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ADA, A FAST-GROWTH MEDIUM FOR *Hymenoscyphus fraxineus*

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SUMMARY

The growth rate of *Hymenoscyphus fraxineus* was evaluated on five different media under laboratory conditions. Statistically significant differences were found among them, where *H. fraxineus* demonstrated the highest growth rate on ADA medium (a newly designed nutritive medium). This study suggests that ADA is a suitable medium for growing *H. fraxineus*.

1 INTRODUCTION

Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz & Hosoya is an invasive species that causes ash dieback in Europe. The disease is extremely severe and drives high mortality rates for *Fraxinus excelsior* and *Fraxinus angustifolia* over a vast area of the European continent. As this disease garners exceptional interest within the scientific community throughout Europe, establishing efficient fungal growth *in vitro* is currently of great importance for laboratories investigating this disease. Malt extract agar (MEA) has been used to grow *H. fraxineus* since its first appearance in Europe and remains the most frequently used medium for cultivating *H. fraxineus*. Isolation of *H. fraxineus*, as described by Kowalski (2006), includes placing pieces of shoots onto Petri dishes with 2% MEA (20 g/L) supplemented with 100 mg/L streptomycin sulphate. The growth rates of *H. fraxineus* and *Hymenoscyphus albidus* were also compared on MEA and MEA complemented with 50 g of fresh or frozen ash (*F. excelsio*r) leaflets, which is referred to as AMEA (Kirisits et al, 2013). The AMEA medium significantly increased the fungal growth of both *H. fraxineus* and *H. albidus*. Additionally, differences in *H. fraxineus* growth rates between media were observed while testing vegetative compatibility (Brasier and Webber 2013). In that study, four nutrient media were selected, namely, carrot agar (CA), malt agar (MA), potato dextrose agar (PDA), and ASA (ash sapwood agar) prepared from peeled ash twigs that were dried for 12 h at 80 °C and subsequently milled.

Here, we aimed to identify an efficient growing medium for *H. fraxineus* by comparing five different nutrient media, including one not described previously.

2 MATERIALS AND METHODS

In January and February 2015, we cultured 15 independent isolates of *Hymenoscyphus fraxineus* collected from ten localities of Czech Republic, Austria, Norway, and Latvia (Table 1). Each isolate was cultivated in five replicates with five different nutrient media. Consequently, 375 cultures were measured.

MEA [HiMedia, Mumbai, India] was prepared by mixing 50 g of MEA powder in 1 L of sterile distilled water (SDW), PDA [HiMedia, Mumbai, India] with 39 g of powder in 1 L of SDW. Modified orange serum (MOS) was prepared using malt extract [Sigma-Aldrich, USA], orange-serum agar [Sigma-Aldrich, USA], dextrose [Sigma-Aldrich, USA] and Technical agar No. 3 [Oxoid, Hampshire, UK] (Müller et al. 1994). The AMEA medium was prepared according to Kirisits et al. (2013), supplementing MEA and Technical Agar No. 3 with dried healthy ash leaves. The ADA medium (ash dust agar) was designed based on the ESA (elm sapwood agar) medium (Brasier, 1981), which is used for growing *Ophiostoma ulmi.* However, given that *H. fraxineus* invades not only sapwood but also the inner bark, the ash twigs were not peeled. Fresh ash twigs up to 5 mm in diameter were collected and directly milled with mill Retsch SM300 (Retsch, Haan, Germany) up to 0.5 mm. The medium was prepared with 50 g of ash dust mixed with 30 g of technical agar No. 3 [Oxoid, Hampshire, UK] and diluted in 1 L of SDW. All of the media were autoclaved at 120 °C for 20 min, and 20 mL per plate was poured into 90 mm diameter Petri dishes. The bottles were shaken while pouring the ADA media to ensure wood dust dispersion.

All of the cultures were incubated in the dark at 25 °C. The growth area was calculated using the following equation: $\pi\ (d_1\ d_2)\ 4^{-1}$ (Santamaria et al. 2004), where d_1 and d_2 were the two perpendicular diameters. The diameters of the colony were measured with a ruler weekly during the first two weeks and twice a week during the last four weeks (once in four days, once in three days, periodically). For evaluating the growing media, the mycelial area after 24 days of cultivation was used, i.e., before any of the strains reached the limits of the plate. The media were compared using a General Linear Model (GLM). For multiple comparisons, a Fisher's least significant difference test was performed. All of the analyses were performed using STATISTICA 12 (StatSoft).

3 RESULTS AND DISCUSSION

Significant differences in the *H. fraxineus* growth area were found among the nutrient media. The most significant results were obtained when both the tested strain ('Strain') and the type of growing medium ('Medium') were included as variables in the GLM model and by log-transformation of the mycelial area values. The variable 'Strain' ($DF=12/292$, $F=11.96$, $p<0.001$) and the variable 'Medium' ($DF=4/292$, $F=18.01$, $p<0.001$) were both highly significant in the model, with a standard error of the estimate=0.84. Post-hoc analysis of the variable 'Medium' revealed that the ADA medium had the highest mycelium area, followed by significantly lower areas with AMEA ($p=0.03$), MOS, MEA, and PDA ($p<0.001$) (Fig. 1).

This test did not identify significant differences between AMEA and MOS or between MEA and PDA. Our results are consistent with Kirisits et al. (2013), who observed that *H. fraxineus* and *H. albidus* exhibited significantly higher growth on AMEA compared with MEA. Similar observations were also reported in Brasier and Webber (2013), where most of the cultures grew faster on ASA than on MA (MEA), and CA and PDA grew slowly and compactly. However, in that study, these differences were not statistically supported. In our experiment, AMEA and MOS support similar growth rates, suggesting that MOS is an appropriate alternative for cultivating *H. fraxineus* in the laboratory.

Either ADA or AMEA are composed of material from healthy ashes showing certain degree of resistance. This could lead us to expect a limitation of the growth of *H. fraxineus* on these media, however, it appears that the presence of healthy ash material (twigs in ADA and leaves in AMEA) is beneficial for the fungal growth. As trees of *F. excelsior* show genetic diversity in resistance to *H. fraxineus* (Cleary et al. 2014, McKinney et al. 2014), it would be very interesting to further examine if this genotypic variation may also influence the growth rate of *H. fraxineus* on these two media.

Consistent with the description of the mycelial morphology in Kowalski and Bartnik (2010), Kirisits et al. (2013), and Brasier and Webber (2013), our cultures greatly differed in colour, texture, and shape (Fig. 2). However, no clear relationship between mycelial colour and the nutrient medium was observed. The mycelial characteristics of each isolate varied even when incubated under the same conditions. Nevertheless, the cultures grown on ADA medium were extremely uniform, where the cultures lacked any distinguishable differences. The mycelium was quite transparent, thn, and white, and covered the surface with slightly protruding white dots. Cultures on ADA not only grew superficially but also appeared to extensively penetrate into the medium on visual assessment. The uniform appearance of the mycelia cultivated on ADA precludes the identification of morphological differences.

The description of the *Hymenoscyphus fraxineus* growth rates and its mycelial characteristics using different nutrient media furthers our knowledge of cultivation methods. In addition, the comparison of five nutrient media provides information for researchers to be able to properly select one of them for distinct intended purposes.

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Table 1. List of isolates of *H. fraxineus* used in this experiment.

Isolates	Year of collection	Season	Locality	Country	Substrate
MeU_1653	2013	Spring	Brno	Czech Republic	Necrotic lesion
MeU_1656	2012	Winter	Brno	Czech Republic	Necrotic lesion
MeU_1657	2012	Winter	Brno	Czech Republic	Necrotic lesion
MeU_1714	2013	Spring	Třemošnice - Počátky	Czech Republic	Necrotic lesion
MeU_1714	2013	Spring	Třemošnice - Počátky	Czech Republic	Necrotic lesion
MeU_1721	2013	Spring	Seč Javorka	Czech Republic	Necrotic lesion
MeU_1723	2013	Spring	Seč - Javorka	Czech Republic	Necrotic lesion
MeU_1731	2013	Spring	Lužické mountains,	Czech Republic	Necrotic lesion
MeU_1732	2013	Spring	Lužické mountains	Czech Republic	Necrotic lesion
MeU_1734	2013	Spring	Podolí	Czech Republic	Necrotic lesion
BB/1/19	2009	Spring	Bisamberg, Lower Austria	Austria	Necrotic lesion
BIZ/1	2010	Autumn	Bizau, Vorarlberg	Austria	Necrotic lesion
VER/2	2009	Summer	Verditz, Carinthia	Austria	Necrotic lesion
LIE 1/7/2	2008	Spring	Liepaja	Latvia	Necrotic lesion
NOR1/23	2010	Summer	Oslo	Norway	Necrotic lesion

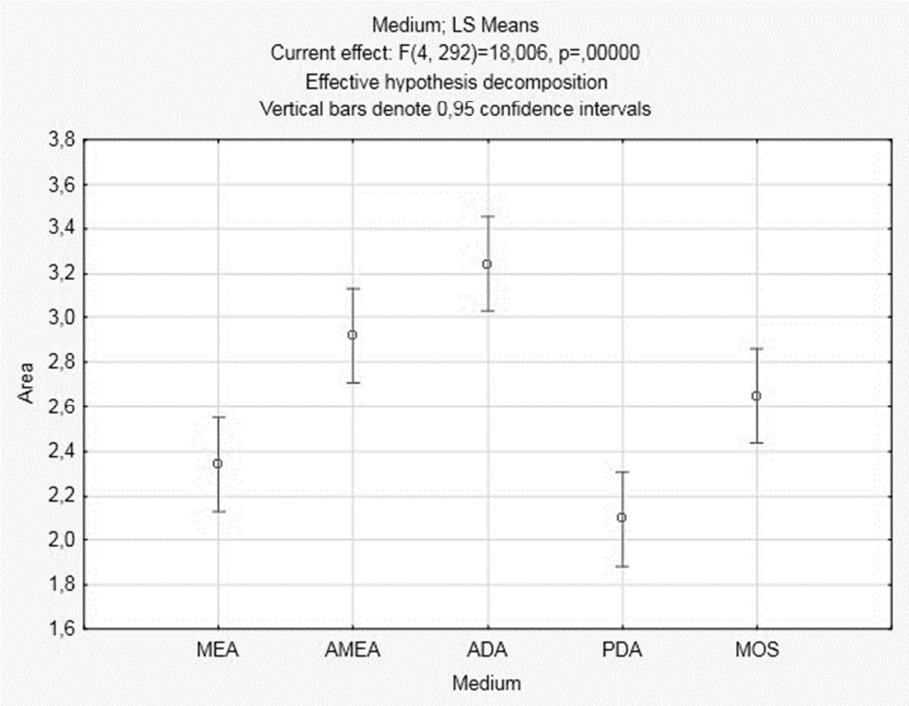

Fig. 1. GLM-estimated mycelial area (cm^2) of the five tested growing media.

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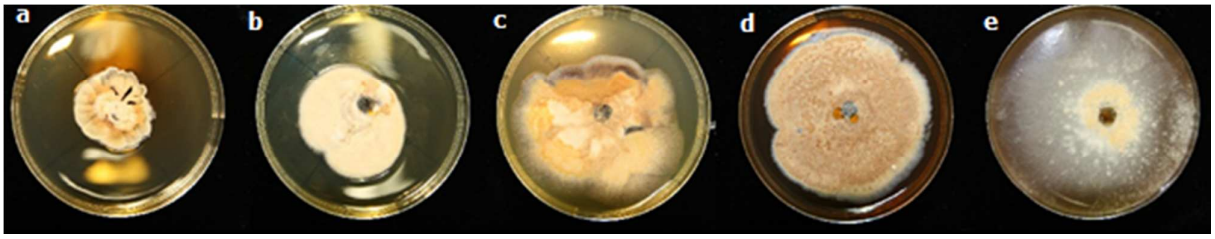

Fig 2. Isolate 1732 (repetition 1) cultured on PDA (a), MEA (b), MOS (c), AMEA (d) and ADA (e) media after 6 week of cultivation.