Decomposition potentialities of *Epichloë bromicola* from *Elymus racemifer*

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Abstract

Fungal endophytes of the genus *Epichloë* possess weak saprotrophic abilities, but no quantitative studies have been conducted regarding their abilities to decompose grass litter. In the present study, *Epichloë bromicola* strains were isolated from *Elymus racemifer*, a common perennial grass in Japan, and assessed for their ability to decompose sterilised grass leaves under pure culture conditions. These abilities were compared with those of two non-systemic endophytic fungi, *Phomopsis* sp. and *Biscogniauxia maritima,* sympatrically encountered in the live tissues. Mass loss of leaves caused by 16 strains of *E. bromicola* on potato dextrose agar at 20ºC for 12 weeks ranged from 0.4% to 10.8% of the original leaf mass, with a mean of 4.9%. This value was not significantly different from that of *Phomopsis* sp. (mean mass loss of 5.6%) but was significantly lower than that of *B. maritima* (mean mass loss of 20.8%) measured under the same culture conditions. The low decomposing ability of *E. bromicola* suggests that this fungus is not responsible for significant decomposition of grass litter, which is consistent with the current belief that *Epichloë* endophytes obtain photosynthetic product from live plant tissues but rarely elongate hyphae on dead tissues.

Keywords: decomposition, grass leaf litter, mass loss, pure culture test

Introduction

The fungal endophytes of the genus *Epichloë* (Clavicipitaceae, Ascomycota) systemically infect aboveground parts of many grass species within the Poaceae. These fungi not only have mutualistic associations with host plants by promoting primary productivity and tolerance to water stress and providing resistance to herbivores and pathogens (Clay and Schardl 2002; Schardl et al. 2009) but also affect belowground processes such as plant residue decomposition and soil chemistry (Omacini et al. 2004; Lemons et al. 2005; Gundel et al. 2016; Song et al. 2022). Previous studies have shown that these vertically transmitted endophytes are capable of utilising only simple carbon sources (White et al. 1991; Li et al. 2008) and rarely persist in dead grass tissues (Christensen and Voisey 2007; Hatano et al. 2024), suggesting weak saprotrophic abilities. Exploring the saprophytic capacity of *Epichloë* endophytes will help understanding their functional and ecological relevance in belowground processes. However, no quantitative studies have been conducted regarding the potentialities of *Epichloë* endophytes as decomposers of grass litter.

The purposes of the present study were to investigate the ability of *Epichloë* strains isolated from leaf sheaths of *Elymus racemifer* to decompose sterilised leaves in pure culture and to compare it with those of two non-systemic endophytic fungi (*Phomopsis* sp. and *Biscogniauxia maritima*) sympatrically encountered in the live tissues. *Elymus racemifer* is a perennial grass that is commonly found along roadsides on farmland and in cities in Japan and harbours *E. bromicola* (Yanagida et al. 2005; Hatano et al. 2024). Two additional strains were included in the pure culture decomposition tests for comparison: *Epichloë uncinata* NBRC32642 and *Trametes versicolor* NBRC30340 (Polyporaceae, Basidiomycota). *Epichloë uncinata* NBRC32642 is a systemic endophyte of *Festuca pratensis*. A ligninolytic white-rot fungus of wood, *T. versicolor* NBRC30340 is a standard strain for the decomposition test of timber under the Japanese Industrial Standard (JIS) and has been used for pure culture decomposition tests of various substrates, including grass leaves (Osono 2020).

Materials and Methods

Fungal materials

A total of 23 fungal strains were examined in the pure culture decomposition test, including 16 strains

ISSN 2463-2872 (Print) ISSN 2463-2880 (Online) https://doi.org/10.33584/jnzg.2025.18.3752

of *E. bromicola*, three strains of *Phomopsis* sp. (Diaporthaceae, Ascomycota), two strains of *B. maritima* (Xylariaceae, Ascomycota), one strain of *E. uncinata*, and one strain of *T. versicolor*. The strains of *E. bromicola*, *Phomopsis* sp., and *B. maritima* were isolated from leaf sheaths of healthy-looking *E. racemifer* plants collected along roadsides near Kyotanabe Campus of Doshisha University, Kyoto, Japan in June 2017. The leaf sheaths were surface disinfected and plated on 1% malt extract agar (MEA, malt extract 1% and agar 2% [w/v]) for the isolation of fungal strains. These fungal taxa occurred in the leaf sheaths of 21.6% (22 samples), 6.9% (7 samples), and 2.9% (3 samples) of 102 individual samples examined, respectively. *Phomopsis* and *Biscogniauxia* species have been encountered as non-systemic endophytes and are considered to be latent pathogens and saprobes, respectively (Udayanga et al. 2011; Luchi et al. 2015).

Representative strains of *E. bromicola* (E02, E03, E05, and E06) and *B. maritima* (B32 and B33) were analysed for the sequence of the rDNA ITS region according to Yoneda et al. (2024) for taxonomic assignment. Sequence data were deposited in the DNA Data Bank of Japan (DDBJ) (LC866402–LC866407). To confirm the taxonomic identity of the representative strains, we conducted a phylogenetic analysis based on the sequence of the rDNA ITS region. The sequences were aligned using the MAFFT version 7 (Katoh et al. 2019). Phylogenetic analyses of the aligned sequences were performed by the maximum likelihood (ML) method implemented in RaxML-NG version 1.2.2 (Kozlov et al. 2019). A ML tree was created by the Figtree version 1.4.4 (Rambaut, 2018). The strains of the genus *Phomopsis* were identified by micro-morphological observations of alpha and beta conidia and conidiomata (Barnett and Hunter 1998). In addition, *E. uncinata* NBRC32642 and *T. versicolor* NBRC30340 were obtained from a culture collection (Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan). All strains were maintained on slants of 1% MEA at 20°C in darkness.

Pure culture decomposition test

First, a preliminary test was conducted to determine the optimal growth temperature for representative *E. bromicola* strains (E01, E02, E05, and E07), according to Hatano et al. (2022). Mycelial disks (6 mm diameter) were taken from the edge of cultures on Petri dishes containing potato dextrose agar (PDA) and incubated at 20°C for 2 weeks. They were then transferred to the middle of Petri dishes (9 cm diameter) containing 20 mL of 1% MEA. The plates were incubated at one of seven temperatures (5, 10, 15, 20, 25, 30, and 35°C) in the dark. The colony diameter was measured in two directions at right angles to each other at 1-week intervals for six weeks, and the colony growth rate was calculated by regressing the colony diameter against the days after inoculation. Five plates were prepared for each isolate and at each temperature.

Attached senescent leaves of *E. racemifer* without obvious attack by insects or microbes were collected in Kyoto in July 2018, oven-dried at 40°C to a constant mass and used as a substratum for pure culture decomposition tests according to Osono et al. (2021). The 23 fungal strains were inoculated to leaves (0.25 g) sterilised by exposure to ethylene oxide gas at 60°C for 6 hours and placed on the surface of Petri dishes (9 cm in diameter) containing PDA medium supplemented with 50 mg/L chloramphenicol (Atect Co., Ltd, Osaka, Japan). Inocula for each plate were cut from the margin of previously inoculated Petri dishes on 1% MEA with a sterile cork borer (6 mm diam) and placed on the agar adjacent to the leaves, with one plug per plate. The plates were incubated at 20°C for 12 weeks in the dark. The incubation temperature of 20°C was determined by referring to the standardised protocol of a pure culture decomposition test (Osono 2020) and the results of preliminary surveys showing that the optimal temperature of hyphal growth was 20-25°C for four strains of *E. bromicola* (E01, E02, E05, and E07) (Figure 1).

After incubation, the leaves were retrieved, oven-dried at 40°C to a constant mass and weighed. Five plates were prepared for each test, and four uninoculated plates served as control. Mass loss of leaves was determined as a percentage of the original mass, taking the mass loss of leaves in the uninoculated (19.5%) and incubated control (2.2%) treatments into account. Prior to these tests, the sterilised leaves were placed on 1% MEA, and after incubation for 8 weeks at 20°C in darkness, no microbial colonies had developed on the plates. This verified the effectiveness of the sterilisation method used in the present study.

Statistical analyses

We analysed the effect of fungal species on the mass loss of leaves using a generalised linear mixed model (GLMM). The fungal species was included as a fixed model, whereas the 23 strains were treated as random effects. The Tukey Honest Significant Differences test (Tukey HSD) was used for multiple comparisons. Error structure and link function of the GLMM were beta distribution and logit, respectively. P value was calculated with a likelihood ratio test by chi-square approximation. R version 4.3.1 (R Core Team, 2023)

was used to perform the analysis.

Results

The phylogenetic analysis grouped the sequences into two distinct clades corresponding to each species (Figure 1). The four *E. bromicola* strains (E02, E03, E05, and E06) formed one clade, while the two *B. maritima* strains (B32 and B33) formed another. The branch lengths suggest greater genetic diversity among the *E. bromicola* strains compared to the *B. maritima* strains.

The optimal temperature for hyphal growth for the four strains of *E. bromicola* was between 20-25°C (Figure 2).

Mass loss of leaves caused by the 23 assessed strains ranged from 0.4% to 27.2% of the original leaf mass, with a mean of 4.9% for *E. bromicola*, 5.6% for *Phomopsis* sp., and 20.8% for *B. maritima* (Figure 3a). The 16 strains of *E. bromicola* were variable in their potentialities to decompose leaves, with the mass loss values ranging from 0.4% to 10.8% (Figure 3a). *Epichloë uncinata* NBRC32642 caused a mass loss of 4.7%, which was a similar level to the mean value for *E. bromicola*. *Trametes versicolor* NBRC30340 caused the greatest mass loss, 27.2% (Figure 3a). The mass loss of leaves was significantly different among fungal species (GLMM, $P < 0.001$). The mean value of mass loss caused by *T. versicolor* was significantly higher than those caused by *E. bromicola* (Tukey HSD, $P < 0.001$), *E. uncinata* ($P < 0.001$), and *Phomopsis* sp. ($P < 0.001$). Similarly, the mass loss caused by *B. maritima* was significantly higher than that of *E. bromicola* ($P < 0.001$), *E. uncinata* ($P < 0.01$), and *Phomopsis* sp. ($P < 0.001$). In contrast, there were no significant differences in mass loss between *T. versicolor* and *B. maritima* ($P = 0.46$). Furthermore, the mean values of mass loss were not significantly different among strains of *E. bromicola*, *E. uncinata*, and *Phomopsis* sp. ($P = 0.99$ for all comparisons) (Figure 3b).

Discussion

In the present study, we quantitatively demonstrated that the *Epichloë* strains examined have the ability to cause a loss of mass of sterilised leaves under the pure culture conditions. These results indicate that the decomposition potentialities of *E. bromicola* and *E. uncinata* were close to those of sympatric non-systemic endophyte *Phomopsis* sp. and were relatively low when compared with those of *B. maritima*, which was considered as a saprobe, and white-rot fungus *T. versicolor*. The results are consistent with previous findings that the decomposing ability was generally lower in non-ligninolytic *Phomopsis* sp. than in ligninolytic fungi in Xylariaceae and Basidiomycota (Osono 2020).

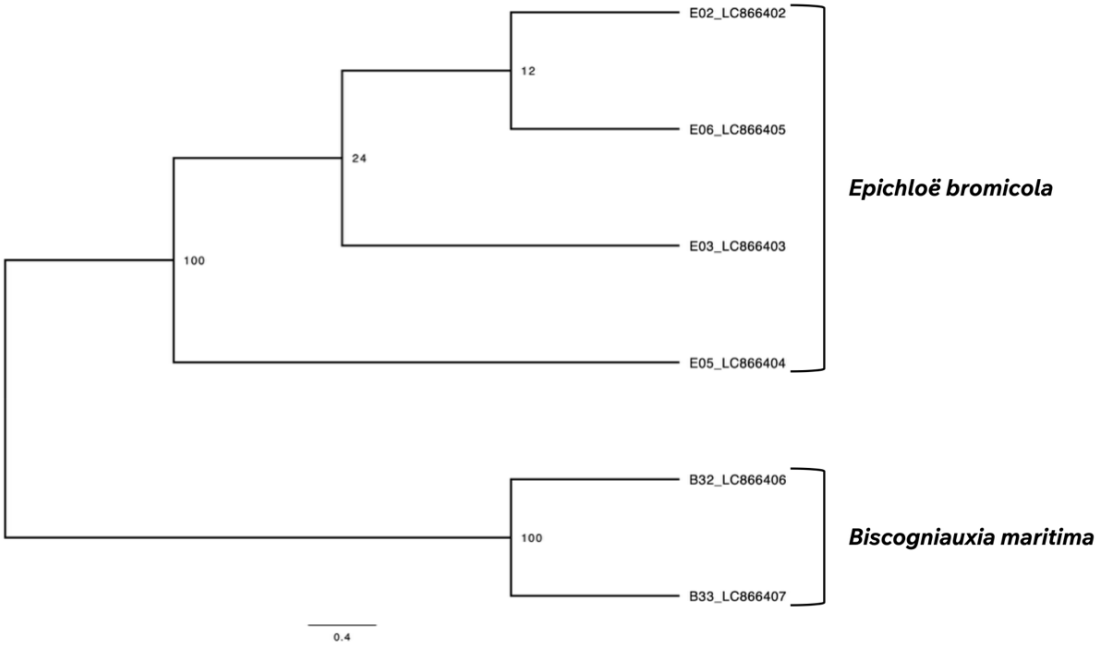

Figure 1 Maximum likelihood tree of *Epichloë bromicola* (strains E02, E03, E05, and E06) and *Biscogniauxia maritima* (strains B32 and B33) based on the ITS region gene sequences. The values shown at the nodes are the confidence levels from 100 replicate bootstrap samplings.

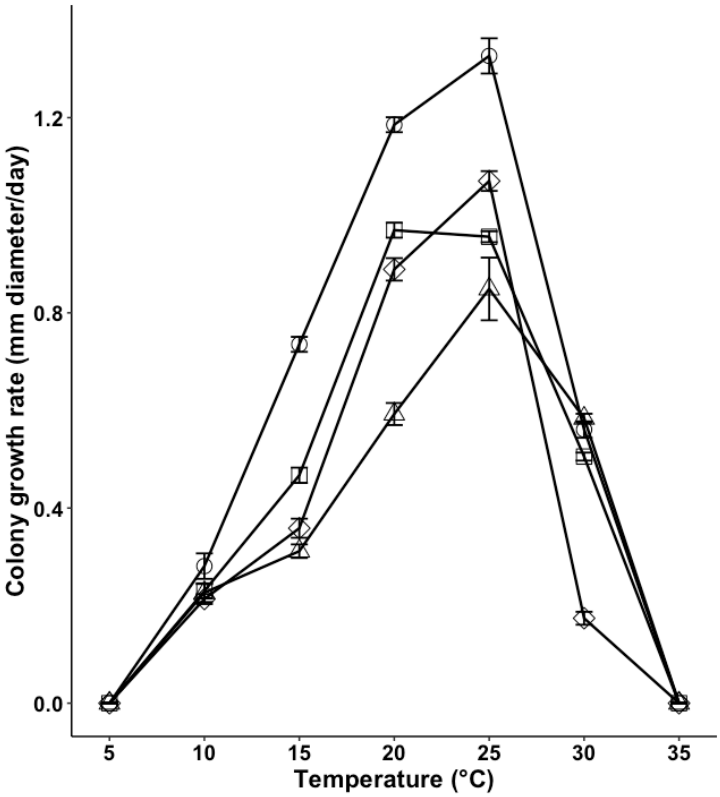

Figure 2 Colony growth rate of four strains of *Epichloë bromicola* (square, E01; circle, E02; triangle, E05; diamond, E07) in relation to constant incubation temperatures. Data points represent means ± SE (n = 5).

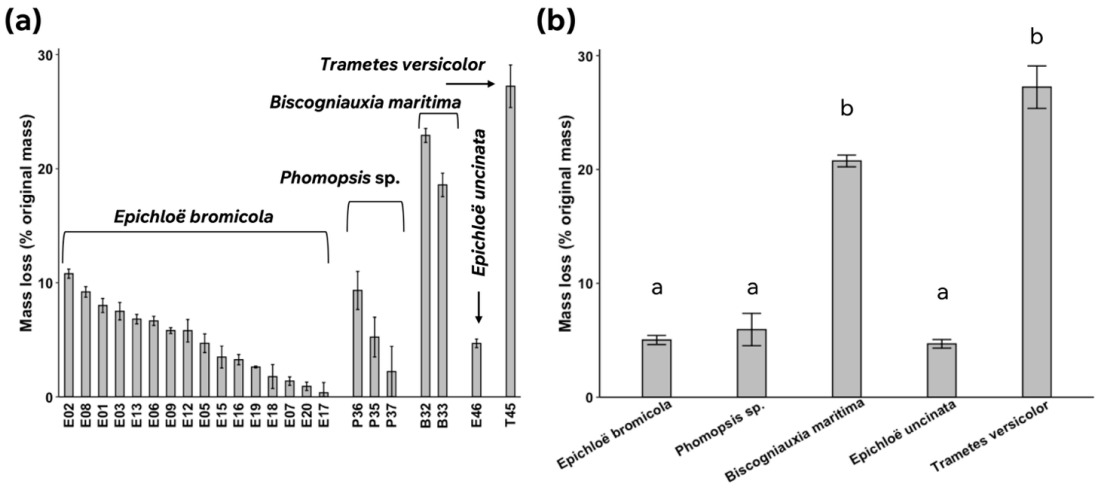

Figure 3 Percent loss of original mass of sterilised leaves of *Elymus racemifer* incubated for 12 weeks. (a) Fungal strains tested include *Epichloë bromicola* (E01, E02, E03, E05, E06, E07, E08, E09, E12, E13, E15, E16, E17, E18, E19, E20), *Phomopsis* sp. (P36, P35, P37), *Biscogniauxia maritima* (B32, B33), *Epichloë uncinata* (E46), and *Trametes versicolor* (T45). (b) Mean mass loss for each fungal species. Bars represent means ± SE. In (a), n = 5 for each strain. In (b), n is the total number of replicates per species (ranging from 5 to 80). Different letters above the bars in (b) indicate significant differences among species according to Tukey HSD test ($P < 0.05$).

It should be borne in mind that the pure culture decomposition test was done on PDA medium enriched in nutrients for fungal growth. External nutrients are often added in pure culture to compensate for nutrient-poor substrata such as wood (Eriksson et al. 1990; Worrall et al. 1997) and can enhance the fungal decomposition ability (Osono 2020). This implies a potential over-estimation of mass loss values for the strains of *E. bromicola* and other taxa examined in the present study. Nonetheless, the results of the present study demonstrated that the decomposition potentiality of *E. bromicola* was similar to or lower than the decomposition potentialities of sympatric non-systemic endophytes. This agrees with a previously reported notion that *Epichloë* endophytes grow by obtaining photosynthetic product from live plant tissues but do not elongate hyphae on dead tissues (Christensen and Voisey 2007; Hatano et al. 2024) and that there is more selection pressure on the mutualistic function in live tissues than on the saprotrophic function in dead tissues for vertically transmitted endophytes.

While these findings are conclusive at a descriptive level, future research could provide a mechanistic understanding of these functional limitations. For example, genomic and transcriptomic analyses of *Epichloë* strains could help determine whether they lack genes encoding key lignocellulolytic enzymes (e.g., cellulases, laccases) or other factors involved in cellulose or lignin decomposition, such as those producing reactive oxygen species. It is also important to consider that *Epichloë* endophytes can alter the chemical composition of their host's tissues (Gundel et al. 2016; Song et al. 2022). In the present study, the senescent leaves were collected from wild populations of *E. racemifer* without pre-screening for *Epichloë* infection status. Therefore, our results demonstrate the intrinsic decomposition potential of the fungi on a standardised substrate, but do not account for these potential indirect effects. A valuable direction for future research would be to conduct decomposition experiments that directly compare litter from known infected and uninfected plants. Such studies would help to disentangle the direct, albeit limited, decomposition activity of *Epichloë* endophytes from their indirect influence on litter chemistry, providing a more complete understanding of their overall role in grassland nutrient cycling.

Conclusion

The present study showed that *Epichloë* strains have limited decomposition ability under pure culture conditions, comparable to that of the non-systemic endophyte *Phomopsis* sp., and lower than that of ligninolytic fungi such as *B. maritima* and *T. versicolor*. These findings support the view that *Epichloë* endophytes are adapted to mutualistic growth in live plant tissues rather than saprotrophic activity on dead tissues, reflecting evolutionary pressures favouring vertical transmission.

ACKNOWLEDGEMENTS

We thank Dr. E. Nakajima for critical reading of the manuscript. This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan [No. 18K05731].

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