of probe fluorescence was low, but still easily discernible at the extreme apices of hyphae on the growing periphery of cultures (Fig. 4). A

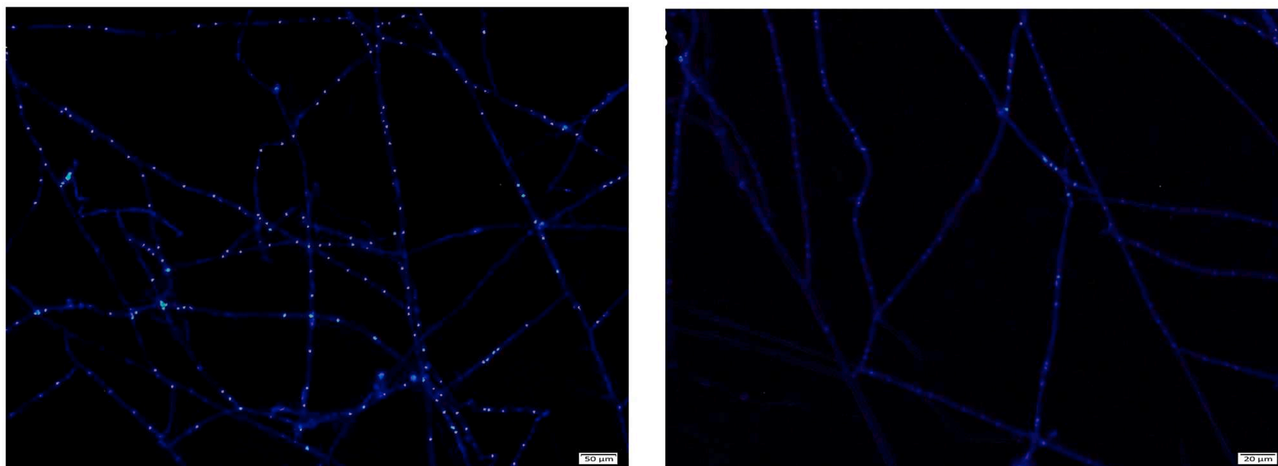

Fig. 2. Assessment of permeabilisation of hyphae with DAPI. DAPI nuclear stain is added to porous hyphae and viewed with a fluorescence microscope under a UV filter set. Bright dotted areas represent fluorescence from stained nuclei. Fluorescence was monitored using a fluorescence light microscope. Images represent replicates of DAPI stained nuclei at two magnification, where scale bars are provided for measure.

fluorescent gradient, brightest at the apex and lessening down the hypha, was apparent (Fig. 4). Additionally, branching hyphae were found to contain fluorescent signal, where the primary hypha, at the point of origin of the branching, did not (Fig. 4). High fluorescent signal was also captured in a swelling that was beginning to, but had not yet formed, a new hyphal branch (Fig. 4). MVX-1153 and MVX 2735 which had tested negative for AbV6 by RT-PCR (Table 1), also showed very low fluorescent signal for AbV6 detection at levels similar to the non-MVX control strain in all replicate preparations (n = 25) (Fig. S3). The negative control (RNase-treated) is the only culture that did not display any level of AbV6 detection using FISH (Table 2).

3.3. FISH detection of AbV16 in A. bisporus strains

FISH was used to localise AbV16 in the mycelium of all strains but was detected in only MVX-2735 and MVX-1153 (Fig. 5A, B). MVX-1153, which was positive for AbV16 on original testing and also by RT-PCR in 2017, showed high fluorescent signal when probed for AbV16 (Fig. 5B). Signals were found in the reticulating network of mycelium towards the centre of cultures and not at the growing edges/hyphal apices. AbV16 showed a highly compartmentalised sequential pattern of signal distribution in the hyphae (Fig. 5B). FISH did not reveal any fluorescence in any replicate (n = 25) of the non-MVX control strain. A

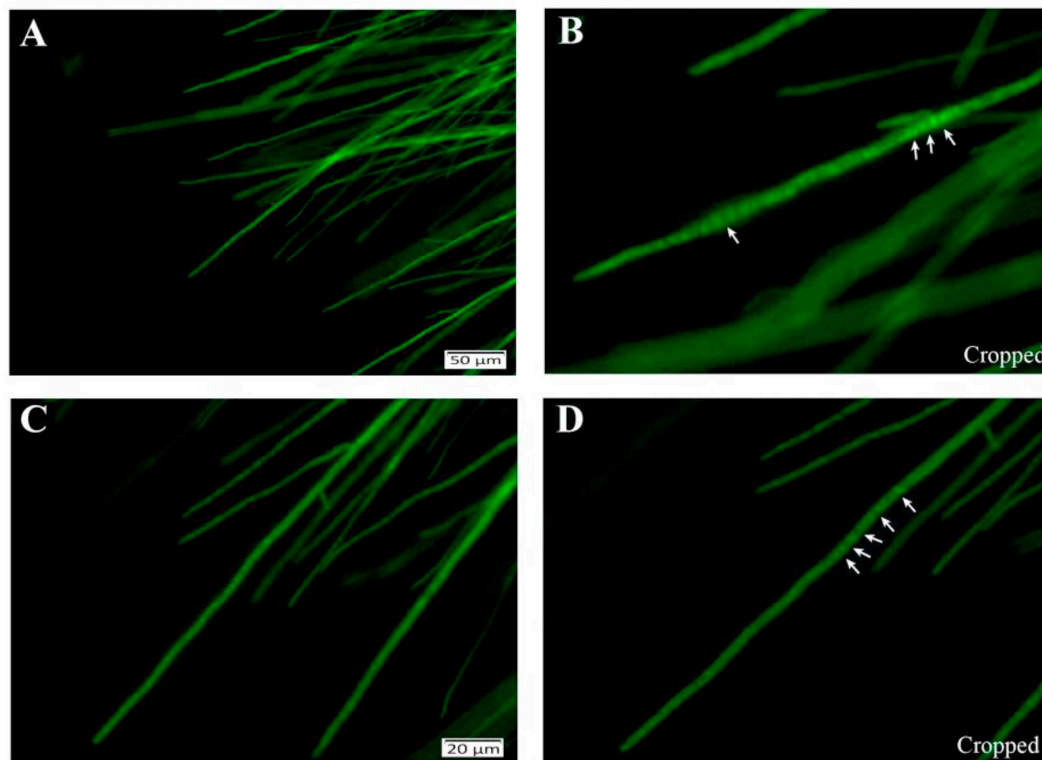

Fig. 3. FISH of MVX-1283 with a FITC-labelled AbV6 probe. High degree of fluorescence is detected in hyphae towards the periphery of the cultures. A and C: Images taken of MVX-1283 culture in different areas and at different magnification. B and D: Close up of area shown with arrows in images A and C, respectively. White arrows point to areas where a halo-effect is seen whereby vacuoles have low fluorescent signal but are surrounded by strong circles of fluorescent signal. Fluorescence was monitored using a fluorescence light microscope. Scale bars are provided in original images for measure.

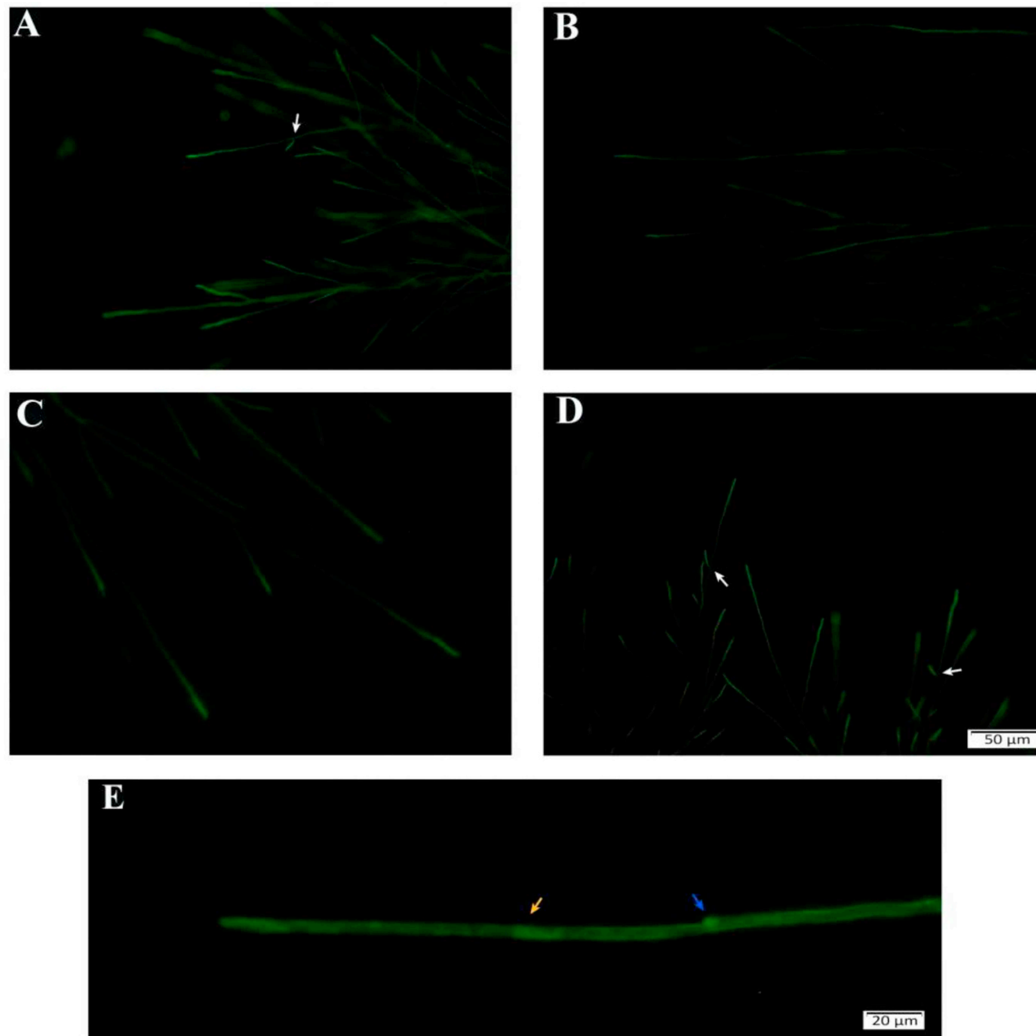

Fig. 4. FISH of non-MVX strain, hyphae with a FITC-labelled AbV6 probe. Fluorescence was observed for AbV6 most intensely at the extreme apices of the hyphae (A-D). A–D represent different replicates of the non-MVX strain probed for AbV6 all with similar patterns of AbV6 detection. White arrows in A & D point to areas where hyphal branches exhibiting fluorescent signal can be seen developing from a point on the primary hypha where there is no signal. (E) Image of a hypha where the right arrow shows a newly forming branch (swelling) where fluorescence is notably high. The left arrow highlights a cellular compartment where AbV6 is congregating at the septum in the direction of the hyphal apex. Fluorescence was monitored using a fluorescence light microscope. Scale bars are provided for measure (A–D scale: 50 µm).

Table 2
FISH detection of MVX in each strain of *A. bisporus* tested.

Strain	Untreated		RNase-Treated	
	AbV6	AbV16	AbV6	AbV16
Non-MVX (A15)	+	−	−	−
MVX-1283	+ +	−	−	−
MVX-2735	+	+	−	−
MVX-1153	+	+ +	−	−

Fluorescence detection is rated as follows; (+ +), High levels of fluorescence; (+), Low levels of fluorescence and (−), no fluorescence detected.

very faint fluorescent signal was detected in replicates of MVX-2735 (Fig. 5A), which had been positive for AbV16 when originally collected but it appeared to have lost this virus when tested by RT-PCR in 2017. MVX 1283 and the non-MVX control did not display any level of AbV16 detection using FISH (Table 2).

4. Discussion

The method for FISH was adapted from previous studies involving this technique on filamentous fungi (Teertstra et al., 2004; Villa et al., 2009), and was used in a novel application to target two disease phenotype-associated viruses of MVX in *A. bisporus* mycelium. This approach facilitated *in situ* cultivation, permeabilisation, hybridisation of FISH probes and visualisation by fluorescence light microscopy of mycelium on a singular platform, without the need for cutting, embedding, or any form of translocation of material (Fig. 1). As such, this technique allows for *in vitro* growth of hyphae and FISH of nucleic acid targets with minimal disturbance to the mycelium architecture. Furthermore, by adapting methods previously evaluated for non-destructive permeabilisation of hyphal cell walls (Villa et al., 2009), the need for lytic enzymes to facilitate DNA probe penetration into fungal hyphae was avoided (Fig. 2). This is highly beneficial as destructive methods delocalise fluorescence signals (Teertstra et al., 2004).

Using the FISH technique described above, and an AbV6 specific

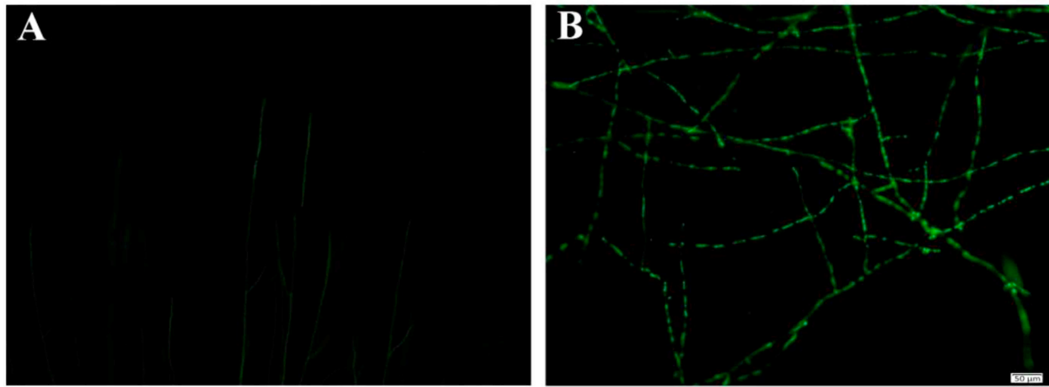

Fig. 5. FISH of MVX strains with a FITC-labelled AbV16 probe. (A) MVX-2735 showing low amounts of fluorescent signal. (B) MVX-1153 showing a high amount of fluorescent signal. AbV16 can be seen in a compartmentalised manner throughout the interconnecting hyphae. Localisation of signal was found at a distance from hyphal apices towards the centre of the culture mycelium. Fluorescence was monitored using a fluorescence light microscope. Scale bars are provided for measure (A and B: 50 µm).

probe, a very strong signal was obtained in the peripheral hyphae of the AbV6-positive strain, MVX-1283 (Fig. 3). The RNase-treated negative control produced no fluorescence, giving confidence that the observed fluorescent signal was an accurate reflection of where the AbV6 virus was located in the mycelium, and clearly showing it to be concentrated in the growing edge of the colony. The level of fluorescence was strong enough to reveal a pattern whereby putative vacuoles (visible as sequentially arranged, equally sized and circular structures in hyphae) appeared to lack fluorescence, but the circumference of these putative vacuoles showed peaks in fluorescent signal, suggesting a possible 'halo-effect' (Fig. 3B, D). However, greater resolution of cellular compartments is needed to better characterise the observed pattern of signal. If a halo-effect on vacuoles was confirmed, it could suggest that the fluorescence is due the congregation of vesicles surrounding the tonoplast of the vacuole (Klionsky et al., 1990).

Contrary to what was expected, AbV6 signal was also detected in the non-MVX control (Fig. 4) as well as in MVX 1153 (Fig. S3) and MVX 2735 (Table 2), all of which had tested negative by RT-PCR in 2017 and had not been shown to contain AbV6 when they were originally collected (Table 1). The signal of AbV6 in these strains was much weaker than that in MVX 1283, and was confined to the apical compartments of hyphae, with signal intensity highest at the extreme apex and lessening in a gradient pattern down the length of the hypha (Fig. 4A–D). Subapical regions in hyphae are areas of high pressure where a variety of molecules congregate such as ribosomes, actin and vesicles. Endomembrane processes feed vesicles from these areas into the extreme apex and surround the Spitzenkörper, during the process of apical growth (Grove and Bracker, 1970). As such, FISH of non-MVX hyphae also suggests synchronous virus localisation with vesicle transport, although this hypothesis warrants further study for validation.

In a study of MVX viruses using a deep sequencing approach, Deakin et al. (2017) detected low levels of AbV6 in mushrooms from a 'non-diseased commercial culture' grown as a control crop and thus, low level presence of this virus may be 'normal'. Similarly, a low-level presence of AbV6 was detected in 'sample 2735' (MVX-2735) by Deakin et al. (2017), similar to the FISH results presented here (Table 2). Other dsRNA elements and virus-like particles have been reported in healthy *A. bisporus* fruitbodies in the past (Akarapisan, 2000; Grogan et al., 2003; Romaine and Schlagnhaufer, 1989; Romaine et al., 1994), but most testing has been on mushrooms rather than mycelium. Virus presence may vary between mushroom fruitbodies and the vegetative mycelium giving rise to them. The FISH method may be useful to visualise viruses *in situ* in different tissues to explore virus dynamics at whole organism level.

The second virus targeted for observation by FISH was AbV16, which is associated with the brown cap symptom (Eastwood et al., 2015; Fleming-Archibald et al., 2015). Neither the RNase-treated control cultures nor the non-MVX strain produced fluorescence when probed for AbV16. High levels of AbV16 were detected in MVX-1153 (Fig. 5B). In contrast to the results for AbV6, AbV16 fluorescence signals were found at high levels within the central areas of the colony at a distance from the culture peripheries (Fig. 5B). Patterns of signal distribution suggest that AbV16 is highly compartmentalised and packaged within the mycelium of MVX-1153 (Fig. 5B). RNA virus packaging in vesicles of *A. bisporus* has been reported previously (Romaine et al., 1994).

MVX-2735 cultures showed very low levels of fluorescence for AbV16, with very localised occurrence (Fig. 5A). Recent studies also suggest that AbV16 was 'lost' from this inoculum (Deakin et al., 2017) and by RT-PCR (this study). The loss of the virus could be due to long-term culture maintenance of the strain as the more recently isolated strain MVX-1153 had much greater AbV16 signal intensity. The FISH technique highlights how, although AbV16 was not recently detected in MVX-2735 by either deep sequencing (Deakin et al., 2017) or by RT-PCR (this study), the AbV16 virus was still present within the MVX-2735 mycelium, but at a very low level. This highlights the benefits of the FISH technique in detecting virus presence at low levels and could be a very useful tool in studying viral dynamics in cultures where many viruses may be present in the same mycelium.

The characterisation and localisation of AbV6 and AbV16 within host *A. bisporus* mycelium is ground-breaking and would not be possible with typical molecular techniques. These results validate the application of FISH to study MVX. Furthermore, the reproducible and non-destructive methods described provide an application for use in other species of filamentous fungi for the study of intracellular mycoviruses.

5. Conclusion

In this work, FISH was successfully adapted to target two viruses within the mycelium of *A. bisporus*. The full FISH workflow was achieved on singular microscope slides, with a reproducible, cost-effective and non-destructive method. The detection, localisation and patterns of distribution of two different mycoviruses, AbV6 and AbV16, at both high and low levels of presence within the host mycelium, were characterised. The localisation patterns observed may suggest movement of viruses through vesicle-hijacking mechanisms, in particular the observed congregation of AbV6 at hyphal apices and putatively surrounding vacuoles. This work demonstrates how FISH can be applied to studies of mycoviruses within fungal host mycelium.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgments

EOC is funded by a Teagasc Walsh Scholarship Scheme (grant reference number 10564231). This work was also funded by the Teagasc Overseas Travel Award (2017).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2020.105913.

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