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In Vitro Interactions between *Eutypella parasitica* and Some Frequently Isolated Fungi from the Wood of the Dead Branches of Young Sycamore Maple (*Acer pseudoplatanus*)

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Abstract: The ten most frequently isolated fungi from the wood of the dead branches of *Acer pseudoplatanus* L. were tested in dual cultures to evaluate their in vitro antagonistic activity against *Eutypella parasitica* R.W. Davidson and R.C. Lorenz, the causative agent of a destructive disease of maples in Europe and North America. The tested fungi, treated also as challenge isolates, were *Diaporthe* sp., *Eutypa* sp., *Eu. maura*, *E. parasitica*, *Fusarium avenaceum*, *Neocucurbitaria acerina*, *Neonectria* sp., *Peniophora incarnata*, *Petrakia irregularis*, and *Phomopsis pustulata*. The antagonistic ability of each challenge isolate was evaluated by calculating an index of antagonism (*AI*) based on the interaction type in the dual cultures. The results of competition between the fungal isolates were quantified after re-isolations from the interaction zone (*s*). The dual cultures revealed two main types of competitive interactions: Deadlock, consisting of mutual inhibition after mycelial contact or at a distance, and replacement, reflecting in the inhibition of *E. parasitica*, followed by partial overgrowth by the replacing fungus. Statistical analysis showed significant differences in average *AI* and *s* of challenge isolates between different dual culture assays. Based on the results of the antagonism index, *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* had the highest inhibitory effect on *E. parasitica* growth and were recognized as the most promising candidates for further biocontrol studies of *E. parasitica*. The mycelium of *E. parasitica* at the interaction zones remained mostly viable, except in dual cultures with *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp., where re-isolations did not yield any colony of the *E. parasitica* isolate. Based on the results, we assume that *E. parasitica* is a weak competitor, which invests less energy in direct mycelial competition. We discuss the potential of the observed antagonists as a possible biocontrol of Eutypella canker of maple. Nevertheless, additional experiments should be performed for a solid conclusion about competitive ability of *E. parasitica* and usefulness of antagonists as biocontrol.

Keywords: *Eutypella parasitica*; dual culture; hyphal interaction; deadlock; replacement; competition; antagonism; inhibition; re-isolation; biocontrol

1. Introduction

Interactions play a significant role in shaping the community structure of fungal organisms [1]. They are an important determinant of the distribution, growth pattern, and abundance of fungal species in any natural fungal community [2,3] and can also be used for developing biocontrol strategies. Interactions in the natural environment are complex [1] and have been studied using a variety of

techniques, including observations of hyphal interactions, tests of inhibition on hyphal growth, and examination of reaction types [3]. Some fungal endophytes are known for their defensive power against various tree pathogens [4], but there are also concerns about fungi having the opposite function of contributing to the development of tree diseases [5–7]. Endophytes can influence the presence of pathogens in a tree by different mechanisms, i.e., antibiosis by metabolites, competition for nutrients or space, mycoparasitism, and indirect effects including induced systemic resistance of the tree [8,9].

Interactions between fungal mycelia commonly result in distinctive changes along interaction zones [10]. The outcome of interspecific fungal interactions depends on species compatibility [11], as well as the microclimate and physio-chemical structure of the substrate [12]. The fungal interaction outcome is a complex phenomenon and may be one of the following: (1) Deadlock, i.e., neither isolate enters the territory of the other, or (2) replacement, i.e., one isolate is partially or entirely replaced by the other [13]. Deadlock usually occurs due to each isolate excreting and/or detecting "non-native" chemical compounds that inhibit growth [14]. This type of interaction commonly occurs following, but sometimes also prior to, mycelial contact [15]. Partial replacement occurs when one fungus initially gains headway but subsequently stops, or when both species make some entry into the territory of the other [12]. In contrast, complete replacement results from one individual fungus completely engulfing the other. In addition to deadlock and replacement, Boddy [12] also used a third possible outcome of an interaction—intermingling, i.e., a neutral interaction that results in the fusion of colonies for compatible genotypes or spatial intermixing for incompatible genotypes.

The fungus *Eutypella parasitica* R.W. Davidson and R.C. Lorenz, the causative agent of Eutypella canker of maple, was reported for the first time in Europe from Slovenia [16]. Later, the disease was also reported from other regions in Europe [17–23]. It is believed to originate from North America [24] and to represent a considerable risk for naturally distributed maples in Europe [22,25]. The fungus *E. parasitica* most likely enters the trunk through branch stubs or bark wounds [26] where it competes with other fungal species. The fungal species *Eutypa* sp. Tul. and C. Tul., *Eutypa maura* (Fr.) Sacc., *Fusarium avenaceum* (Fr.) Sacc., *Neocucurbitaria acerina* Wanas., Camporesi, E.B.G. Jones and K.D. Hyde, *Diaporthe* sp. Nitschke, *E. parasitica*, *Neonectria* sp. Wollenw., *Petrakia irregularis* Aa, *Phomopsis pustulata* (Sacc.) Died., and *Peniophora incarnata* (Pers.) P. Karst. are some of the most frequently isolated species from the wood of the dead branches of young sycamore maple [27]. However, little is known about how they interact and compete for the same substrate.

The aim of the present study was to investigate the in vitro activity of the ten most frequently isolated fungal species from the wood of the dead branches of *Acer pseudoplatanus* L. [27] against *E. parasitica* in dual culture experiments.

2. Materials and Methods

2.1. Interactions between E. parasitica and Ten Fungal Isolates in Dual Cultures

The dual culture technique was used to test the possible antagonistic effect of ten fungal isolates isolated from the wood of the dead branches of *A. pseudoplatanus* [27] against an isolate of *E. parasitica* (ZLVG 805, see Table 1). The reference isolate of *E. parasitica* was obtained from Eutypella canker of maple (46.0533° N, 14.4914° E, 338 m a.s.l.) on *A. pseudoplatanus* and is referred to as the "response isolate" throughout the paper. Other fungal isolates used in the experiment are referred to as "challenge isolates", including one additional isolate of *E. parasitica* (ZLVG 791).

Interactions between isolates were performed in plastic Petri dishes (Ø = 90, *h* = 15 mm) containing 3.9% (w/v) potato dextrose agar (PDA; Becton Dickinson, Sparks, Maryland, United States). Agar discs (Ø = 5 mm) taken from the margin of actively growing, one-week-old cultures were placed 4 cm apart from each other on the PDA. The cultures were incubated at 24 °C in the dark (I-190 CK incubator; Kambič, Semič, Slovenia).

For the self-inhibition test, two discs of *E. parasitica* (ZLVG 805) taken from the same colony were used. The control consisted of pairing *E. parasitica* (ZLVG 805) with a sterile agar disc. Each combination

was replicated three times, following examples in the literature [4,28–30]. A total of 36 interactions were examined, including the self-inhibition assays and control pairings.

Table 1. Fungal isolates [1] used in dual cultures assays.

Fungal Isolate	Collection Number [2]	Number of Days [3]
Diaporthe sp. Nitschke	ZLVG 788	12
Eutypa sp. Tul. & C. Tul.	ZLVG 790	7
Eutypa maura (Fr.) Sacc.	ZLVG 789	5
Eutypella parasitica R.W. Davidson & R.C. Lorenz	ZLVG 791	12
Eutypella parasitica R.W. Davidson & R.C. Lorenz [4]	ZLVG 805	6
Fusarium avenaceum (Fr.) Sacc.	ZLVG 792	12
Neocucurbitaria acerina Wanas., Camporesi, E.B.G. Jones & K.D. Hyde	ZLVG 794	—
Neonectria sp. Wollenw.	ZLVG 795	6
Peniophora incarnata (Pers.) P. Karst.	ZLVG 797	5
Petrakia irregularis Aa	ZLVG 798	14
Phomopsis pustulata (Sacc.) Died.	ZLVG 799	7

[1] The ten most frequently isolated fungi from the wood of the dead branches of *A. pseudoplatanus* [27] as challenge isolates and the response isolate of *E. parasitica*. [2] ZLVG—Culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute. [3] Number of days required for each fungal isolate to form a two-centimeter-diameter colony at 24 °C in the dark, also referred to as growth rate. [4] Response isolate of *E. parasitica* paired with the ten challenge fungal isolates.

Based on differences in the growth rate of each fungal isolate in comparison to the response isolate of *E. parasitica*, the isolates were inoculated into Petri dishes on different starting days to give them a chance to meet in the center of the plate (Table 1). We therefore carried out a preliminary test of the growth rate of the fungal isolates. Agar discs (Ø = 5 mm) taken from the margin of actively growing cultures were cut and placed in the center of 90-mm diameter plastic Petri dishes containing 3.9% PDA and incubated at 24 °C in the dark (I-190 CK incubator; Kambič, Semič, Slovenia). We counted the number of days required for each fungal isolate to form a two-centimeter-diameter colony (Table 1). Slower growers were placed on the agar in advance of *E. parasitica*, while faster growers were placed on the agar after *E. parasitica*.

The antagonistic ability of each fungal isolate against *E. parasitica* was examined daily and scored using the Badalyan, Innocenti, and Garibyan [13] rating for a macroscopically determined type of interaction. A rating scale for the three main and four sub-types of interactions was used (Table 2).

Table 2. Types and subtypes of fungal interactions with corresponding scores [13].

Label	Type of Interaction	Score
A	Deadlock [1] at mycelial contact	1
B	Deadlock at a distance, without mycelial contact	2
C	Replacement [2]	3
C_{A1}	Partial replacement after initial deadlock with mycelial contact	3.5
C_{B1}	Partial replacement after initial deadlock at a distance	4
C_{A2}	Complete replacement after initial deadlock with mycelial contact	4.5
C_{B2}	Complete replacement after initial deadlock at a distance	5

[1] Mutual inhibition in which neither isolate was able to overgrow the other. [2] Overgrowth without initial deadlock.

An antagonism index (*AI*) [13] was calculated for each challenge fungal isolate using the equation:

$$AI = \sum n \times i, \quad (1)$$

where n is the number of each type or sub-type of interaction in dual cultures and i is the corresponding score (Table 2). *AI* is a qualitative measure defined as the ability of a fungus to dominate and

compete with other species [31]. Higher *AI* denotes higher competitive and inhibitory ability of a challenge isolate.

2.2. Re-Isolations from Dual Cultures

Ten days after mycelium contact or ten days after no mycelium growth on the connective line between both colonies, the observed results of the competition were quantified after fungal re-isolation. Five agar discs (Ø = 5 mm) were cut from the interaction zone or from the margin of the *E. parasitica* colony (in the case of no mycelium contact), and two agar discs (one per isolate) were cut from areas with a presumed growth of only one individual fungus (Figure 1) [32]. To confirm the viability of *E. parasitica* in co-cultures, discs were placed on 3.9% (*w/v*) PDA plates and incubated at 24 °C in the dark (I-190 CK incubator; Kambič, Semič, Slovenia). The outgrown mycelium cultures were compared to the original isolates, and the identity of *E. parasitica* cultures was additionally confirmed by using *E. parasitica* specific primers (EpF/R). The methodology followed that described by Piškur, et al. [33], except for DNA extraction, which was done using a NucleoSpin®Plant II (Macherey Nagel, Düren, Germany) following the manufacturer's instructions, after homogenizing the fungal material with a Lysing Matrix A tube (MP Biomedicals, Solon, OH, USA) using a Precellys Evolution device (Bertin Technologies, Montigny-le-Bretonneux, France). Reactions that yielded a single and strong fragment size of 341 bp on the electrophoresis gel were classified as *E. parasitica* positive [33].

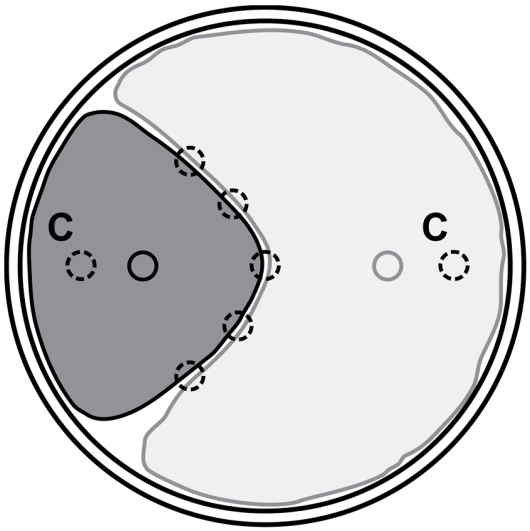

Figure 1. Design of the re-isolation test from a Petri dish with competing mycelia: Five discs taken from the interaction zone and two control discs (C) from the colony margins. The gray culture is the response isolate of *E. parasitica*, and the white culture is the challenge isolate (illustration by S. Zidar, Slovenian Forestry Institute).

The number of successful re-isolations per individual isolate in each interaction was counted. The re-isolation success of isolate A (challenge isolate) in a dual culture assay with isolate B (*E. parasitica*) was quantified as the sum of re-isolated fungal isolates from each Petri dish. These sums were log-transformed according to Koukol, Mrnka, Kulhankova, and Vosatka [32]:

$$s = \ln((A+1)/(B+1)), \quad (2)$$

where s is the score quantifying the re-isolation success of a challenge isolate in the interaction, and A and B are the mean number of successfully re-isolated challenge and response fungal isolates, respectively (n = max 5). The estimated value of s ranges between −1.79 and +1.79. Higher values of s indicate higher average re-isolation success, while lower values of s indicate lower average re-isolation success of a challenge isolate in competition with *E. parasitica*.

A non-parametric Kruskal–Wallis test was used to compare average antagonistic ability and average re-isolation success of a challenge isolate between different dual culture assays. Afterwards, a post-hoc multiple comparison Dunn test with Benjamini–Hochberg adjustment was used. To test the assumption of normality, the Shapiro–Wilk's test was applied, and for testing the homogeneity of variances, the Levene's test was used.

All calculations and graph design were performed in Microsoft Excel version 16.0.12527.20612. Statistical analyses were performed in the R software environment for statistical computing [34] with the "car" [35], "FSA" [36], and "rcompanion" packages [37]. Mycelial interactions were photographed using a Panasonic Lumix DMC-FZ7 digital camera (Panasonic, Osaka, Japan).

3. Results

3.1. Interactions between E. parasitica and Ten Fungal Isolates in Dual Cultures

Macroscopic examination of dual cultures revealed that almost all challenge isolates made hyphal contact with the response isolate, i.e., *E. parasitica*, within the time of the experiment. *Peniophora incarnata* was the most aggressive challenge isolate, almost completely replacing *E. parasitica* (Figure 2g). Moreover, we did not observe any macroscopic changes in the mycelium of *E. parasitica* in any of the tested dual cultures. In co-cultures of *F. avenaceum*, *N. acerina*, *Pe. irregularis*, and *Ph. pustulata*, a large quantity of spores around the agar disc of the response isolate was observed.

Dual culture assays showed a diverse pattern of interaction types between the response and challenge fungal isolates (Table 3). In four assays we found an interaction in which *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* partially replaced *E. parasitica* after initial deadlock with mycelium contact (interaction type C_{A1}) (Figure 2b,c,f,g). In five other assays, the challenge and the response isolate inhibited each other's growth after initial mycelial contact and neither isolate was able to overgrow the other (interaction type A) (Figure 2a,d,e,h,i). In just two cases (*Diaporthe* sp. and *N. acerina*), we observed deadlock at a distance, without mycelial contact (interaction type B), but since this was not the predominant type of the specific assay, we did not use it as a representative type of interaction. However, we did not observe any case in which *E. parasitica* overgrew the challenge isolate. The types of interactions were reflected in the calculated antagonism index (*AI*) (Table 3). Most of the challenge isolates achieved low *AI* values. Of the challenge isolates, *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* had the strongest antagonistic effect (Table 3).

Table 3. Interaction type and antagonism index (*AI*) of *E. parasitica* in dual culture assays.

Fungal Isolate	Interaction Type [2]	*AI*	Statistic Group [4]
Diaporthe sp.	A [3]	4	ab
Eutypa sp.	C_{A1}	10.5	a
Eutypa maura	C_{A1}	10.5	a
Eutypella parasitica (ZLVG 791)	A	3	b
Eutypella parasitica (ZLVG 805) [1]	A	3	b
Fusarium avenaceum	A	3	b
Neocucurbitaria acerina	A [3]	4	ab
Neonectria sp.	C_{A1}	10.5	a
Peniophora incarnata	C_{A1}	10.5	a
Petrakia irregularis	A	3	b
Phomopsis pustulata	A	3	b

[1] Self-inhibition assay. [2] Type of interaction followed the classification of Badalyan, Innocenti, and Garibyan [13], see Table 2. [3] In one out of three Petri dishes, the B interaction type was determined. [4] Different letters indicate significant differences ($p < 0.05$).

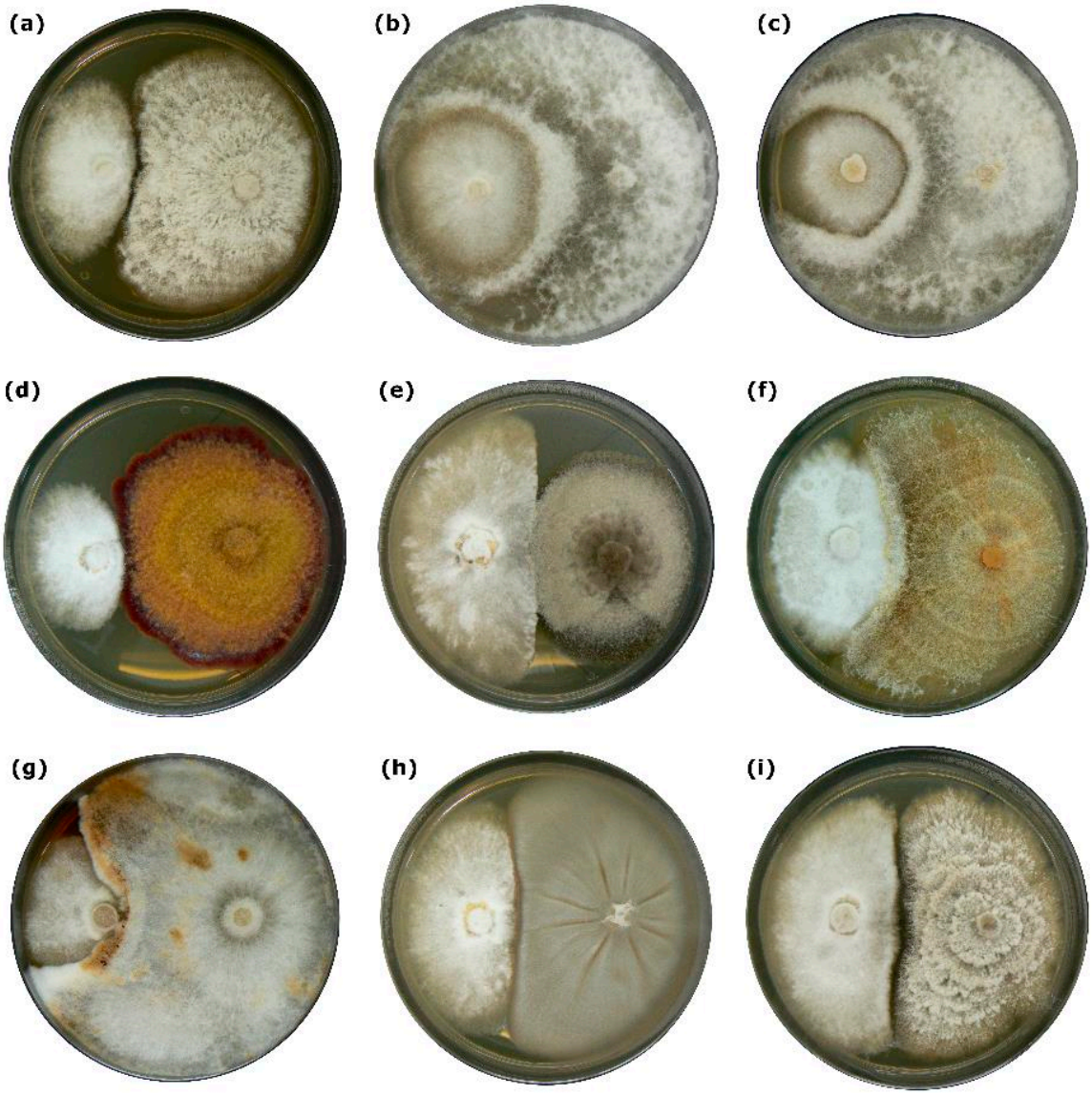

Figure 2. Mycelial interactions after 18 days of co-incubation between the response isolate of *E. parasitica* (left) and the challenge isolate (right): (**a**) *Diaporthe* sp.; (**b**) *Eutypa* sp.; (**c**) *Eu. maura*; (**d**) *F. avenaceum*; (**e**) *N. acerina*; (**f**) *Neonectria* sp.; (**g**) *P. incarnata*; (**h**) *Pe. irregularis*; and (**i**) *Ph. pustulata*. Note: In other replicates different types of interactions could be observed (Table 3).

In co-cultures with *Eutypa* sp., *Eu. maura*, *Neonectria* sp., *P. incarnata*, *Pe. irregularis*, and *Ph. pustulata*, the formation of a distinctive interaction zone could be observed (Figure 2b,c,f–i). Contact of fungal isolates with each other resulted in yellowish brown, sometimes even black, pigmentation and formation of dense mycelium. Dense mycelium at the interaction zone of interaction type A was usually produced by both isolates (Figure 2e), while in interaction type C_{A1}, dense aerial mycelium, was produced only by the overgrowing isolate (Figure 2b,c,f,g). Changes in pigmentation of the interaction zone were best observed from the reverse side of the Petri dishes.

In a self-inhibition test and dual culture assay with a different isolate of *E. parasitica*, colonies almost uniformly showed interaction type A (Figure 3), where neither isolate was able to overgrow the other. From six replicated co-cultures (three for self-inhibition test and three for dual culture assay), a dense mycelium at the interaction zone was formed only in one case. For the control pairings of *E. parasitica* and sterile agar discs there was no reason to determine the interaction type.

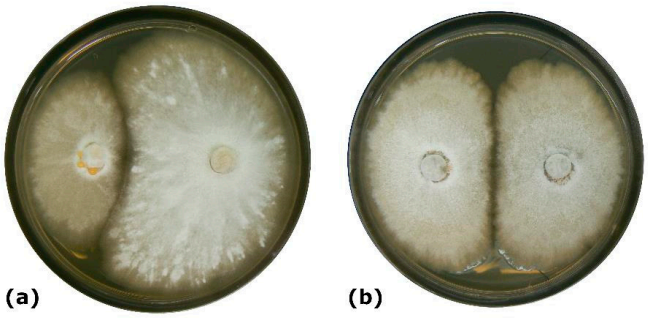

Figure 3. Mycelial interactions after 18 days of co-incubation between: (**a**) Response (left) and challenge (right) isolate of *E. parasitica* and (**b**) two response isolates of *E. parasitica* (self-inhibition).

The results of the Kruskal–Wallis statistical test revealed significant differences ($p < 0.001$) in the average antagonistic ability of challenge isolates between different dual culture assays. Pairwise comparisons revealed statistically significant differences ($p < 0.05$) for the average *AI* score between *E. parasitica* ZLVG 791 (a dual culture assay) on one hand and *Eutypa* sp., *Eu. maura*, *Neonectria* sp. and *P. incarnata* on the other. The same results were obtained with *E. parasitica* ZLVG 805 (a self-inhibition test) (Table 3).

3.2. Re-Isolations from Dual Cultures

Out of 189 re-isolated discs, 2.1% did not develop any colony, while in 24.3% of cases both challenge and response isolates were re-isolated from the interaction zone. No re-isolations of *E. parasitica* were yielded after interaction with *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp., which were the most successful isolates regarding the number of successful re-isolations. In contrast, *Diaporthe* sp., *N. acerina*, and *Pe. irregularis* were re-isolated less successfully (Figure 4).

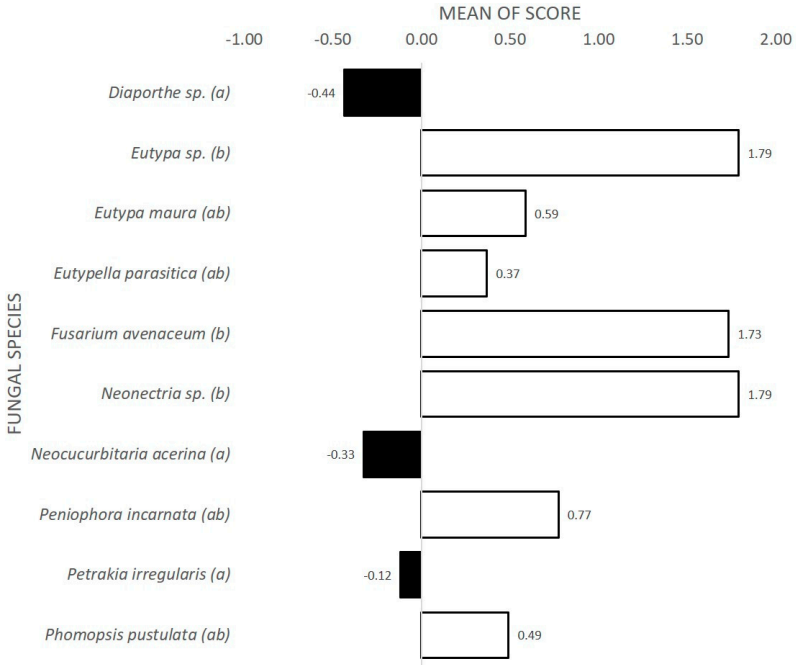

Figure 4. Mean scores (s, Equation (2), [−1.79, +1.79]) of re-isolation of fungal isolates from dual cultures quantifying the re-isolation success of a challenge isolate in the interaction zone. Bars above zero refer to higher average re-isolation success, while bars below zero refer to lower average re-isolation success of the challenge isolate in competition with *E. parasitica*. Different letters in parenthesis, indicate significant differences ($p < 0.05$).

Seven out of ten challenge isolates showed positive re-isolation success (Figure 4). For *E. parasitica* in the self-inhibition test, the average value of *s* was determined at 0.00 because we could not differentiate among the same isolate after re-isolation. Similarly, for the control pairings of *E. parasitica* and sterile agar discs, we did not obtain the average value of *s* because there was no challenge isolate which could influence the growth of *E. parasitica*.

The results of the Kruskal–Wallis statistical test revealed significant differences ($p < 0.01$) in the average re-isolation success of challenge isolates between different dual culture assays. Pairwise comparisons revealed statistically significant differences ($p < 0.05$) for the average re-isolation score between *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp. on one hand and *Diaporthe* sp., *N. acerina*, and *Pe. irregularis* on the other. No statistically significant differences ($p < 0.05$) were obtained for any of the pairwise comparisons with *E. parasitica*.

4. Discussion

4.1. Interactions between E. parasitica and Ten Fungal Isolates in Dual Cultures

Based on the *AI* values, fungal isolates can be divided into three categories according to Badalyan, Innocenti, and Garibyan [13]: (1) Active, with $AI > 15$; (2) moderately active, with *AI* between 10 and 15; and (3) weakly active, with $AI < 10$. A lower index of antagonism indicated that the challenge isolate was weaker in terms of its inhibition of the response isolate [3]. Our experiment revealed mostly weakly active challenge isolates. Only *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* were moderately active in inhibition according to the index of antagonism.

Because of the occasionally very similar macroscopic appearance of co-incubated mycelia, it was difficult to determine the type of interaction or to select the isolate which overgrew the other. It was especially difficult to differentiate between interaction type A and B. However, *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* were the only challenge isolates that overgrew the response isolate (interaction type C_{A1}). This result suggests that these isolates have the greatest potential as biocontrol agents, based on the *AI* values. Consistent with the general results of other research on fungal interactions [30], two types of interactions were observed.

Eutypella parasitica did not overgrow any of the challenge isolates, suggesting the relative non-aggressiveness of the response isolate. Based on the obtained *AI* values, we assume that *E. parasitica* is usually a weak competitor compared to other fungal species which are present in the dead branches of *A. pseudoplatanus*. This is consistent with our previous results [27], where the quick progression of *E. parasitica* from the wood of the outer part of the dead branch into the wood of the trunk was observed. However, the outcome of interactions in nature varies depending on the size and quality of the resource, i.e., wood and microclimate [12].

Different interaction types in replicates of the same dual cultures (in our case *Diaporthe* sp. and *N. acerina*) are frequently observed [4,10,38,39]. A very diverse set of bioactive substances may be produced during interspecific mycelial interactions [3,40] and may have an effect on inhibition abilities, pigment production in the interaction zone, colony color, etc. [2,3,13–15,41]. Further studies of secondary metabolites and their role in antagonistic activity toward *E. parasitica* are therefore needed. Studies of interaction patterns in vitro may produce effective strategies for biological control [3], but extrapolating findings from the laboratory to natural habitats should be done carefully. In the natural environment, inhibition would be influenced by a plethora of additional factors, i.e., inoculum potential, germination efficiency, growth rate, substrate utilization patterns, microclimate, etc. [3]. This study can serve as a guide to the possible outcome of different interactions in nature, but these interactions should be further studied in the natural environment, which was beyond the scope of the study.

4.2. Re-Isolations from Dual Cultures

Re-isolations were not always successful from dual culture assays (co-cultures with *F. avenaceum* and *P. incarnata*). If we re-isolated both isolates from the interaction zone, we assumed that the challenge isolates were not very active competitors. In contrast, re-isolation of only the challenge isolate from the interaction zone suggested the elimination of the response isolate of *E. parasitica* and obvious higher success in competition. This was consistent with higher average values of *s*. Negative values of *s* were calculated for co-cultures with *Diaporthe* sp., *N. acerina*, and *Pe. irregularis*, suggesting their weaker competition success.

Re-isolations after deadlock at mycelial contact (interaction type A) resulted mostly in the growth of both isolates, even from the same discs. Koukol, Mrnka, Kulhankova, and Vosatka [32] reported the same re-isolation results after deadlock at a distance (interaction type B), which was not the general case in our study. Re-isolations from assays with interaction type B yielded both isolates in only two out of ten cases, while in the eight other cases, re-isolations yielded only *E. parasitica* colonies. The mycelium of *E. parasitica* remained viable and grew from re-isolation discs even after being partially replaced by *P. incarnata*. In contrast, re-isolations from dual cultures with *Eutypa* sp., *F. avenaceum* and *Neonectria* sp. did not yield any colony of the response isolate from interaction zone, which suggests replacement of the response isolate and its weaker competitive ability against those challenge isolates. The fact that *Eutypa* sp., *F. avenaceum*, and *Neonectria* sp. were able to outcompete the response isolate of *E. parasitica* suggests that *E. parasitica* probably invests less energy in direct mycelial competition against those challenge isolates, as reported by Koukol, Mrnka, Kulhankova, and Vosatka [32]. Although no research has addressed this until now, the re-isolation tests offered a reliable tool to confirm the re-isolation success of an individual isolate after contact with a competitor's mycelium [32].

4.3. Summary of In Vitro Interactions between E. parasitica and Challenge Isolates

Based on *AI*, the inhibition effect of *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* on the response isolate was significantly higher in comparison with other challenge isolates. These results were consistent with the expected very successful re-isolation of these challenge isolates from the interaction zones. Based on *s*, *Eutypa* sp. and *Neonectria* sp. were good inhibitors of *E. parasitica* growth and destroyed it in the interaction zone. Similarly, *Eu. maura* and *P. incarnata* had high *AI* values and achieved relatively high values of *s*. At the interaction zone of those assays, slightly macroscopically changed mycelium was observed, which could be the first sign of response isolate elimination. In contrast, *N. acerina* and *Diaporthe* sp. had relatively low, insignificant values of *AI* and consequently also lower values of *s*, suggesting the higher success of *E. parasitica* in competition with these isolates. Dual cultures of *E. parasitica* and *F. avenaceum* with poor inhibitory effect resulted in poor re-isolation of the response isolate. This is additional proof of the extreme complexity of interspecific interactions, which sometimes result in extraordinary and unexpected outcomes that are of great interest for further research.

The results presented in this study are the first insight into the complex interactions between the maple pathogen *E. parasitica* and some of the most frequently isolated fungal species from the wood of the dead branches of young sycamore maple.

Eutypella canker of maple caused by *E. parasitica* can cause significant economic and resource loss. Biological control of *E. parasitica* would therefore be an excellent way to avoid or at least minimize the negative effects of the pathogen. Preliminary tests of possible antagonism in the laboratory are crucial for achieving a basic understanding of interactions and searching for potential biological control agents. The significance of the obtained results *in vivo* remains to be investigated. The exact microclimate and the whole fungal community present in natural environment were not able to be fully mimicked in the laboratory conditions. Furthermore, studies of secondary metabolites and their role in antagonistic activity would be beneficial.

Finally, we would like to point out a drawback of this study in the light of the interpretation and generalization of the obtained results. Because of the low number of repetitions and only one

tested reference isolate of *E. parasitica*, the results should be treated with caution. Further tests are needed to verify the universality of the obtained findings, with wider range of response isolates of *E. parasitica* tested.

5. Conclusions

The interactions between the ten most frequently isolated species from the wood of the dead branches of *A. pseudoplatanus* and *E. parasitica* in dual cultures on PDA revealed two main types of competitive interactions: Deadlock, consisting of mutual inhibition after mycelial contact or at a distance, and replacement, resulting in the inhibition of *E. parasitica*, followed by partial overgrowth by the replacing fungus. The results of the antagonism index suggested that *Eutypa* sp., *Eu. maura*, *Neonectria* sp., and *P. incarnata* were the most competitive and had the highest inhibition of *E. parasitica* growth. These isolates are promising candidates for use as biocontrol agents but additional experiments with different *E. parasitica* isolates should be done for confirmation and clarification of our results. Re-isolations revealed that the mycelium of *E. parasitica* at the interaction zones remained mostly viable, except in dual cultures with *Eutypa* sp., *Neonectria* sp., and *F. avenaceum*. Based on the results of the antagonism index (*AI*) and re-isolation success (*s*) in our preliminary study, we can assume that *E. parasitica* is a weak competitor which invests less energy in direct mycelial competition. Nevertheless, additional tests and supplementary experiments with a wider range of *E. parasitica* isolates should be performed for a solid conclusion. The results provide a general insight into the antagonistic activities of the ten most frequently isolated fungi from the wood of the dead branches of *A. pseudoplatanus* against *E. parasitica*, as well as a basis for further research.

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References

1. Falconer, R.E.; Brown, J.L.; White, N.A.; Crawford, J.W. Modelling interactions in fungi. *J. R. Soc. Interface* **2008**, *5*, 603–615. [CrossRef]
2. Badalyan, S.M.; Innocenti, G.; Garibyan, N.G. Interactions between xylotrophic mushrooms and mycoparasitic fungi in dual-culture experiments. *Phytopathol. Mediterr.* **2004**, *43*, 44–48.
3. Yuen, T.K.; Hyde, K.D.; Hodgkiss, I.J. Interspecific Interactions among Tropical and Subtropical Freshwater Fungi. *Microb. Ecol.* **1999**, *37*, 257–262. [CrossRef]
4. Hanácková, Z.; Havrdová, L.; Černý, L.; Zahradník, D.; Koukol, O. Fungal Endophytes in Ash Shoots—Diversity and Inhibition of *Hymenoscyphus fraxineus*. *Balt. For.* **2017**, *23*, 89–106.
5. Bengtsson, S.B.K.; Barklund, P.; von Brömssen, C.; Stenlid, J. Seasonal Pattern of Lesion Development in Diseased *Fraxinus excelsior* Infected by *Hymenoscyphus pseudoalbidus*. *PLoS ONE* **2013**, *9*, e76429. [CrossRef] [PubMed]
6. Bakys, R.; Vasaitis, R.; Barklund, P.; Ihrmark, K.; Stenlid, J. Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathol.* **2009**, *58*, 284–292. [CrossRef]

7. Heilmann-Clausen, J.; Boddy, L. Inhibition and Stimulation Effects in Communities of Wood Decay Fungi: Exudates from Colonized Wood Influence Growth by Other Species. *Microb. Ecol.* **2005**, *49*, 399–406. [CrossRef]
8. Lacava, P.T.; Azavedo, J.L. Biological Control of Insect-Pest and Diseases by Endophytes. In *Advances in Endophytic Research*; Verma, V., Gange, A., Eds.; Springer: New Delhi, India, 2014; pp. 231–256.
9. Viterbo, A.; Inbar, J.; Hadar, Y.; Chet, I. Plant Disease Biocontrol and Induced Resistance via Fungal Mycoparasites. In *The Mycota IV. Environmental and Microbial Relationships*; Kubicek, C.P., Druzhinina, I.S., Eds.; Springer: Berlin/Heidelberg, Germany, 2007; pp. 127–146.
10. Rayner, A.D.M.; Griffith, G.S.; Wildman, H.G. Induction of metabolic and morphogenetic changes during mycelial interactions among species of higher fungi. *Elicitors Plant Microb. Metab.* **1994**, *22*, 389–394. [CrossRef]
11. Hietala, A.M.; Korhonen, K.; Sen, R. An Unknown Mechanism Promotes Somatic Incompatibility in *Ceratobasidium bicorne*. *Mycologia* **2003**, *95*, 239–250. [CrossRef] [PubMed]
12. Boddy, L. Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiol. Ecol.* **2000**, *31*, 185–194. [CrossRef]
13. Badalyan, S.M.; Innocenti, G.; Garibyan, N.G. Antagonistic activity of xylotrophic mushrooms against pathogenic fungi of cereals in dual culture. *Phytopathol. Mediterr.* **2002**, *41*, 200–225.
14. White, N.A.; Boddy, L. Extracellular enzyme localization during interspecific fungal interactions. *Fems Microbiol. Lett.* **1992**, *98*, 75–80. [CrossRef]
15. Evans, J.A.; Eyre, C.A.; Rogers, H.J.; Boddy, L.; Muller, C.T. Changes in volatile production during interspecific interactions between four wood rotting fungi growing in artificial media. *Fungal Ecol.* **2008**, *1*, 57–68. [CrossRef]
16. Jurc, D.; Ogris, N.; Slippers, B.; Stenlid, J. First report of Eutypella canker of *Acer pseudoplatanus* in Europe. *Plant Pathol.* **2006**, *55*, 577. [CrossRef]
17. Cech, T.L. Erstnachweis von *Eutypella parasitica* in Österreich. *Forstsch. Aktuell* **2007**, *40*, 10–13.
18. Ogris, N.; Diminić, D.; Piškur, B.; Kraigher, H. First report of *Eutypella parasitica* causing cankers on field maple (*Acer campestre*) in Croatia. *Plant Pathol.* **2008**, *57*, 785. [CrossRef]
19. Cech, T.L.; Schwanda, K.; Klosterhuber, M.; Straßer, L.; Kirisits, T. Eutypella canker of maple: First report from Germany and situation in Austria. *For. Pathol.* **2016**, *46*, 336–340. [CrossRef]
20. Jurc, D.; Ogris, N.; Piškur, B.; Csóka, G. First report of Eutypella canker of maple (*Eutypella parasitica*) in Hungary. *Plant Dis.* **2016**, *100*, 1241. [CrossRef]
21. Černý, K.; Hrabětová, M.; Svobodová, I.; Mrázková, M.; Kowalski, T. *Eutypella parasitica* naturalised in Bohemian and Polish Silesia. *For. Pathol.* **2017**. [CrossRef]
22. Bregant, C. *Eutypella Parasitica*: Primo Rinvenimento e Monitoraggio in Fruili Venezia Giulia. Master Thesis, Università degli Studi di Padova, Legnaro, Italy, 2018.
23. Jurc, D.; Piškur, B.; Ogris, N.; Brglez, A.; Linaldeddu, B.T.; Bregant, C.; Montecchio, L. First report of Eutypella canker caused by *Eutypella parasitica* on *Acer campestre* in Italy. *Plant Dis.* **2019**, *104*. [CrossRef]
24. Davidson, R.W.; Lorenz, R.C. Species of *Eutypella* and *Schizoxylon* associated with cankers of maple. *Phytopathology* **1938**, *28*, 733–745.
25. Ogris, N.; Jurc, D.; Jurc, M. Spread risk of Eutypella canker of maple in Europe. *Bull. Oepp/Eppo Bull.* **2006**, *36*, 475–485. [CrossRef]
26. French, W.J. Eutypella Canker on Species of *Acer* in New York State. Ph.D Thesis, State University College of Forestry at Syracuse University, Syracuse, NY, USA, 1967.
27. Brglez, A.; Piškur, B.; Ogris, N. *Eutypella parasitica* and other frequently isolated fungi in wood of dead branches of young sycamore maple (*Acer pseudoplatanus*) in Slovenia. *Forests* **2020**, *11*, 467. [CrossRef]
28. Tian, Y.; Tan, Y.; Liu, N.; Yan, Z.; Liao, Y.; Chen, J.; de Saeger, S.; Yang, H.; Zhang, Q.; Wu, A. Detoxification of Deoxynivalenol via Glycosylation Represents Novel Insights on Antagonistic Activities of *Trichoderma* when Confronted with *Fusarium graminearum*. *Forests* **2016**, *8*, 335. [CrossRef]
29. Trivedi, S.; Srivastava, M.; Pandey, S.; Kumar, V.; Singh, A. Antagonism and Hyphal Relationship between *Trichoderma* spp. and *Fusarium oxysporum-Rhizoctonia bataticola* causing Wilt Complex in Chickpea. *J. Pure Appl. Microbiol.* **2016**, *10*, 1591–1598.

30. López-González, R.C.; Gómez-Cornelio, S.; De la Rosa-García, S.C.; Garrido, E.; Oropeza-Mariano, O.; Heil, M.; Partida-Martínez, L.P. The age of lima bean leaves influences the richness and diversity of the endophytic fungal community, but not the antagonistic effect of endophytes against *Colletotrichum lindemuthianum*. *Fungal Ecol.* **2017**, *26*, 1–10. [CrossRef]
31. Morón-Ríos, A.; Gómez-Cornelio, S.; Ortega-Morales, B.O.; De la Rosa-García, S.; Partida-Martínez, L.P.; Quintana, P.; Alayón-Gamboa, A.; Cappello-García, S.; Gonzáles-Gómez, S. Interactions between abundant fungal species influence the fungal community assemblage on limestone. *PLoS ONE* **2017**, *12*, e0188443. [CrossRef]
32. Koukol, O.; Mrnka, L.; Kulhankova, A.; Vosatka, M. Competition of *Scleroconidioma sphagnicola* with fungi decomposing spruce litter needles. *Can. J. Bot.* **2006**, *84*, 469–476. [CrossRef]
33. Piškur, B.; Ogris, N.; Jurc, D. Species-specific primers for *Eutypella parasitica*, the causal agent of Eutypella canker of maple. *Plant Dis.* **2007**, *91*, 1579–1584. [CrossRef]
34. R Core Team. *R: A Language and Environment for Statistical Computing, 3.6.1*; R Foundation for Statistical Computing: Vienna, Austria, 2019; Available online: http://www.R-project.org (accessed on 17 September 2020).
35. Fox, J.; Weisberg, S. *An R Companion to Applied Regression*, 3rd ed.; Sage: Thousand Oaks, CA, USA, 2019. Available online: https://socialsciences.mcmaster.ca/jfox/Books/Companion/ (accessed on 17 September 2020).
36. Ogle, D.H.; Wheeler, P.; Dinno, A. *FSA: Fisheries Stock Analysis. R Package Version 0.8.25*. 2019. Available online: https://github.com/droglenc/FSA (accessed on 17 September 2020).
37. Mangiafico, S. *Rcompanion: Functions to Support Extension Education Program Evaluation. R Package Version 2.1.7*. 2019. Available online: https://cran.r-project.org/web/packages/rcompanion/rcompanion.pdf (accessed on 17 September 2020).
38. Kusari, P.; Kusari, S.; Spiteller, M.; Kayser, O. Endophytic fungi harbored in *Cannabis sativa* L.: Diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers.* **2013**, *60*, 137–151. [CrossRef]
39. Rezgui, A.; Vallance, J.; Ben Ghnaya-Chakroun, A.; Bruez, E.; Dridi, M.; Djidjou Demasse, R.; Rey, P.; Sadfi-Zouaoui, N. Study of *Lasidiodiplodia pseudotheobromae*, *Neofusicoccum parvum* and *Schizophyllum commune*, three pathogenic fungi associated with the Grapevine Trunk Diseases in the North of Tunisia. *Eur. J. Plant Pathol.* **2018**, *152*, 127–142. [CrossRef]
40. Prior, R.; Feife, A.; Begerow, D. Antagonistic activity of the phyllosphere fungal community. *Sydowia* **2017**, *69*, 183–198.
41. Asthana, A.; Shearer, C.A. Activity of *Pseudohalonectria* and *Ophioceras*. *Mycologia* **1990**, *82*, 554–561. [CrossRef]