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Effect of culture media on radial growth and fructification of ophiostomatoid fungi isolated from bark beetles

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Abstract

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Ophiostomatoid fungi are important associates of bark beetles (Scolytinae) and cause sapstain and tracheomycosis in trees. We examined the growth and fructification of seven species from the genera *Ophiostoma, Graphilbum, Leptographium*, and *Grosmannia*, isolated from bark beetle species of the genera *Ips, Orthotomicus*, and *Pityogenes* collected from Scots pine (*Pinus sylvestris*). Ten isolates were cultivated on water agar (WA), malt extract agar (MEA), MEA with tyrosol (T), and MEA with vitamins (biotin, thiamine, pyridoxine) to determine the optimal medium for growth and fructification. Radial growth varied significantly among media and isolates. The fastest radial growth was observed on MEA supplemented with tyrosol at 0.5 µL per 250 mL (T0.5). Fructification of *Ophiostoma minus* and *Leptographium piceaperdum* was significantly higher on the nutrient-poor WA, MEA with tyrosol, and vitamin-enriched MEA than on plain MEA. The results highlight the importance of medium composition for fungal fructification and provide a basis for optimizing fungal development under different conditions.

Keywords

bark beetles, culture media, ophiostomatoid fungi, radial growth

Introduction

Ophiostomatoid fungi (Ascomycota, Ophiostomatales), also known as blue-stain fungi, belong to the families Ophiostomataceae and Ceratocystidaceae (Seifert et al., 1993). They are known for causing sapwood blue staining and contributing to the decline of host trees. These fungi live in association with bark beetles (Coleoptera: Scolytinae), especially those with mycangia, which also serve as their vectors in forests (Jankowiak and Hilszański, 2005; Linnakoski et al., 2010; Repe et al., 2013; Chang et al., 2017, 2021; Yamaoka, 2017; Aas et al., 2018; Pastirčáková et al., 2018; Song et al., 2024; Wang et al., 2024a, 2025a). Ophiostomatoid fungi participate in diverse ecological interactions and serve multiple roles within coniferous forest ecosystems. They significantly influence the health and fitness of host trees. Their introduction into the phloem can induce necrotic lesions and facilitate colonization of the sapwood, potentially disrupting water and nutrient transport systems. Such disruptions may result in the decline or mortality of the host tree (Six and Wingfield, 2011; Six, 2012; Yamaoka, 2017; Wang et al., 2024a, 2025b). Additionally, numerous species within the genus *Ophiostoma* are known to cause surface discoloration characterized by pigmented mycelial growth, which manifests as dark streaks or spots commonly referred to as bluestain or sapstain. Conifers are attacked by fungi that belong to the genera *Ophiostoma*, *Cera-*

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tocystis, *Ceratocystiopsis*, and their related anamorphic genus *Leptographium* (Lieutier et al., 2009; Wang et al., 2024b).

Despite their ecological and economic relevance, inducing the fructification of ophiostomatoid fungi under laboratory conditions remains difficult. The composition of the culture medium significantly affects fungal development, including growth rate, colony morphology, and reproductive structures (Lal et al., 2019; Miyashira et al., 2010; Nussbaum et al., 2023; Sharma and Pandey, 2010). Numerous studies have investigated the growth and sporulation of ophiostomatoid fungi on artificial media (Jacobs and Wingfield, 2001; Kirisits, 2004). Specific additives such as vitamins and bioactive compounds can further influence fungal physiology. Thiamine supports growth, sporulation, and enzyme production (Abeysinghe et al., 2020; Ge et al., 2017; Jin et al., 2021; Kijpornyongpan and Aime, 2021; Nazemi et al., 2015; Singh et al., 1980; Yousef et al., 2017), while biotin functions as a cofactor in carboxylation reactions and contributes to perithecia and ascospore formation (Deacon, 2006; Linnakoski et al., 2016). Tyrosol, a phenolic compound involved in microbial communication, can also affect fungal vitality (Jakab et al., 2019; Kolařík et al., 2025; Márton et al., 2023; Rodrigues and Černáková, 2020).

In light of recent bark beetle outbreaks and their impact on European forests (Jankowiak and Hilszański, 2005; Grodzki, 2007; Krokene, 2015; Faria and Inácio, 2023; Repe et al., 2013), it is essential to understand the biology and ecology of ophiostomatoid fungi, particularly regarding their growth under different cultural conditions. Optimizing culture media is a key to improving fungal identification, reproduction, and preservation (Chan et al., 2021; Trollip et al., 2021; Wang et al., 2018).

The aim of this study was to evaluate the effects of different culture media and selected additives (vitamins and tyrosol) on radial growth and reproductive structure formation in ophiostomatoid fungi isolated from bark beetles associated with Scots pine (*Pinus sylvestris* L.) under laboratory conditions.

Materials and methods

Isolation of fungi

Fungal cultures used in this study were isolated from adults of four bark beetle species (*Ips acuminatus* (Gyllenhal, 1827), *Ips sexdentatus* (Börner, 1776), *Orthotomicus longicollis* (Gyllenhal, 1827), and *Pityogenes chalcographus* (Linnaeus, 1761)) collected from naturally infested Scots pines (*Pinus sylvestris*) in the Malacky region (48°26′N, 17°04′E; 200 m asl) in western Slovakia. The beetles were individually sampled with sterile forceps from galleries beneath the bark, stored separately in sterile 1.5 mL microtubes, and transported to the laboratory. Each live beetle was allowed to crawl on an agar surface in a 60 mm Petri dish for 24 hours at 20 °C in the dark. The agar medium consisted of 2% malt extract agar (MEA, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), supplemented with the addition of agar (4 g L^{-1}, Agar-Agar, Kobe I, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to increase the firmness of the medium and cycloheximide (250 mg L^{-1}), streptomycin sulphate (600 mg L^{-1}), and tetracycline hydrochloride (100 mg L^{-1}). Afterwards, the beetles were removed from the dishes, and the agar plates were incubated at 20 °C in the dark. The Petri dishes were checked daily, and all fungal colonies that grew on the agar plates were transferred to

Table 1. A list of fungal isolates used for testing the effect of culture media on radial growth and fructification, including molecular identification data

Isolate number	Fungal species	Bark beetle species	GenBank accession numbers		
			ITS	*tub*	*tef1-α*
PS353	*Graphilbum acuminatum* Jankow. & H. Solheim	*Ips acuminatus*	PV600485	PV606478	PV606485
PS345	*Graphilbum furuicola* Jankow. & H. Solheim	*I. sexdentatus*	PV600483	–	PV606483
PS348	*G. furuicola*	*Orthotomicus longicollis*	PV600484	–	PV606484
PS386	*Grosmannia chlamydata* (K. Jacobs, M.J. Wingf. & H. Solheim) M.L. Yin, Z.W. de Beer & M.J. Wingf.	*Pityogenes chalcographus*	PV600489	–	PV606489
PS384	*Leptographium piceaperdum* K. Jacobs & M.J. Wingf.	*P. chalcographus*	PV600488	PV606480	PV606488
PS375	*Ophiostoma ips* (Rumbold) Nannf.	*I. sexdentatus*	PV600487	–	PV606487
PS328	*Ophiostoma minus* (Hedgc.) Syd. & P. Syd.	*I. sexdentatus*	PV600481	PV606476	–
PS327	*O. minus*	*O. longicollis*	PV600480	PV606475	PV606481
PS329	*O. minus*	*O. longicollis*	PV600482	PV606477	PV606482
PS372	*Ophiostoma piceae* (Münch) Syd. & P. Syd.	*P. chalcographus*	PV600486	PV606479	PV606486

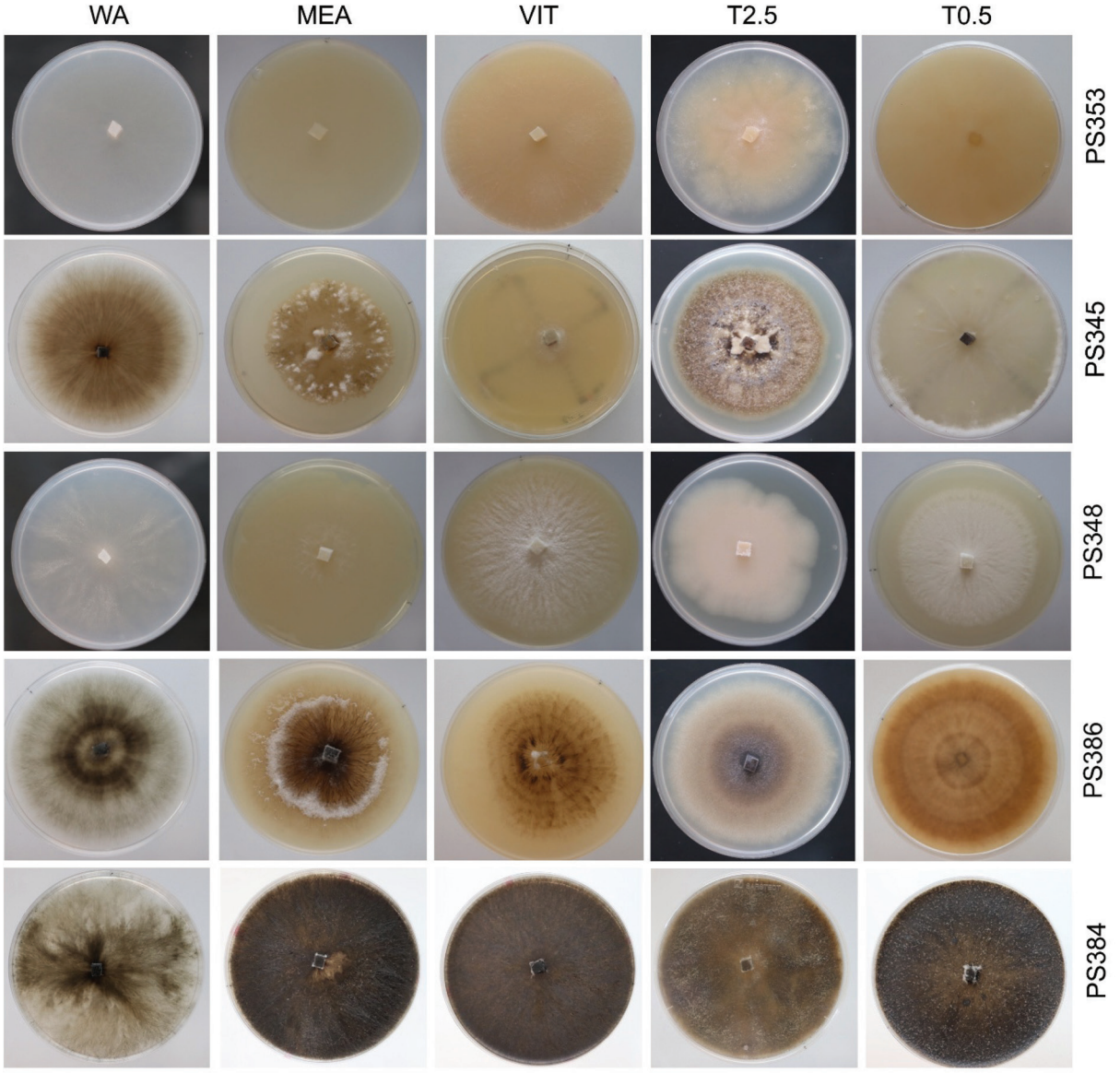

Fig. 1. Colony morphology of tested isolates of two fungal species PS353 (*Graphilbum acuminatum*), PS345 (*Graphilbum furuicola*), PS348 (*G. furuicola*), PS386 (*Grosmannia chlamydata*), and PS384 (*Leptographium piceaperdum*) growing for two weeks on the following culture media: Water agar (WA), Malt extract agar (MEA), Malt extract agar supplemented with vitamins (VIT), Malt extract agar with tyrosol concentrations of 0.5 µL (T0.5) and 2.5 µL (T2.5) per 250 mL of culture media. The inoculated plates were incubated at 24 ± 1 °C in complete darkness.

fresh MEA plates without antibiotics to isolate pure cultures. Fungi that developed on the medium surface were isolated, subcultured, and maintained in pure culture at 8 °C. Bark beetle specimens were stored in 96% ethanol for species identification, which was done based on external morphological features using taxonomic keys by Pfeffer (1995).

Molecular identification of fungal isolates

DNA was extracted from pure fungal cultures using the EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit (Bio Basic Inc., Markham, ON, Canada) according to the manufacturer's protocol. DNA was resuspended in 50 µL of elution buffer and stored at –20 °C. DNA barcoding was performed by sequencing the following molecular markers: the internal transcribed spacer (ITS) region of ribosomal DNA, partial genes of translation elongation factor (*tef1-α*) and β-tubulin (*tub*). The ITS region (ITS1-5.8S-ITS2), *tef1-α*, and *tub* genes were amplified using the following primer pairs: ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993), EF1F/EF2R (Jacobs et al., 2004), Bt2a/Bt2b (Glass and Donaldson, 1995), and T10/Bt2b (O'Donnell and Cigelnik, 1997). DNA amplification was performed in 20 µL of PCR reaction mixture containing approximately 2 ng μL^{-1} of template DNA, 5 × HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia), forward and reverse primers (10 pmol μL^{-1}), and molecular-grade water. PCR conditions for the primer pairs ITS1F/ITS4 and Bt2a/Bt2b were performed according to the

Table 2. Mycelia growth and colony characteristics of fungal isolates on the tested culture media

Isolate number	Fungal species	Media type[a]	Colour and texture of the colony	Notes
PS353	*Graphilbum acuminatum*	WA	Hyaline and glabrous	Agar plate evenly overgrown by mycelium
		MEA	Creamy and glabrous	Agar plate evenly overgrown by mycelium
		VIT	Opaque creamy and glabrous	Agar plate evenly overgrown by mycelium
		T0.5	Opaque creamy and glabrous	Agar plate evenly overgrown by mycelium
		T2.5	Opaque creamy and glabrous	Agar plate partly overgrown, irregular shape of colony with rhizoidal margin
PS345	*Graphilbum furuicola*	WA	Partially hyaline, light brown and silky	Colony with a brown radial zone
		MEA	Light brown with white powdery segments	With concentric zone in the middle of colony
		VIT	Partially hyaline and silky	Agar plate almost completely overgrown by mycelium
		T0.5	Partially hyaline and silky with white powdery segments	Agar plate evenly overgrown by mycelium
		T2.5	Greyish brown and velvety	With brown concentric zone in the middle of colony
PS386	*Grosmannia chlamydata*	WA	Partially hyaline with dark grey radial segments	Agar plate almost completely overgrown by mycelium
		MEA	Opaque creamy with black and white centric powdery segments	Agar plate evenly overgrown by mycelium
		VIT	Opaque creamy and velvety with brown concentric zones	Agar plate evenly overgrown by mycelium
		T0.5	Opaque creamy and velvety with brown concentric zones	Agar plate evenly overgrown by mycelium
		T2.5	Opaque creamy with light brown concentric zones and dark centre	Agar plate almost completely overgrown by mycelium
PS384	*Leptographium piceaperdum*	WA	Partially hyaline with dark grey rhizoidal segments	Agar plate evenly over grown by mycelium
		MEA	Dark grey and velvety	Agar plate evenly overgrown by mycelium
		VIT	Dark grey and velvety	Agar plate evenly overgrown by mycelium
		T0.5	Dark grey and velvety with white granular elements	Agar plate evenly overgrown by mycelium
		T2.5	Greyish brown and velvety	Agar plate evenly overgrown by mycelium
PS375	*Ophiostoma ips*	WA	Hyaline and light brown, glabrous	Agar plate evenly overgrown by mycelium, with a concentric zone in the middle of the colony
		MEA	Opaque greyish brown and silky	Agar plate evenly overgrown by mycelium, with concentric zones
		VIT	Opaque light brown and silky	Agar plate evenly overgrown by mycelium
		T0.5	Opaque brown, hyaline at the edges of the Petri dish	Agar plate evenly overgrown by mycelium, with concentric zones
		T2.5	Dark brown and greyish powdery on the surface	Agar plate evenly overgrown by mycelium
PS328	*Ophiostoma minus*	WA	Hyaline and silky with blackish rhizoidal texture	Agar plate evenly overgrown by mycelium
		MEA	Opaque brown and greyish velvety surface	Agar plate evenly overgrown by mycelium, with concentric zones
		VIT	Opaque light brown and velvety	Agar plate evenly overgrown by mycelium, with concentric zones
		T0.5	Black with a creamy irregularly shaped zone in the middle of the colony	Agar plate evenly overgrown by mycelium, with concentric zones, furrowed
		T2.5	Opaque brown with light brown zones at the edges, cottony	Agar plate evenly overgrown by mycelium, with concentric zones, furrowed

Table 2. Continued

Isolate number	Fungal species	Media type[a]	Colour and texture of the colony	Notes
PS327	*O. minus*	WA	Hyaline grey and velvety	Agar plate evenly overgrown by mycelium
		MEA	Opaque greyish black with white powdery segments	Agar plate evenly overgrown by mycelium
		VIT	Opaque brown and velvety with white powdery segments	Agar plate evenly overgrown by mycelium, with concentric zones, furrowed
		T0.5	Opaque grey and velvety with light brown powdery segments	Agar plate evenly overgrown by mycelium with concentric zones, furrowed
		T2.5	Opaque light brown and velvety with radial dark brown zones	Agar plate evenly overgrown by mycelium with concentric zones, furrowed
PS329	*O. minus*	WA	Hyaline and silky with blackish rhizoidal texture	Agar plate evenly overgrown by mycelium
		MEA	Opaque light brown and greyish velvety surface	Agar plate evenly overgrown by mycelium
		VIT	Opaque light brown and greyish velvety surface	Agar plate evenly overgrown by mycelium
		T0.5	Brown with a creamy irregularly shaped zone at the edges of the colony	Agar plate evenly overgrown by mycelium, with concentric zones, furrowed
		T2.5	Opaque greyish brown and velvety	Agar plate evenly overgrown by mycelium, with concentric zones, furrowed
PS372	*Ophiostoma piceae*	WA	Hyaline and glabrous	Agar plate evenly overgrown by mycelium
		MEA	Opaque creamy and silky	Agar plate evenly overgrown by mycelium
		VIT	Hyaline and light brown	Agar plate almost completely overgrown by mycelium
		T0.5	Opaque and orange brown with white radial segments	Agar plate evenly overgrown by mycelium with radial rings
		T2.5	Opaque creamy orange and velvety with a white powdery central zone	Not reaching the edges of the Petri dish, with a concentric zone in the middle of the colony

[a]Media type: water agar (WA), malt extract agar (MEA), MEA supplemented with vitamins (VIT), MEA supplemented with tyrosol at a concentration of 0.5 µL (T0.5), MEA supplemented with tyrosol at a concentration of 2.5 µL (T2.5) per 250 mL of culture media.

method described by Pastirčáková et al. (2018). PCR conditions for EF1F/EF2R and T10/Bt2b followed the protocols of Jacobs et al. (2004) and O'Donnell and Cigelnik (1997), respectively. The PCR products were visualized by horizontal electrophoresis on 1% (w/v) TBE agarose gels stained with Simply Safe (EURx Ltd., Gdansk, Poland) and enzymatically purified using the EPPiC FAST kit (A&A Biotechnology, Gdańsk, Poland). The amplified products were sequenced using the Sanger method at Macrogen Europe B.V., Amsterdam, The Netherlands. Only forward sequencing was performed. The obtained sequences were compared against the NCBI GenBank nucleotide database using the BLASTn algorithm (Zhang et al., 2000). Fungal species were identified based on 99–100% sequence identity. All sequences have been deposited in the NCBI GenBank database.

Culture media

Five different types of culture media were tested: 1) water agar (WA) consisting of 5 g agar, 0.025 g streptomycin sulphate, and 0.05 g cycloheximide; 2) malt extract agar (MEA) comprising 8.4 g malt extract agar, 1 g agar, 0.025 g streptomycin sulphate, 0.05 g cycloheximide; 3) MEA supplemented with vitamins (VIT), which included 0.05 g biotin, 0.13 g thiamine, 0.35 g pyridoxine; 4) MEA supplemented with tyrosol at a concentration of 0.5 µL (T0.5); 5) MEA supplemented with tyrosol at a concentration of 2.5 µL (T2.5). All components were dissolved in 250 mL of deionized water. The culture media were prepared following the manufacturer's instructions, with vitamins added according to the protocol described by Jacobs and Wingfield (2001). The culture media were poured into 90 mm Petri dishes.

Measurement of radial growth rates

A total of ten fungal isolates from seven species were selected for the experiment (Table 1). To measure radial growth rates, we inoculated the centre of each 90 mm diameter agar plate with a 5×5 mm mycelial fragment from the tested isolate. The inoculated plates were incubated at 24 ± 1 °C in the dark, and the radial growth was measured with a digital calliper every 24 hours until the plates were covered with fungal growth or until the colony stopped growing. The mycelial fragment used for this experiment was excised from

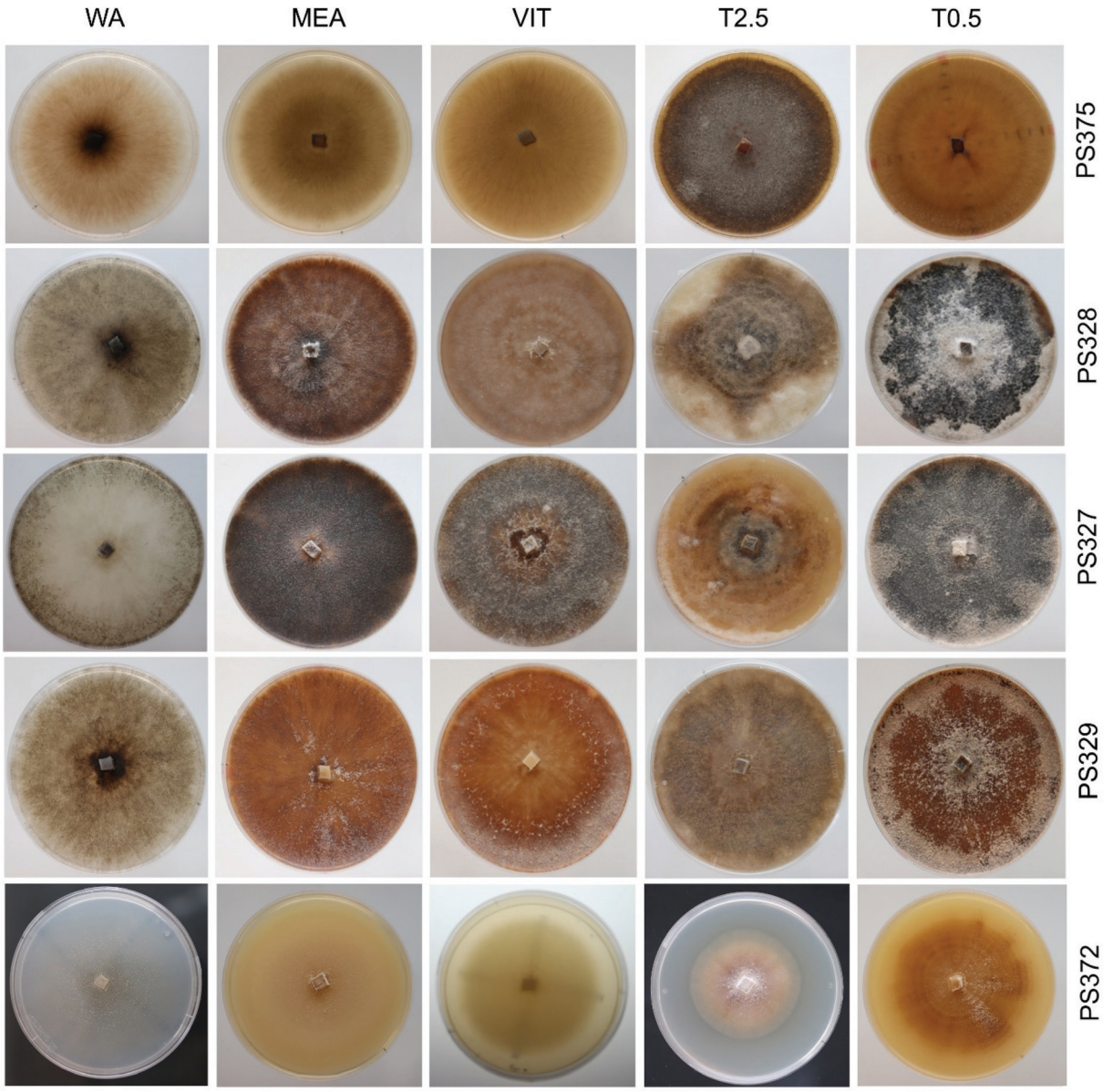

Fig. 2. Colony morphology of tested fungal isolates of five species PS375 (*Ophiostoma ips*), PS328 (*O. minus*), PS327 (*O. minus*), PS329 (*O. minus*), and PS372 (*O. piceae*) growing for two weeks on the following culture media: Water agar (WA), Malt extract agar (MEA), Malt extract agar supplemented with vitamins (VIT), Malt extract agar with tyrosol concentrations of 0.5 µL (T0.5) and 2.5 µL (T2.5) per 250 mL of culture media. The inoculated plates were incubated at 24 ± 1 °C in complete darkness.

the actively growing margin of the colony on a Petri dish. Two perpendicular lines crossing at the centre of the Petri dish (creating four segments) were marked on the underside of each dish. The diameter of the fungal colonies was measured along both perpendicular lines to allow for average calculations in cases of irregular growth. Five replicates (one Petri dish per replicate) were used for each fungal isolate and medium type tested. The radial growth rate for each isolate and culture medium was calculated from each line and expressed as mm per day.

Microscopic observations

Reproductive structure formation (fructification) of fungal cultures grown on the tested culture media was analysed using a standard light microscope (Olympus BX51, Tokyo, Japan). Measurements of reproductive structures were taken from fungal cultures grown at 24 °C in the dark after 3 weeks of incubation. Morphological characteristics were described and measured, with 30 relevant microscopic structures measured for each, using the software QuickPHOTO MICRO 3.1. At 100× magnification, immature perithecia (protoperithecia) were measured as spherical bodies without visible excretory structures. Measurements of perithecia (base, neck diameter) were made at 40× with a stereo microscope Olympus SZ51. The fungal structures were photographed using a digital camera Canon DS126621. The density of protoperithecia and perithecia produced on the surface of agar plates was analysed as an indicator of fructification using a stereo microscope.

Table 3. Pairwise comparisons of radial growth rates of ten fungal isolates on five culture media[a]

Isolate[b]/contrast	diff	SE	df	t	p	Isolate[b]/contrast	diff	SE	df	t	p
PS327						PS353					
MEA – T0.5	–0.38	1.35	895	–0.28	1.0000	MEA – T0.5	–2.49	1.74	1,811	–1.43	0.8102
MEA – T2.5	**4.33**	**1.10**	**420**	**3.96**	**0.0009**	**MEA – T2.5**	**5.73**	**1.21**	**602**	**4.73**	**<0.0001**
MEA – VIT	0.16	1.30	783	0.12	1.0000	MEA – VIT	–1.50	1.54	1,316	–0.98	0.9811
MEA – WA	1.96	1.20	582	1.64	0.6588	MEA – WA	0.11	1.47	1,164	0.07	1.0000
T0.5 – T2.5	**4.72**	**1.16**	**517**	**4.07**	**0.0005**	**T0.5 – T2.5**	**8.22**	**1.47**	**1,133**	**5.61**	**<0.0001**
T0.5 – VIT	0.54	1.35	895	0.40	1.0000	T0.5 – VIT	0.98	1.74	1,811	0.57	0.9998
T0.5 – WA	2.34	1.25	689	1.87	0.4737	T0.5 – WA	2.60	1.69	1,680	1.54	0.7326
T2.5 – VIT	**–4.18**	**1.10**	**420**	**–3.81**	**0.0016**	**T2.5 – VIT**	**–7.24**	**1.21**	**602**	**–5.96**	**<0.0001**
T2.5 – WA	**–2.37**	**0.97**	**262**	**–2.45**	**0.1398**	**T2.5 – WA**	**–5.62**	**1.13**	**467**	**–4.97**	**<0.0001**
VIT – WA	1.80	1.20	582	1.51	0.7582	VIT – WA	1.61	1.47	1,164	1.10	0.9591
PS328						PS372					
MEA – T0.5	–0.84	1.56	1,378	–0.54	0.9999	MEA – T0.5	–0.43	0.83	143	–0.51	0.9999
MEA – T2.5	**4.47**	**1.17**	**536**	**3.82**	**0.0015**	**MEA – T2.5**	**6.51**	**0.77**	**104**	**8.47**	**<0.0001**
MEA – VIT	–0.04	1.41	1,009	–0.03	1.0000	MEA – VIT	–0.26	0.82	133	–0.32	1.0000
MEA – WA	1.44	1.25	689	1.15	0.9447	MEA – WA	1.32	0.77	104	1.72	0.6012
T0.5 – T2.5	**5.31**	**1.35**	**890**	**3.93**	**0.0009**	**T0.5 – T2.5**	**6.94**	**0.81**	**129**	**8.55**	**<0.0001**
T0.5 – VIT	0.80	1.56	1,378	0.51	0.9999	T0.5 – VIT	0.16	0.86	161	0.19	1.0000
T0.5 – WA	2.28	1.43	1,056	1.60	0.6873	T0.5 – WA	1.75	0.81	129	2.16	0.2834
T2.5 – VIT	**–4.51**	**1.17**	**536**	**–3.86**	**0.0013**	**T2.5 – VIT**	**–6.78**	**0.80**	**120**	**–8.52**	**<0.0001**
T2.5 – WA	**–3.03**	**0.98**	**276**	**–3.09**	**0.0221**	**T2.5 – WA**	**–5.19**	**0.75**	**93**	**–6.95**	**<0.0001**
VIT – WA	1.48	1.25	689	1.18	0.9336	VIT – WA	1.59	0.80	120	2.00	0.3886
PS329						PS375					
MEA – T0.5	–0.46	1.42	1,050	–0.32	1.0000	MEA – T0.5	–1.68	1.26	700	–1.34	0.8658
MEA – T2.5	2.96	1.17	542	2.53	0.1125	MEA – T2.5	1.50	0.89	186	1.68	0.6267
MEA – VIT	0.19	1.26	698	0.15	1.0000	MEA – VIT	–1.04	1.03	333	–1.01	0.9775
MEA – WA	2.82	1.15	508	2.44	0.1394	MEA – WA	–1.43	1.03	333	–1.39	0.8351
T0.5 – T2.5	3.42	1.31	794	2.62	0.0863	T0.5 – T2.5	3.18	1.25	678	2.55	0.1056
T0.5 – VIT	0.65	1.38	962	0.47	1.0000	T0.5 – VIT	0.65	1.35	894	0.48	1.0000
T0.5 – WA	3.28	1.29	757	2.54	0.1063	T0.5 – WA	0.25	1.35	894	0.18	1.0000
T2.5 – VIT	–2.77	1.13	465	–2.46	0.1339	T2.5 – VIT	–2.53	1.02	315	–2.49	0.1252
T2.5 – WA	–0.14	1.01	303	–0.14	1.0000	**T2.5 – WA**	**–2.93**	**1.02**	**315**	**–2.88**	**0.0414**
VIT – WA	2.62	1.11	434	2.38	0.1659	VIT – WA	–0.40	1.14	493	–0.35	1.0000
PS345						PS384					
MEA – T0.5	**–5.64**	**0.87**	**174**	**–6.46**	**<0.0001**	MEA – T0.5	–1.46	1.99	2,334	–0.74	0.9980
MEA – T2.5	–0.88	0.80	122	–1.10	0.9596	MEA – T2.5	2.90	1.51	1,267	1.92	0.4343
MEA – VIT	**–4.24**	**0.86**	**161**	**–4.95**	**<0.0001**	MEA – VIT	–0.98	1.82	1,983	–0.54	0.9999
MEA – WA	**–4.26**	**0.82**	**133**	**–5.22**	**<0.0001**	MEA – WA	3.48	1.41	1,011	2.47	0.1273
T0.5 – T2.5	**4.76**	**0.83**	**144**	**5.71**	**<0.0001**	T0.5 – T2.5	4.37	1.83	2,019	2.38	0.1601
T0.5 – VIT	1.40	0.89	186	1.58	0.7062	T0.5 – VIT	0.48	2.09	2,503	0.23	1.0000
T0.5 – WA	1.39	0.85	155	1.63	0.6694	T0.5 – WA	4.94	1.74	1,820	2.83	0.0458
T2.5 – VIT	**–3.36**	**0.82**	**132**	**–4.12**	**0.0007**	T2.5 – VIT	–3.88	1.64	1,578	–2.37	0.1674
T2.5 – WA	**–3.38**	**0.77**	**107**	**–4.37**	**0.0003**	T2.5 – WA	0.57	1.17	542	0.49	0.9999
VIT – WA	–0.02	0.83	143	–0.02	1.0000	VIT – WA	4.46	1.54	1,336	2.89	0.0386
PS348						PS386					
MEA – T0.5	–0.97	0.95	246	–1.02	0.9750	MEA – T0.5	–1.17	1.14	480	–1.03	0.9726
MEA – T2.5	**5.06**	**0.81**	**129**	**6.24**	**<0.0001**	**MEA – T2.5**	**3.76**	**0.85**	**158**	**4.40**	**0.0002**
MEA – VIT	–0.87	0.84	151	–1.03	0.9736	MEA – VIT	–1.24	1.05	354	–1.18	0.9345
MEA – WA	0.45	0.84	151	0.54	0.9999	MEA – WA	0.37	0.87	170	0.43	1.0000
T0.5 – T2.5	**6.04**	**0.90**	**195**	**6.72**	**<0.0001**	**T0.5 – T2.5**	**4.93**	**1.06**	**375**	**4.64**	**<0.0001**
T0.5 – VIT	0.10	0.93	221	0.11	1.0000	T0.5 – VIT	–0.06	1.22	633	–0.05	1.0000
T0.5 – WA	1.42	0.93	221	1.53	0.7411	T0.5 – WA	1.55	1.08	393	1.44	0.8068
T2.5 – VIT	**–5.93**	**0.78**	**111**	**–7.60**	**<0.0001**	**T2.5 – VIT**	**–5.00**	**0.97**	**261**	**–5.15**	**<0.0001**
T2.5 – WA	**–4.61**	**0.78**	**111**	**–5.91**	**<0.0001**	**T2.5 – WA**	**–3.39**	**0.77**	**107**	**–4.38**	**0.0003**
VIT – WA	1.32	0.82	132	1.62	0.6801	VIT – WA	1.61	0.98	277	1.64	0.6609

[a]Media type: water agar (WA), malt extract agar (MEA), MEA supplemented with vitamins (VIT), MEA supplemented with tyrosol at a concentration of 0.5 µL (T0.5), MEA supplemented with tyrosol at a concentration of 2.5 µL (T2.5). [b]Fungal isolates: PS327–PS329 (*Ophiostoma minus*), PS345 and PS348 (*Graphilbum furuicola*), PS353 (*G. acuminatum*), PS372 (*Ophiostoma piceae*), PS375 (*O. ips*), PS384 *(Leptographium piceaperdum*), PS386 (*Grosmannia chlamydata*). Pairwise differences (diff), their standard errors (SE), Kenward-Roger adjusted degrees of freedom (df), test statistics (t), and associated probabilities (p) are shown. Differences statistically significant at $\alpha = 0.05$ are highlighted in bold. Note that the table is arranged in two columns.

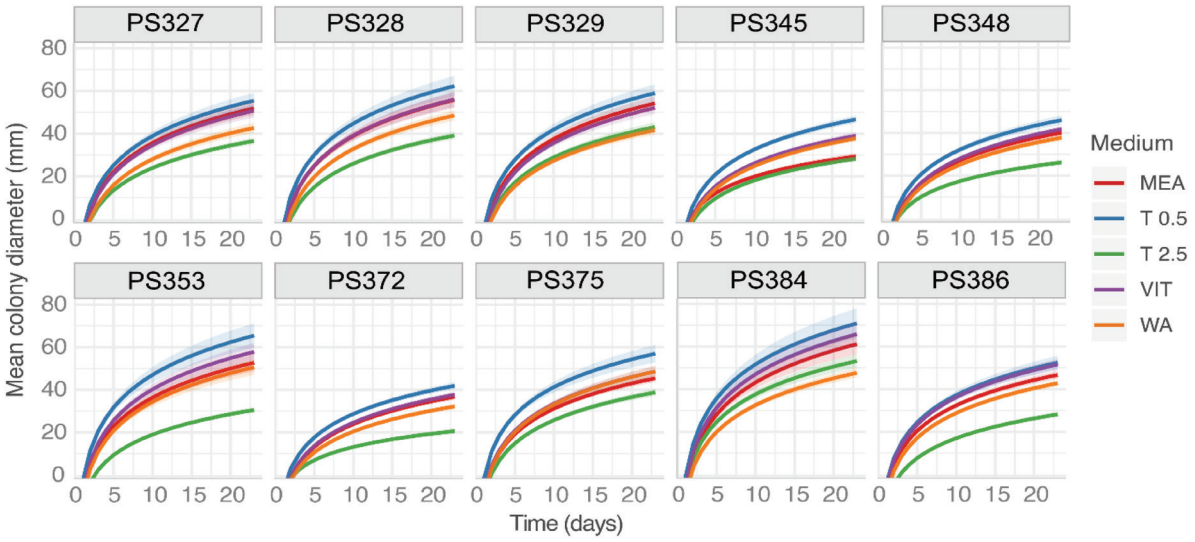

Fig. 3. Comparison of radial growth rates among ten isolates across five culture media. LMM-based predictions (lines) are shown with 95% confidence intervals. The names of fungal species: PS327 (*Ophiostoma minus*), PS328 (*O. minus*), PS329 (*O. minus*), PS345 (*Graphilbum furuicola*), PS348 (*G. furuicola*), PS353 (*Graphilbum acuminatum*), PS372 (*O. piceae*), PS375 (*O. ips*), PS384 (*Leptographium piceaperdum*), PS386 (*Grosmannia chlamydata*). The names of culture media: water agar (WA), malt extract agar (MEA), MEA supplemented with vitamins (VIT), MEA supplemented with tyrosol at a concentration of 0.5 µL (T0.5), MEA supplemented with tyrosol at a concentration of 2.5 µL (T2.5).

Fungal isolates and their bark beetle associations

A total of ten fungal isolates representing seven ophiostomatoid species were obtained from adult bark beetles collected in Scots pine galleries (Table 1). The isolates belonged to the genera *Graphilbum*, *Leptographium*, *Grosmannia*, and *Ophiostoma* and were associated with four bark beetle species (*Ips acuminatus*, *I. sexdentatus*, *Orthotomicus longicollis* and *Pityogenes chalcographus*). Molecular identification using ITS, β-tubulin, and TEF1-α markers confirmed the taxonomic placement of all isolates. Colony morphology of the fungal isolates was influenced by the type of culture media (Figs 1–2). Colony characteristics of fungal isolates on the tested culture media are given in Table 2.

Data analysis

The linear mixed-effects models (LMM) (Pinheiro and Bates, 2000) were used to assess the effect of culture media on the radial growth rate of isolates. LMM included fixed effects for medium type, isolate, and time, as well as their interactions, while repeatedly measured radial growth was treated as a random slope. As growth approached a plateau over time, a log transformation of time was applied to better capture the saturating pattern in the model. To test the significance of the fixed effects, the Kenward-Roger approximation was used to estimate the denominator degrees of freedom (Kuznetsova et al., 2017). To compare radial growth rates among culture media within each isolate, a series of pairwise slope contrasts with Sidak adjustment for multiple comparisons was used (Lenth, 2016).

LMM were also used to test the effect of culture media on the radial growth of three fungal genera (*Ophiostoma*, *Leptographium* and *Graphilbum*). In this genus-level analysis, the same model structure as above was used, with isolates treated as random effects to account for within-genus variability.

The differences in fructification between culture media and isolates were compared using Generalized Linear Models (GLM, McCullagh and Nelder, 1989). Six isolates (PS345, PS348, PS353, PS372, PS375, and PS386) did not fructify in any of the tested media and were therefore excluded from the analysis due to invariability. A GLM with a generalized Poisson distribution (Joe and Zhu, 2005) and a logarithmic link function was used to test the effects of culture media and isolates. The two-way interaction was omitted from the model due to the excessive number of zeros in certain treatment combinations, which caused convergence problems.

Statistical analysis was performed in R v4.2.2 (R Core Team, 2022) using the packages emmeans (Lenth, 2023), ggplot2 (Wickham, 2016), glmmTMB (Brooks et al., 2017), lme4 (Bates et al., 2015), and lmerTest (Kuznetsova et al., 2017).

Results

Effect of culture media on the radial growth rate

Statistical analysis (LMM) revealed that culture media significantly influenced radial growth rate, but this effect

depended on the isolate (time × media × isolate interaction: $F(36, 321.7) = 3.03$, $p < 0.0001$). Although there was no general pattern across all isolates, T0.5 consistently supported the highest radial growth, while all other media usually outperformed T2.5 (Table 3, Fig. 3).

Pairwise comparisons revealed significant differences in growth among culture media and isolates (Table 3). The growth on medium MEA was significantly faster compared to medium T2.5 for isolates PS327, PS353, PS328, PS372, PS348, and PS386. In contrast, the comparisons of medium T2.5 were significantly lower compared to medium VIT among isolates PS327, PS353, PS328, PS372, PS345, PS348, and PS386. In comparison with medium T2.5, the growth was also significantly lower on medium WA for isolates PS353, PS328, PS372, PS375, PS345, PS348, and PS386. The pairwise comparison of two different concentrations of tyrosol, the growth on medium T0.5 was significantly faster than in medium T2.5 for isolates PS327, PS353, PS328, PS372, PS345, PS348, and PS386.

The difference was recorded at the isolate PS345 in comparison with the medium MEA where the growth was significantly lower than on medium T0.5 and the growth on medium MEA was also significantly lower than on medium VIT, the growth on medium MEA was also significantly lower than on medium WA. In contrast, the growth of isolate PS384 on medium VIT was significantly faster compared with medium WA. Isolates PS329 and PS375 showed fewer significant contrasts, suggesting media composition may have a less pronounced effect on their radial growth compared to other isolates, or that their growth rates are less sensitive to tyrosol or vitamin supplementation under the conditions tested. This suggests isolate-specific responses, possibly reflecting differences in nutritional preferences or tolerance to additives.

The genus-level analysis showed that culture media significantly influenced radial growth rate, but this effect depended on the fungal genus (time × media × genus interaction: $F(8, 229.5) = 2.20$, $p = 0.0284$). Although there was no general pattern across all genera, T0.5 consistently supported the highest radial growth, while all other media usually outperformed T2.5 (Table 4, Fig. 4).

Among the fungal isolates examined, the genus *Graphilbum* showed greater variation in growth across media. Significantly higher growth was recorded on T0.5 compared to T2.5, and also grew better on MEA compared to T2.5. In contrast, reduced growth was on T2.5 relative to VIT and WA. The genus *Leptographium* exhibited significantly higher growth on T0.5 compared to T2.5, but in contrast of medium T2.5, significantly faster growth was on MEA, while its lowest growth occurred on T2.5 relative to VIT and WA. In comparison, the VIT medium supported growth significantly better than WA. The genus *Ophiostoma* exhibited significantly higher growth on T0.5 compared to T2.5. Additionally, growth stimulation was observed on MEA relative to T2.5. In contrast, growth on T2.5 was significantly lower than on VIT and WA. In contrast, reduced growth was on T2.5 relative to WA.

Table 4. Pairwise comparisons of radial growth rates of three fungal genera on five culture media[a]

Genus[b]/contrast	diff	SE	df	t	p
Graphilbum					
MEA – T0.5	**–2.99**	**0.69**	**217**	**–4.35**	**0.0002**
MEA – T2.5	**2.84**	**0.61**	**149**	**4.70**	**0.0001**
MEA – VIT	**–2.37**	**0.65**	**178**	**–3.63**	**0.0037**
MEA – WA	–1.69	0.64	167	–2.64	0.0873
T0.5 – T2.5	5.84	0.64	184	9.07	<0.0001
T0.5 – VIT	0.63	0.69	213	0.91	0.9889
T0.5 – WA	1.31	0.67	200	1.94	0.4257
T2.5 – VIT	**–5.21**	**0.60**	**145**	**–8.66**	**<0.0001**
T2.5 – WA	**–4.53**	**0.59**	**134**	**–7.71**	**<0.0001**
VIT – WA	0.68	0.64	163	1.07	0.9656
Leptographium / Grosmannia					
MEA – T0.5	–0.88	1.06	507	–0.83	0.9948
MEA – T2.5	**3.81**	**0.85**	**214**	**4.46**	**0.0001**
MEA – VIT	–1.17	0.99	392	–1.18	0.9344
MEA – WA	1.64	0.84	223	1.95	0.4161
T0.5 – T2.5	**4.69**	**1.00**	**419**	**4.67**	**<0.0001**
T0.5 – VIT	–0.30	1.12	648	–0.26	1.0000
T0.5 – WA	2.52	0.99	440	2.54	0.1086
T2.5 – VIT	**–4.98**	**0.93**	**312**	**–5.36**	**<0.0001**
T2.5 – WA	**–2.17**	**0.76**	**157**	**–2.85**	**0.0490**
VIT – WA	**2.81**	**0.92**	**327**	**3.07**	**0.0233**
Ophiostoma					
MEA – T0.5	–0.89	0.59	309	–1.51	0.7598
MEA – T2.5	**3.91**	**0.50**	**193**	**7.78**	**<0.0001**
MEA – VIT	–0.44	0.56	264	–0.78	0.9966
MEA – WA	1.02	0.53	219	1.95	0.4194
T0.5 – T2.5	**4.80**	**0.56**	**270**	**8.61**	**<0.0001**
T0.5 – VIT	0.46	0.60	343	0.76	0.9974
T0.5 – WA	**1.92**	**0.58**	**293**	**3.32**	**0.0100**
T2.5 – VIT	**–4.34**	**0.52**	**223**	**–8.42**	**<0.0001**
T2.5 – WA	**–2.89**	**0.48**	**175**	**–5.99**	**<0.0001**
VIT – WA	1.46	0.54	249	2.71	0.0693

[a]Media type: water agar (WA), malt extract agar (MEA), MEA supplemented with vitamins (VIT), MEA supplemented with tyrosol at a concentration of 0.5 µL (T0.5), MEA supplemented with tyrosol at a concentration of 2.5 µL (T2.5).
[b]The genus *Graphilbum* includes the species *G. acuminatum* and *G. furuicola*. The *Leptographium/Grosmannia* group includes *Leptographium piceaperdum* and *Grosmannia chlamydata*. The genus *Ophiostoma* includes *O. ips*, *O. minus*, and *O. piceae*. *Leptographium* and *Grosmannia* were pooled due to their close phylogenetic relationship.
Pairwise differences (diff), their standard errors (SE), Kenward-Roger adjusted degrees of freedom (df), test statistics (t), and associated probabilities (p) are shown. Differences statistically significant at $\alpha = 0.05$ are highlighted in bold.

In summary, the results support the positive impact of tyrosol supplementation, especially at a concentration of 0.5 µL/250 mL (T0.5), in promoting fungal radial growth across most tested isolates. These findings underscore the importance of media composition in culturing fungal species and suggest that supplementing MEA with tyrosol may be broadly advantageous for facilitating rapid mycelial expansion in ophiostomatoid fungi. Differences in isolate response also highlight the need for tailored medium selection in future mycological studies.

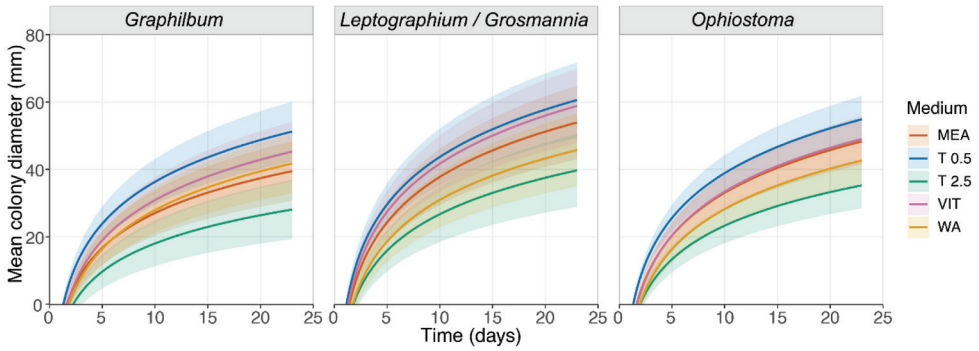

Fig. 4. Comparison of radial growth rates among three fungal genera across five culture media. LMM-based predictions (lines) are shown with 95% confidence intervals (shaded areas), illustrating the uncertainty in predicted mean colony diameters. The genus *Graphilbum* contains species (*Graphilbum acuminatum*, *Graphilbum furuicola*), genus *Leptographium* (*Leptographium piceaperdum*, *Grosmannia chlamydata*), and genus *Ophiostoma* contain species (*Ophiostoma ips*, *O. minus*, *O. piceae*). *Leptographium* was included together to *Leptographium/Grosmannia* to reflect the pooled treatment of these closely related ophiostomatoid genera. The culture media: Water agar (WA), Malt extract agar (MEA), Malt extract agar supplemented with vitamins (VIT), Malt extract agar with tyrosol concentrations of 0.5 µL (T0.5) and 2.5 µL (T2.5) per 250 mL.

Influence of culture media on reproductive structure formation

Reproductive structure production varied substantially among isolates and media (Table 5). WA and T0.5 media promoted the formation of protoperithecia in *Ophiostoma minus* isolates, whereas MEA favoured the development of perithecia in *Leptographium piceaperdum*. Vitamin-supplemented MEA (VIT) supported protoperithecia formation in two *O. minus* isolates. T2.5 medium generally induced fewer reproductive structures. Morphological diversity was observed in the generative forms, including *Pesotum, Sporothrix*, *Leptographium*-like conidiophores, and synnemata. Microscopic measurements showed variation in the size of protoperithecia across isolates and culture media (Table 6).

Statistical analysis (GLM) showed significant differences among isolates ($\chi^2(9) = 409$, $p < 0.0001$) and media ($\chi^2(4) = 200$, $p < 0.0001$) in production of reproductive structures. Fructification was highest on WA, particularly for isolates PS328 and PS329, followed by MEA and T0.5, depending on the isolate. Both T2.5 and VIT generally supported very little to no fructification. Isolates PS327, PS328 and PS329 showed comparable levels of fructification, whereas isolate PS384 produced fewer structures on most media except MEA, where it formed high densities protoperithecia (Fig. 5).

Macromorphological characteristics of the studied isolates

The colony morphology of the tested fungal isolates differed across various culture media. On nutrient-rich media, the isolates generally formed denser, more pigmented colonies (especially on culture media VIT, MEA, and T0.5). In contrast, growth on low-nutrient media (especially WA) was usually lighter, more transparent, and had a visibly finer texture. The most distinct alterations in mycelial appearance, including pigmentation, structural characteristics and colony margins were observed on tyrosol-amended media (T0.5 and T2.5) especially isolates PS328 (*Ophiostoma minus*), PS327 (*O. minus*), PS329 (*O. minus*), PS372 (*Ophiostoma piceae*) showed higher contrast than isolates PS375 (*Ophiostoma ips*), PS353 (*Graphilbum acumina-*

Table 5. Vegetative and generative structures[a] produced by fungal isolates on five culture media after one month of incubation

Isolate number	Fungal species	Culture media[b]				
		WA	VIT	MEA	T0.5	T2.5
PS353	*Graphilbum acuminatum*	S	S	H	S	S
PS345	*Graphilbum furuicola*	H	H	S	H	H
PS348	*G. furuicola*	H	H	S	H	H
PS386	*Grosmannia chlamydata*	H	S	S	H	H
PS384	*Leptographium piceaperdum*	P/L	S	PR/L	L	Sy /H
PS375	*Ophiostoma ips*	H	S	H	S	S
PS328	*Ophiostoma minus*	PR/H	H	H	PR/H	PR/H
PS327	*O. minus*	PR/H	PR	H	PR/H	PR
PS329	*O. minus*	PR	PR	H	H/PR	H
PS372	*Ophiostoma piceae*	Pes/Sp	Sp	Pes/Sp	Pes	Pes

[a]Sterile mycelium (S), *Hyalorhynocladiella* (H), Perithecium (P), *Leptographium*-like (L), *Pesotum* (Pes), *Sporothrix* (Sp), Protoperithecium (PR), Synnemata (Sy).
[b]Water agar (WA), Malt extract agar supplemented with vitamins (VIT), Malt extract agar (MEA), Malt extract agar with tyrosol concentrations of 0.5 µL (T0.5) and 2.5 µL (T2.5) per 250 mL of culture media.

Table 6. Morphometric parameters of protoperithecia without a developed neck

Isolate number	Fungal species	Medium type[a]	n	Width (µm) Mean ± std	Min.	Max.	Length (µm) Mean ± std	Min.	Max.
PS384	*Leptographium piceaperdum*	MEA	30	129.60 ± 76.07	46.00	339.00	122.17 ± 70.13	48.00	305.00
PS384	*L. piceaperdum*	WA	30	183.00 ± 55.96	78.00	274.00	185.90 ± 56.06	90.00	278.00
PS329	*Ophiostoma minus*	WA	30	42.63 ± 13.88	20.00	75.00	41.67 ± 11.66	18.00	71.00
PS329	*O. minus*	VIT	30	44.67 ± 10.08	32.00	67.00	43.50 ± 7.65	30.00	59.00
PS329	*O. minus*	T0.5	30	66.73 ± 15.90	35.00	92.00	65.10 ± 17.03	34.00	114.00
PS327	*O. minus*	WA	30	47.47 ± 14.79	18.00	78.00	47.23 ± 14.81	25.00	71.00
PS327	*O. minus*	VIT	30	63.53 ± 19.09	32.00	102.00	62.27 ± 18.85	32.00	102.00
PS327	*O. minus*	T0.5	30	89.60 ± 30.33	40.00	154.00	85.47 ± 29.36	46.00	162.00
PS327	*O. minus*	T2.5	30	54.50 ± 14.84	39.00	108.00	54.13 ± 12.73	38.00	85.00
PS328	*O. minus*	WA	30	74.10 ± 20.47	27.00	113.00	73.77 ± 21.69	29.00	127.00
PS328	*O. minus*	T0.5	30	63.57 ± 12.46	25.00	87.00	61.83 ± 11.95	32.00	86.00
PS328	*O. minus*	T2.5	30	40.70 ± 9.46	25.00	62.00	38.93 ± 8.19	27.00	58.00

Notes: Protoperithecia were measured after 3 weeks from the start of growth. Only one isolate PS384 developed perithecium on medium WA. The measurements: n = 30, perithecium: the mean base (width) 183.27 ± 55.96 µm, (min. 78.00 µm, max. 274.00 µm). Base (length) 185.90 ± 56.06 µm (min. 90 µm, max. 278 µm). Perithecium with neck (length): 305.43 ± 102.46 µm, (min.133.00 µm, max. 468.00 µm). Neck (length): 121.27 ± 50.52 µm, (min. 31 µm, max. 223 µm). Neck (top): 19.87 ± 4.89 µm, (min. 12.00 µm, max. 30.00 µm). Neck (base): 35.57 ± 8.13 µm, (min. 20.00 µm, max. 51.00 µm).
[a]Media type: water agar (WA), malt extract agar (MEA), MEA supplemented with vitamins (VIT), MEA supplemented with tyrosol at a concentration of 0.5 µL (T0.5), MEA supplemented with tyrosol at a concentration of 2.5 µL (T2.5).

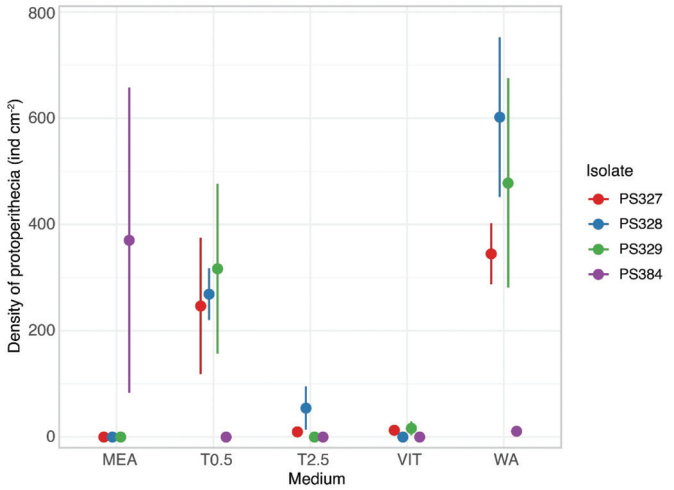

Fig. 5. Comparison of fructification of four fungal isolates on five culture media. Mean densities (dots) are shown with 95% confidence intervals (error bars). Isolates that did not form reproductive structures were excluded from the analysis. "ind cm^{-2}" refers to the number of protoperithecia per square centimetre on the Petri dish. The names of fungal species: PS327 (*Ophiostoma minus*), PS328 (*O. minus*), PS329 (*O. minus*), PS384 (*Leptographium piceaperdum*).

tum), PS345 (*Graphilbum furuicola*), PS348 (*G. furuicola*), PS386 (*Grosmannia chlamydata*), PS384 (*Leptographium piceaperdum*) in contrast with the other media (WA, MEA, VIT). These morphological observations complement the growth data and presented consistent isolate-specific patterns across replicates (Figs. 1–2 and Table 2).

Discussion

This study demonstrates the significant influence of different culture media and selected additives on the radial growth under in vitro conditions and fructification (development of sexual and asexual reproductive structures) of ophiostomatoid fungi isolated from bark beetles associated with Scots pine. Our results revealed strong medium-dependent variability in both mycelial growth and reproductive structure formation across several fungal species, providing important insights for mycological research and practical applications. The significant medium × isolate interaction confirmed isolate-specific differential responses.

Understanding fungal growth dynamics is essential for assessing competitive potential, dispersal strategies, and colonization of weakened trees under nutrient-limited conditions (Lieutier et al., 2009). In line with this, the nutrient-poor WA medium promoted the initiation of protoperithecia but restricted radial growth, whereas MEA supported faster growth but did not stimulate pro-

toperithecia formation.

MEA is widely used for isolating ophiostomatoid fungi from bark beetles and for morphological studies (Harrington et al., 2001; Jacobs et al., 2006; Liu et al., 2017). For enhanced perithecia production, sterilized pine twigs with bark can be placed on MEA (Chang et al., 2021), an approach recommended by several authors (Yun et al., 2009; Jankowiak et al., 2018; Trollip et al., 2021). Although our study did not apply such substrates, we tested MEA supplemented with vitamins (VIT medium), as suggested by previous research. Thiamine, for example, has been shown to play a crucial role in fungal development. Jin et al. (2021) demonstrated its essential function for conidial formation and hyphal growth in *Beauveria bassiana* (Bals.-Criv.) Vuill., while Yousef et al. (2017) showed that thiamine supplementation enhanced both growth and sporulation of *Trichoderma* spp., thereby improving biocontrol efficacy. Biotin has also been identified as a key factor. Lilly and Barnett (1947) demonstrated its indispensability for the development of reproductive structures in *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. In agreement with these findings, our VIT medium (containing thiamine, biotin, and pyridoxine) supported protoperithecia formation in two isolates of *Ophiostoma minus* (PS237 and PS329), outperforming plain MEA.

While tyrosol is typically regarded as non-toxic at biologically relevant concentrations, multiple studies have demonstrated inhibitory or cytotoxic effects at supraphysiological levels (i.e., higher concentrations), (Kovács and Jakab et al., 2025; Márton et al., 2023). The role of tyrosol, primarily studied in *Candida* spp. as a signalling molecule affecting biofilm formation and hyphal morphology (Jakab et al., 2019; Rodrigues and Černáková, 2020; Márton et al., 2023; Kolařík et al., 2025), also proved relevant here. On low-concentration tyrosol medium (T0.5), three isolates of *O. minus* developed protoperithecia with faster radial growth, whereas higher tyrosol concentration (T2.5) slowed growth but still supported reproductive structure formation.

Our results underscore the critical role of culture media composition in shaping growth and reproductive strategies of both pathogenic and non-pathogenic ophiostomatoid fungi. In particular, WA, VIT, and low-concentration tyrosol media supported protoperithecia formation, while plain MEA generally did not. One isolate (PS384) was exceptional, producing perithecia on WA. These findings emphasize that careful selection of media is essential for experimental work and may provide practical guidance for optimizing fungal cultivation under different research and applied contexts.

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