Efektywność transferu wertykalnego grzybów endofitycznych

Ewa Węgrzyn

Rozprawa doktorska

Wydział Biologii

Uniwersytet im. Adama Mickiewicza w Poznaniu

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The efficiency of vertical transmission of endophytic fungi

Ewa Węgrzyn

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prof. dr hab. Marleny Lembicz

Zakład Botaniki Systematycznej i Środowiskowej
Wydział Biologii
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Spis treści

Streszczenie

Problem

Transfer wertykalny to mechanizm, który zapewnia rozprzestrzenianie się endofitów grzybowych z wykorzystaniem nasion rośliny w populacji, czyli tym samym zapewnia trwanie endofitów w populacji gatunku rośliny. Przez długi czas w badaniach zakładano, że wszystkie nasiona zarażonych roślin (a w efekcie przyszłe potomstwo gospodarza) zawierają w swoim wnętrzu strzępki endofitów grzybowych. Okazało się, że tak nie jest. Dotąd nie wiadomo jednak (1) czy jest to proces uniwersalny, (2) czy występuje u roślin dziko rosnących nie będących trawami oraz (3) jakie czynniki wpływają na efektywność transferu wertykalnego.

Hipotezy

Testowano trzy hipotezy dotyczące wpływu siedliska, stadium życiowego rośliny, gradientu wysokości na efektywność transferu wertykalnego endofitów grzybowych występujących w dziko rosnących roślinach. Hipoteza pierwsza - efektywność transferu wertykalnego i jego zróżnicowanie taksonomiczne i ilościowe jest istotnie większe w przypadku nasion pochodzących z roślin występujących na siedlisku antropogenicznym. Hipoteza druga - w trakcie przemieszczania się mykobiomu na drodze roślina dojrzała – nasiona – siewki transfer wertykalny jest mniej efektywny, tj. ma miejsce zmniejszanie się liczby taksonów tworzących mykobiom, a nasiona pochodzące bezpośrednio z owoców i osadzone jeszcze na roślinie matecznej będą charakteryzować się mniejszym zróżnicowaniem taksonomicznym mykobiomu w porównaniu do mykobiomu siewek, które powstały z nasion znajdujących się w glebie. Hipoteza trzecia - gradient wysokości istotnie wpływa na skład grzybów endofitycznych wraz z wysokością. Różnorodność endofitów grzybowych maleje wraz ze wzrostem wysokości, a transfer wertykalny endofitów od rośliny matecznej do nasion jest niedoskonały.

Metody badań

Przeprowadzono detekcję oraz identyfikację molekularną grzybów endofitycznych w nasionach/siewkach/liściach badanych roślin. Detekcję przeprowadzono na dwa sposoby: materiał był traktowany 4,5%-owym roztworem podchlorynu sodu przez 60

minut lub rośliny były sterylizowane w 75%-owym roztworze etanolu przez 30 sekund, 4,5%-owym roztworze podchlorynu sodu przez 3,5 minuty, i ponownie w roztworze etanolu przez 15 sekund. Badany materiał umieszczono na szalkach z pożywką PDA z chloramfenikolem. W celu identyfikacji molekularnej endofitów, izolaty grzybów pogrupowano w morfotypy w oparciu o cechy makroskopowe, takie jak wygląd i kolor grzybni. DNA zostało wyizolowane z mycelium za pomocą kitu Quick-DNA Fungal/Bacterial Miniprep Kit. Do identyfikacji molekularnej użyto starterów ITS1F oraz ITS4.

Wyniki

Stwierdzono, że efektywność transferu wertykalnego grzybów endofitycznych jest niedoskonała i istotnie zależna od rodzaju siedliska i stadium życiowego rośliny. Największe zróżnicowanie taksonomiczne grzybów endofitycznych stwierdzono w nasionach kosaćca występującego na siedliskach antropogenicznych. Stadium życiowe rośliny wpływa także na efektywność pionowego przemieszczania się mykobiomu, najwięcej endofitów grzybowych stwierdzono w liściach dojrzałych roślin, nie stwierdzono ich obecności w nasionach pochodzących bezpośrednio z zebranych owoców malin. Nie stwierdzono natomiast istotnego wpływu gradientu wysokości n.p.m na efektywność transferu wertykalnego. Rośliny niezależnie od wysokości mają podobny skład mykobiomu.

Abstract

The problem

Vertical transfer is a mechanism, which allows for the dispersal of fungal endophytes, by using plant seeds of a given population, thus ensuring the existence of the endophytes within a population of a plant species. For a long time many research studies assumed that all the seeds of the infected plants (thus, consequently, the future offspring of the host) contain the fungal endophyte hyphae inside. This turned out not to be the case. However, it is not yet known (1) whether it is a universal process, (2) whether it can be found in the wild plants, which are not grasses, and (3) what factors can impact the effectiveness of the vertical transfer.

The hypotheses

Three hypotheses regarding the influence of the habitat, the plant life stage and the height gradient on the effectiveness of the vertical transfer of the fungal endophytes found in wild plants were tested. The first hypothesis - the effectiveness of the vertical transfer, i.e. its taxonomic and quantitative diversity is significantly greater in the case of the seeds from the plants found in the anthropogenic habitat. The second hypothesis - during the travel of the mycobiome along the path: mature plant - seeds - seedlings, the vertical transfer is less effective, i.e. the number of the taxa forming the mycobiome decreases and the seeds, coming directly from the fruits and still embedded in the mother plant, will be characterized by a lesser taxonomic diversity of the mycobiome, compared to the mycobiome of the seedlings which developed from the seeds in the soil. The third hypothesis - with altitude, the height gradient substantially affects the composition of the endophytic fungi. The diversity of the fungal endophytes decreases as the altitude increases, and the vertical transfer of the endophytes from the mother plant to the seeds is imperfect.

Testing methods

A detection and molecular identification of the endophytic fungi within the seeds/seedlings/leaves of the studied plants was carried out. The detection was carried out in two ways: the material was treated with a 4.5% sodium hypochlorite solution for 60 minutes or: the plants were sterilized in 75% ethanol for 30 seconds, 4.5% sodium hypochlorite for 3.5 minutes and again in ethanol for 15 seconds. The tested material

was placed on the plates with the PDA medium containing chloramphenicol. For the molecular identification purposes of the endophytes, the fungal isolates were grouped into morphotypes, based on their macroscopic features such as mycelium shape and colour. DNA was isolated from the mycelium using the Quick-DNA Fungal/Bacterial Miniprep Kit. The primers ITS1F and ITS4 were used for the molecular identification.

The results

It was found that the efficiency of the vertical transfer of the endophytic fungi is imperfect and depends significantly on the type of the habitat and the life stage of the plant. The greatest taxonomic diversity of the endophytic fungi was observed in the seeds of the irises found in the anthropogenic habitats. The life stage of the plant also affects the efficiency of the vertical travel of the mycobiome; the greatest number of the fungal endophytes were found in the leaves of mature plants - their presence was not found in the seeds that came directly from the harvested raspberry fruits. However, there was no significant effect of the height gradient on the effectiveness of the vertical transfer. Plants, regardless of altitude, have a similar mycobiome composition.

1. Wprowadzenie

Stan wiedzy

Endofity grzybowe to endosymbionty występujące w prawie każdej roślinie (Petrini 1986). Wszystkie endofityczne grzyby, które zamieszkują tkanki roślinne i mają symbiotyczny związek z rośliną – gospodarzem, tworzą mikrobiom grzybowy (inaczej nazywany mykobiomem (Peay i in. 2016). Dzieli się on na mikrobiom podstawowy, zwykle zależny od genotypu rośliny, nabywany przez potomstwo od matki, oraz mikrobom satelitarny, nabywany w miejscu występowania (Compant i in. 2019). Mikrobiom grzybowy zmienia się w ciągu życia rośliny, a jego różnorodność taksonomiczna zależy od wielu czynników, w tym od gatunku rośliny, jej genotypu, stadium życiowego czy środowiska życia. Z kolei od składu taksonomicznego mikrobiomu grzybowego zależy kondycja rośliny, w tym takie jej cechy jak np. wielkość biomasy, wielkość nasion i ich zdolność do kiełkowania. Ponadto odpowiedni skład gatunkowy mikrobiomu grzybowego może zapewnić roślinie skuteczną jej obronę przed patogennymi grzybami, bakteriami, wirusami i roślinożercami (np. Sikora i in. 2008, Shearin i in. 2018). Efekty interakcji grzybów endofitycznych z roślinami są także zróżnicowane. Obserwujemy spektrum zmienności efektów fizjologicznych i ekologicznych od korzystnych do niekorzystnych, nawet w przypadku jednego taksonu grzyba (np. Saikkonen i in. 1998, Faeth i Fagan 2002).

Grzyby endofityczne rosną w nadziemnych częściach roślin, pomiędzy komórkami, nie wykazując żadnych widocznych oznak infekcji. Grzyb może więc zasiedlać roślinę w każdej jej fazie życia, tj. nasionach, siewkach, roślinie dojrzałej. Rozmnażają się najczęściej bezpłciowo, grzyb dostaje się do organów rozmnażania generatywnego rośliny i tym samym do zalążka, poprzez stopniowe przerastanie jej tkanek. Po zapłodnieniu zalążek rozwija się w nasiono zawierające w warstwie aleuronowej sieć strzępek grzyba (Philipson i in. 1986). Wraz z początkiem kiełkowania nasion, strzępki w merystemie wierzchołkowym pędu rozpoczynają kolonizację zawiązków liści i przemieszczają się pionowo w rozwijające się części nadziemne rośliny – gospodarza grzyba (Christensen i in. 2007). W ten sposób grzyby endofityczne przedostają się do kolejnych pokoleń rośliny. Jest to tzw. transfer wertykalny (np. Hodgson i in. 2014, Wiewióra i in. 2015, Abdelfattah i in. 2023). Taki wzrost strzępek,

który pozwala im na przemieszczanie się ku górze zasiedlanej rośliny to sposób na wykorzystanie nasion gospodarza do rozprzestrzeniania się.

Transfer wertykalny to mechanizm, który zapewnia rozprzestrzenianie się endofitów z wykorzystaniem nasion rośliny w populacji, czyli tym samym zapewnia trwanie endofitów w populacji gatunku rośliny (Schardl 1996).

Przez długi czas w badaniach zakładano, że wszystkie nasiona zarażonych roślin (a w efekcie przyszłe potomstwo gospodarza) zawierają w swoim wnętrzu strzępki endofitów grzybowych. Okazało się, że tak nie jest. W wielu relacjach endofit-gospodarz, notuje się tzw. transfer niedoskonały: niższą frekwencję grzyba niż spodziewana w pokoleniach potomnych roślin (np. Afkham i Rudgers 2008, Tintjer i in. 2008, Hodgson i in. 2014). Oznacza to, że endofit grzybowy nie zawsze przedostaje się do nasion gospodarza, którego zasiedla. Transfer niedoskonały endofitów zmniejsza częstotliwość ich występowania w populacjach gospodarzy. Brak obecności endofita we wszystkich pokoleniach rośliny i jego ‘ubywanie’ wywoła odmienne efekty ekologiczne interakcji endofit-roślina. Prawdopodobnie jest to też przyczyna odkrywania przez badaczy w naturze różnych, często sprzecznych skutków działania endofita w roślinie (np. Faeth i Sullivan 2003, Olejniczak i Lembicz 2007, Czarnoleski i in. 2013). Dotąd nie wyjaśniono przyczyn spadku efektywności transferu wertykalnego endofitów grzybowych.

W roślinie żywicielskiej istnieją cztery krytyczne etapy wzrostu dla transferu wertykalnego grzybów endofitycznych na przykładzie endofitów z rodzaju *Epichloë:* kiełkowanie (między nasionami a siewką), krzewienie (między siewką a wegetatywnym krzewem), kwitnienie (między wegetatywnym krzewem a rozwijającym się kwiatostanem) i rozrodczość (między rozwijającym się kwiatostanem a nasionami) (Clay i in. 1989, Clay i in. 2002, Gundel i in. 2008). Niepowodzenie pionowego transferu może wystąpić w dowolnym z tych czterech punktów cyklu transferu (Afkham i Rudgers 2008). Gundel i in. (2011) uznali, że niepowodzenie transferu endofitów można podzielić na dwie kategorie: transfer prezygotyczny i postzygotyczny. Niepowodzenie transferu prezygotycznego oznacza brak rozprzestrzeniania się grzyba z rośliny wegetatywnej na nasiona, podczas gdy niepowodzenie transferu postzygotycznego odnosi się do śmierci endofitów podczas przechowywania nasion (Gundel i in. 2008). Wpływ na efektywność transferu wertykalnego grzybów

endofitycznych może mieć zarówno gatunek żywiciela, jego genotyp, etap życia i wiek rośliny, siedlisko, w którym występuje, genotyp samego endofita, jak i warunki biotyczne i abiotyczne środowiska.

Czego nie wiemy?

Wiadomo, że transfer wertykalny grzybów endofitycznych jest procesem niedoskonałym, ale nie wiadomo, (1) czy jest to proces uniwersalny, (2) czy w ogóle występuje i jeśli tak, to jaki jest u gatunków roślin dziko występujących, istnieje bowiem duża luka w wiedzy o całościowym mikrobiomie grzybowym występującym w roślinach dzikich czy zagrożonych, dotychczas głównie badano transfer u roślin uprawnych, (3) nieznane są też czynniki wpływające na efektywność transferu wertykalnego.

2. Obiekty badań

Badania, których wyniki przedstawiono w niniejszej rozprawie doktorskiej, prowadzono na siedmiu gatunkach roślin dziko występujących w terenie: kosaćcu syberyjskim (*Iris sibirica* L.), malinie właściwej (*Rubus ideaus* L.) oraz w pięciu gatunkach roślin górskich takich jak urdzik karpacki (*Soldanella carpatica* Vierh.), podbiałek alpejski (*Homogyne alpina* L.), marchwica pospolita (*Mutellina purpurea* (Poir.) Reduron, Charpin & Pimenov), starzec górski (*Senecio subalpinus* W. D. J. Koch) oraz starzec gajowy (*Senecio nemorensis* L.).

3. Cel pracy i hipotezy badawcze

W badaniach testowano hipotezy dotyczące efektywności transferu wertykalnego, czyli przemieszczania się mykobiomu od rośliny matecznej do jej nasion w różnych gatunkach roślin dziko rosnących (Ramka 1). Uwzględnione zostały trzy czynniki, takie jak rodzaj siedliska, stadium życiowe rośliny, gradient wysokości, oczekując, że będą one miały istotny wpływ na efektywność transferu wertykalnego.

Ramka 1 – Pytania stawiane w poszczególnych badaniach

ROŚLINA	**PYTANIA**
Kosaciec syberyjski	Czy endofity grzybowe występują w nasionach ramet rośliny klonalnej *Iris sibirica?* Czy ich zróżnicowanie taksonomiczne i ilościowe różni się w zależności od siedliska rośliny klonalnej?
Malina właściwa	Czy skład taksonomiczny i ilość mikrobiomu grzybowego różni się w zależności od stadium życiowego w malinie?
Urdzik karpacki Podbiałek alpejski Marchwica pospolita Starzec górski Starzec gajowy	Czy różnorodność mykobiomu roślin i jego transfer do przyszłego potomstwa, czyli nasion zmienia się w gradiencie wysokości?

Przyjęto następujące hipotezy:

Hipoteza pierwsza

Efektywność transferu wertykalnego mykobiomu od rośliny matecznej do jej nasion tj. jego zróżnicowanie taksonomiczne i ilościowe jest istotnie większa w przypadku nasion pochodzących z roślin występujących na siedlisku antropogenicznym.

Hipoteza ta oparta jest na założeniu, że roślina rosnąca na obcym dla siebie siedlisku nabywa nowe grzyby endofityczne będące, tzw. taksonami statelitarnymi, wśród których są endofity wspomagające roślinę, ale także działające na nią negatywnie, najczęściej są to patogeny.

Hipoteza druga

W trakcie przemieszczania się mykobiomu na drodze roślina dojrzała – nasiona – siewki transfer wertykalny jest mniej efektywny, tj. ma miejsce zmniejszanie się liczby taksonów tworzących mykobiom. Nasiona pochodzące bezpośrednio z owoców i osadzone jeszcze na roślinie matecznej będą charakteryzować się mniejszym zróżnicowaniem taksonomicznym mykobiomu w porównaniu do mykobiomu siewek, które powstały z nasion znajdujących się w glebie.

Hipoteza ta oparta jest na założeniu, że siewki pochodzące z nasion z gleby, zdążą nabyć mikrobiom grzybowy satelitarny.

Hipoteza trzecia

Gradient wysokości istotnie wpływa na skład grzybów endofitycznych wraz z wysokością. Różnorodność endofitów grzybowych maleje wraz ze wzrostem wysokości, a transfer wertykalny endofitów od rośliny matecznej do nasion jest niedoskonały.

Hipoteza ta oparta jest na założeniu, że czynniki abiotyczne wpływają na skład mykobiomu. Wraz ze wzrostem wysokości zmienia się temperatura, wilgotność gleby, kompozycja gatunkowa, skraca się okres wegetacyjny, gleba jest płytsza i mniej żyzna. Może mieć to wpływ na skład mykobiomu w roślinie dorosłej. W efekcie także wpływać będzie na efektywność przemieszczania się endofitów do nasion.

4. Metodyka

W celu weryfikacji hipotez przeprowadzono detekcję oraz identyfikację molekularną grzybów endofitycznych w nasionach/siewkach/liściach badanych roślin. Poniższe metody były wspólne dla wszystkich trzech publikacji.

Detekcja endofitów w nasionach maliny właściwej oraz w nasionach roślin z Tatrzańskiego Parku Narodowego

Nasiona były sterylizowane w roztworze 4,5%-owym podchlorynu sodu przez 60 minut, następnie przepłukane w wodzie destylowanej i wyłożone na szalkę z pożywką PDA z chloramfenikolem (100 mg/L). Szalki były przechowywane w cieplarce w temperaturze 25ºC. Pojawiające się mycelium grzybowe było przenoszone na nowe szalki Petriego, aby oddzielić czyste kolonie. W celu potwierdzenia skuteczności procesu sterylizacji, 50 µl wody z płukania rozprowadzono na szalce z pożywką PDA z chloramfenikolem i inkubowano w temperaturze 25ºC przez 14 dni. Procedurę wykonywano w sterylnych warunkach, pod komorą z laminarnym przepływem powietrza.

Detekcja grzyba w nasionach kosaćca syberyjskiego oraz w siewkach i liściach maliny właściwej oraz w liściach roślin z Tatrzańskiego Parku Narodowego

Nasiona, liście siewek oraz liście dorosłych osobników były sterylizowane w 75%-owym roztworze etanolu przez 30 sekund, 4,5%-owym roztworze podchlorynu sodu przez 3,5 minuty, i ponownie w roztworze etanolu przez 15 sekund, następnie

przepłukane w wodzie destylowanej. Następnie liście siewek i dorosłych osobników zostały pocięte na małe ok. 1cm kawałki, które umieszczono na szalkach z pożywką PDA z chloramfenikolem (100 mg/L). Szalki przechowywano w cieplarce w temperaturze 25ºC. Z fragmentów zainfekowanych tkanek, w miejscu przecięcia pojawiało się mycelium grzyba, które było systematycznie przenoszone na nowe szalki Petriego, aby oddzielić czyste kolonie. W celu potwierdzenia skuteczności procesu sterylizacji, 50 µl wody z płukania rozprowadzono na szalce z pożywką PDA z chloramfenikolem i inkubowano w temperaturze 25ºC przez 14 dni. Procedurę wykonywano w sterylnych warunkach, pod komorą z laminarnym przepływem powietrza.

Identyfikacja molekularna

W celu identyfikacji molekularnej endofitów, izolaty grzybów pogrupowano w morfotypy w oparciu o cechy makroskopowe, takie jak wygląd i kolor grzybni. Następnie izolaty reprezentatywne dla każdego morfotypu analizowano metodami molekularnymi. DNA zostało wyizolowane z mycelium za pomocą kitu Quick-DNA Fungal/Bacterial Miniprep Kit według dołączonego protokołu. Do identyfikacji molekularnej grzybów wykorzystywane były sekwencje rDNA - fragmenty ITS (ang. „internal transcribed spacers"). Są to niekodujące sekwencje powtarzalne, zlokalizowane w rDNA pomiędzy wysoce konserwatywnymi regionami 18S, 5.8S a 28S. Odznaczają się one dużą zmiennością między poszczególnymi gatunkami grzybów. Ich długość waha się od 600 do 800 par zasad i stosunkowo łatwo je amplifikować z małych próbek DNA z użyciem specyficznych starterów (Goodwin i Zismann 2001).

Reakcję PCR przeprowadzono z użyciem pary starterów ITS1F i ITS4 dopasowanych do niekodujących sekwencji powtarzalnych ITS1 i ITS2 grzybów z gromady *Ascomycota*. Sekwencja nukleotydowa zastosowanych starterów wygląda następująco:

ITS-1F (F): CTTGGTCATTTAGAGGAAGTAA
ITS-4 (R): TCCTCCGCTTATTGATATGC

PCR przeprowadzono w objętości 25 µl zawierającej 2,5 µl 10X buforu DreamTaq, 2,5 µl 2,5 mM dNTP mix, 0,5 µl każdego startera w stężeniu 10 µM, 0,5 µl

polimerazy DreamTaq, 13,5 µl wody wolnej od nukleaz i 5 µl matrycy DNA. Amplifikację prowadzono w termocyklerze Applied Biosystems 2720 stosując program o następujących parametrach: 2 min w 95ºC; 35 cykli po 30 s w 95ºC, 30 s w 55ºC i 60 s w 72ºC; i na koniec 5 min w 72ºC. Sekwencjonowanie uzyskanego DNA grzybów wykonano w Wydziałowej Pracowni Technik Biologii Molekularnej Wydziału Biologii UAM. W tym celu produkty PCR oczyszczono przy użyciu fosfatazy alkalicznej i egzonukleazy I. Procedura ta miała na celu usunięcie niewykorzystanych starterów oraz nukleotydów. Uzyskane sekwencje analizowano za pomocą oprogramowania Chromas (www.technelysium.com.au), które następnie porównano z tymi opublikowanymi w bazach danych nukleotydów Europejskiego Laboratorium Biologii Molekularnej (EMBL) i w bazach danych NCBI (www.ncbi.nlm.nih.gov), stosując BLAST (Altschul i in. 1990). Pozytywna identyfikacja gatunków została potwierdzona, jeśli miały one ≥98% identyczności sekwencji regionu ITS z sekwencją referencyjną z baz danych (Canals i in., 2014). Uzyskane wyniki przesłano do GenBank.

5. Wyniki

Wyniki przeprowadzonych badań zostały przedstawione w trzech artykułach:

(1) Węgrzyn E, Dominiak-Świgoń M, Górzyńska K, Chmiel J, Świtalski K, Lembicz M (2020). Fungal microbiota in the seeds of the clonal plant *Iris sibirica* – a threatened species in Europe. *Sydowia,* 72: 107-114.

Badania wykazały, że nasiona *Iris sibirica* były zasiedlane przez endofity. Wśród wszystkich zebranych nasion 130 ze 180 nasion było zainfekowane grzybem (72,2%). Otrzymane izolaty pogrupowano w 12 morfotypów, które okazały się być różnymi gatunkami grzybów endofitycznych. Największe zróżnicowanie taksonomiczne grzybów endofitycznych stwierdzono w nasionach kosaćca występującym na siedliskach antropogenicznych.

(2) Wysoczański W, Węgrzyn E, Jaroszewicz B, Lembicz M (2021). Fungal microbiota in seeds, seedlings and mature plants of raspberry (*Rubus ideaus* L.). *European Journal of Plant Pathology*, 1-6.

Badania wykazały, że wielkość i zróżnicowanie taksonomiczne mikrobioty grzybowej maliny jest inne w nasionach, siewkach i roślinie dojrzałej. W 180 nasionach pochodzących ze świeżych owoców maliny nie stwierdzono obecności grzybów endofitycznych. Najwięcej grzybów (27 taksonów) stwierdzono w liściach dojrzałych roślin, natomiast 8 taksonów stwierdzono w siewkach. Łącznie w siewkach i liściach dojrzałych roślin stwierdzono 34 grzyby endofityczne.

(3) Wysoczański W, Węgrzyn E, Olejniczak P, Lembicz M (2023). Mycobiota diversity and its vertical transmission in plants along an elevation gradient in mountains. *Fungal Ecology,* 63: 101244.

Zidentyfikowano łącznie 16 taksonów w pięciu gatunkach roślin. Badania wykazały, że różnice w wysokości n. p. m. nie miały związku ze składem gatunkowym grzybów obserwowanych na danym stanowisku. Oznaczać to może również, że wykryte gatunki grzybów nie wykazują preferencji względem wysokości. Nie stwierdzono również istotnej korelacji pomiędzy składem gatunkowym grzybów obecnych w liściach i w nasionach.

Weryfikacja hipotez

Hipoteza 1.

Efektywność transferu wertykalnego mykobiomu od rośliny matecznej do jej nasion tj. jego zróżnicowanie taksonomiczne i ilościowe jest istotnie większa w przypadku nasion pochodzących z roślin występujących na siedlisku antropogenicznym.

Hipoteza została potwierdzona. Największe zróżnicowanie taksonomiczne grzybów endofitycznych stwierdzono w nasionach kosaćca występującym na siedliskach antropogenicznych. W większości były to gatunki patogenne, mocno powiązane z siedliskiem antropogenicznym.

Hipoteza 2.

W trakcie przemieszczania się mykobiomu na drodze roślina dojrzała – nasiona – siewki transfer wertykalny jest mniej efektywny, tj. ma miejsce zmniejszanie się liczby taksonów tworzących mykobiom. Nasiona pochodzące bezpośrednio z owoców i osadzone jeszcze na roślinie matecznej będą charakteryzować się mniejszym

zróżnicowaniem taksonomicznym mykobiomu w porównaniu do mykobiomu siewek, które powstały z nasion znajdujących się w glebie.
Hipoteza została potwierdzona. Liście dorosłych osobników miały bogaty mikrobiom grzybowy, a z kolei nasiona były pozbawione endofitów. Efektywność transferu wertykalnego endofitów z rośliny dojrzałej do jej nasion była niedoskonała. Mikrobiom grzybowy w siewkach mógł zostać nabyty w glebie.

Hipoteza 3.
Gradient wysokości istotnie wpływa na skład grzybów endofitycznych wraz z wysokością. Różnorodność endofitów grzybowych maleje wraz ze wzrostem wysokości, a transfer wertykalny endofitów od rośliny matecznej do nasion jest niedoskonały.
Pierwsza część hipotezy została odrzucona. Różnorodność endofitów grzybowych jest niezależna od wysokości. Z kolei druga część hipotezy została potwierdzona. Nie stwierdzono istotnej korelacji pomiędzy składem gatunkowym grzybów obecnych w liściach, co potwierdza niedoskonałość transferu wertykalnego od rośliny matki do nasion.

6. Podsumowanie i wnioski

Przeprowadzone badania wykazały, że efektywność transferu wertykalnego grzybów endofitycznych jest niedoskonała i zależna od wielu czynników. Okazało się, że rodzaj siedliska istotnie wpływa na efektywność transferu wertykalnego. W siedliskach antropogenicznych nasiona były prawdopodobnie początkowo zasiedlane przez endofity pochodzące z pierwotnego siedliska rośliny, a następnie przez nowe endofity pozyskane podczas kolonizacji nowego siedliska. Endofity zidentyfikowane w nasionach kosaćca syberyjskiego należą do grupy patogenów roślin. Dwa najczęstsze taksony to *Alternaria alternata* i *Botrytis cinerea*, które są uważane za patogeny roślin (np. Hatta i in. 2002, Staats i in. 2005). Oba gatunki dominują w siedliskach antropogenicznych.

Badania wykazały, że także stadium życiowe rośliny wpływa na efektywność transferu wertykalnego. Skład taksonomiczny endofitycznej mikroflory grzybów zmienia się przez całe życie rośliny. Szczególnie interesujący jest fakt, że w nasionach pobranych bezpośrednio z owoców nie było endofitów grzybowych. Vaughana i in. (1993) donoszą o obecności w dojrzałych owocach malin wielu substancji chemicznych

hamujących rozwój grzybów, w tym takich gatunków, jak np. *A. alternata*, *B. cinerea*. Substancje te mogą być przyczyną nieobecności endofitów grzybowych w nasionach zebranych z owoców maliny, co przerywa transfer werykalny endofitów z dojrzałych roślin na siewki kolejnego pokolenia poprzez nasiona. Powstaje zatem pytanie: w jaki sposób zidentyfikowane endofity grzybowe zainfekowały badane siewki malin? Infekcja nasion mogła mieć miejsce, gdy nasiona zostały zdeponowane w glebie.

Na efektywność transferu wertykalnego nie wpływają natomiast różnice w wysokości n. p. m. Otrzymane wyniki pokazują, że rośliny niezależnie od wysokości mają podobny skład mikrobiomu grzybowego. Wykryte gatunki grzybów nie wykazują preferencji względem wysokości i/lub nie sprzyjają roślinie w funkcjonowaniu na różnych wysokościach. Stwierdzona niedoskonałość transferu wertykalnego może wynikać z dwóch powodów: (1) rozpoznane taksony endofitów nie przemieszczają się często wertykalnie w ciele rośliny od liści do nasion i/lub (2) strategia życia endofitów w roślinach górskich jest inna niż w przypadku ich występowania w roślinach nie żyjących w gradiencie wysokości. W przypadku drugim, endofit mimo, że skolonizowałby nasiona, to w wyniku dominacji rozmnażania bezpłciowego rośliny i trudnych warunków do kiełkowania na wysokości, miałby małe szanse na rozprzestrzenienie się drogą transmisji wertykalnej. Może to być ewolucyjnie utrwalona strategia życia tych endofitów.

Przenoszenie pionowe przez nasiona żywiciela jest mechanizmem umożliwiającym rozprzestrzenianie się endofitów grzybowych w populacji roślin; zapewnia tym samym kontynuację endofitów w populacjach danego gatunku (Hodgson i in. 2014, Wiewióra i in. 2015). Otrzymane wyniki potwierdzają wcześniejsze badania, w których stwierdzono, że większość *„non-systemic”* grzybowych endofitów nie jest przenoszona przez nasiona (Márquez i in. 2012).

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8. Rozprawa doktorska

Publikacja nr 1

Autorzy: Węgrzyn E, Dominiak-Świgoń M, Górzyńska K, Chmiel J, Świtalski K, Lembicz M

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Fungal microbiota in the seeds of the clonal plant *Iris sibirica* – a threatened species in Europe

Ewa Węgrzyn[1], Martyna Dominiak-Świgoń[1], Karolina Górzyńska[1], Julian Chmiel[1], Konrad Świtalski[2] & Marlena Lembicz[1,*]

[1] Adam Mickiewicz University, Faculty of Biology, Department of Systematic and Environmental Botany, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland
[2] Agricultural University, Department of Forest Botany, Wojska Polskiego 28, 60-624 Poznań, Poland

*e-mail: lembicz@amu.edu.pl

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Endophytes, including fungal endophytes, are the first organisms that colonize young plants and are subsequently transmitted to mature plants and their seeds. In a clonal plant with several reproductive episodes, fungal endophytes are transmitted to offspring many times and accumulate in a plant body and population. In our study, we examined the presence of fungal endophytes and their taxonomic diversity in the seeds of the clonal plant *Iris sibirica*. In six studied localities of *I. sibirica* in Poland, which represented three habitat types, 12 taxa of fungal endophytes from Ascomycota were recorded. Two taxa were most abundantly represented: *Alternaria alternata* and *Botrytis cinerea*. The greatest taxonomical differentiation of endophytes was found in the seeds from anthropogenic habitats. The largest number of seeds with endophytes was noted in oak forest habitats (88.33 %), while the smallest number was noted in grassland habitats (55 %). This is the first report on the occurrence of endophytic fungi in seeds of a critically endangered species in Europe.

Key words: fungal endophytes, meta-holobiont, ramets, perennial plant.

A seed is a dormant plant in the embryonic form. It has all of the nutrients essential for the growth of a young organism – i.e., a seedling (Verma & White 2019). Seeds also harbour other organisms, including fungi (Barret et al. 2015). These organisms are an element of symbiotic microorganism communities, often called microbiota. A plant inhabited by symbionts is called a holobiont (Bulgarelli et al. 2013, Vandenkoornhuyse et al. 2015). The majority of studies shows that fungal endophytes positively affect seed germination and seedling growth and survival. It has been shown that seeds with fungal endophytes germinate faster than seeds without them (Schardl et al. 2004).

Seedlings originating from seeds containing some fungal endophytes, especially *Epichloë* fungi, are larger and cope better with such stresses as drought, pathogen presence or herbivore grazing (e.g., Schardl & Phillips 1997, Ormacini et al. 2000, Brem & Leuchtmann 2001, Ernst et al. 2003, Novas et al. 2003, Clarke et al. 2006, Sikora et al. 2008, Wäli et al. 2008, Swarthout et al. 2009, Tayung et al. 2012, Delgado-Sánchez et al. 2013, Shearin et al. 2018). Other plant-endophyte associations result in improved plant adaptation to salt and thermal stress, increased biomass, or resistance to pathogen damage (Redman et al. 2002, Arnold et al. 2003, Waller et al. 2005). Thus, there is a constant search for such fungal endophytes that affect plants positively and may improve their hosts' condition through artificial symbioses (Kauppinen et al. 2016, Bamisile et al. 2018). However, there are also some fungal endophytes that have a negative effect on their hosts. They not only reduce seed size and delay germination but also decrease plant lifespan (e.g., Petroski et al. 1990, Olejniczak & Lembicz 2007).

Endophytes, including endophytic fungi, are the first organisms that colonize young plants. They may be colonized by endophytes contained within the seed or may be newly infected by contagious fungi, which may later become seed-transmitted. These endophytes are transferred by vertical transmission to subsequent plant generations (Shade et al. 2017, Vujanovic & Germida 2017). A perennial plant usually has several reproductive episodes throughout its life history; thus, fungal endophytes may be transmitted to each pool of sexually produced plant offspring. If such transmission is successful, these offspring are inhabited by endophytes originating from their mother plant. In this study,

we focused on the presence of fungal endophytes in seeds produced by the offspring of a perennial clonal plant. These offspring, called daughter ramets, are genetic copies of a mother plant interconnected by rhizomes or stolons both with their mother plant and among themselves (Harper 1986, Vuorisalo & Tuomi 1986). Ramets may reproduce sexually, produce seeds and exchange resources and information (Stuefer et al. 1996, Oborny 2019). A recent study has shown that endosymbionts – e.g., bacteria and fungi – may also be transmitted within the ramet network (Vannier et al. 2016). In clonal plants, endosymbionts use two methods for migration throughout the plant body: vertical transmission by seeds (typical for non-clonal plants) and horizontal transmission through rhizomes and stolons (Vannier et al. 2018).

We attempted to answer two questions: (1) do fungal endophytes occur in the seeds produced by a clonal plant and, if this is the case, (2) does their taxonomic and quantitative diversity differ depending on the habitat of a clonal plant? The object of our study was Siberian iris, *Iris sibirica* L. In Europe, this species is generally classified as Near Threatened (NT) (Allen et al. 2014). However, in many countries, it is listed as Endangered (EN), e.g., in the Czech Republic (Proházka 2001), Slovakia (Eliáš et al. 2015), Hungary (Király 2007), Germany (Metzing et al. 2018), Belarus (Yakovleva 2015), Lithuania (Patalauskaite 2007) and Ukraine (Didukh 2009). In Poland, it is a strictly protected species ranked as Vulnerable (VU) throughout the whole country (Kaźmierczakowa et al. 2016). Despite its legal protection, the number of its few populations continues to decrease (Kostrakiewicz 2008). This species can be found in humid habitats, such as: thickets and their banks, and wet meadows, which due to their floristic composition can be included in the complex *Molinietum medioeuropaeum* Koch 1926 (Bróż & Jędrzejczyk 1994). This study is the first report on the occurrence of fungal endophytes in the seeds of *I. sibirica*.

Materials and methods

Study species

Siberian iris (*I. sibirica*) is a perennial monocotyledonous plant of the family *Iridaceae* (Goldblatt & Manning 2008). It is a clonal plant with a phalanx growth form (Fig. 1). As a result of clonal growth, ramets are produced. These ramets are interconnected by rhizomes with short internodes. The flowering shoots develop successively 2–4 actinomorphic, blue-purple flowers (Szöllősi et al. 2010, Szöllősi et al. 2011). The fruit is a capsule. Siberian iris is a Eurosiberian species with a compact geographic range.

Seed collection

Seeds of *I. sibirica* were collected from six localities, differentiated in terms of habitats (Tab. 1). For each clone at a given locality three flowering shoots with mature capsules were collected. Individual ramet clusters were distant from one another; thus, delimitation of a single ramet cluster was

Tab. 1. Localities, collection date, habitat type and site coordinates of Siberian Iris, *Iris sibirica*, checked for fungal endophytes.

Locality	Collection date	Habitat	Coordinates
Folusz	2018	grassland	52.981851 17.677889
Wielkopolska National Park	2018	grassland	52.251778 16.803446
Miąskowo	2018	oak forest	52.167557 17.421467
Miradz	2018	oak forest	52.549495 18.211673
Botanical Garden in Poznań	2018	anthropogenic habitat	52.419740 16.883692
Sołacz	2018	anthropogenic habitat	52.423038 16.901086

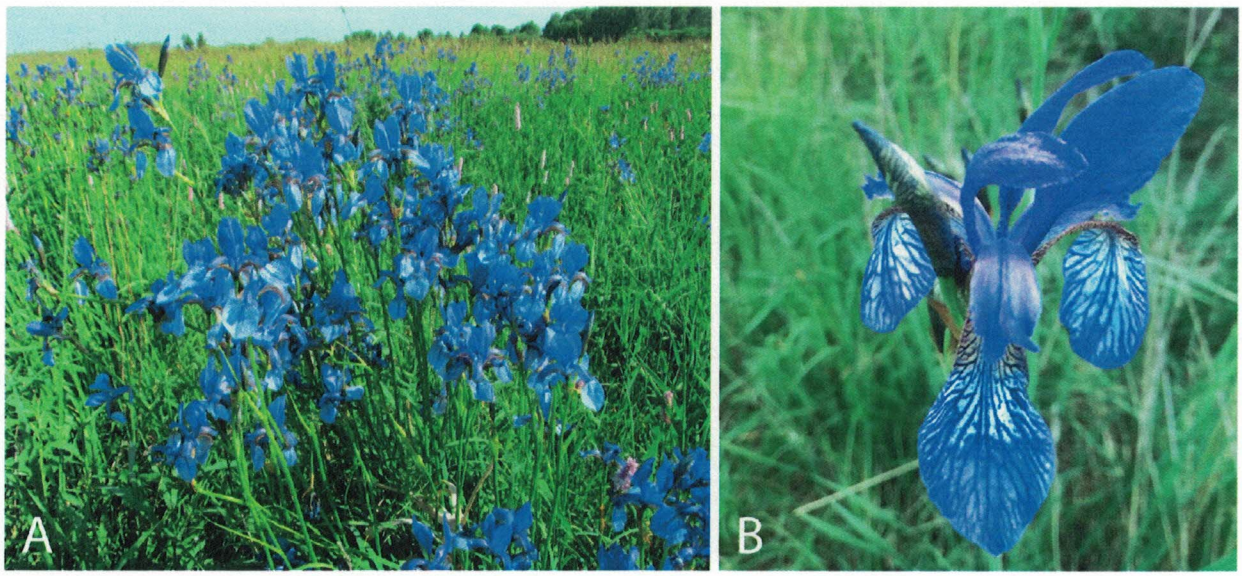


Fig. 1. Siberian iris, *Iris sibirica*, in a purple moor-grass meadow (*Molinietum caeruleae*) – one of the natural habitats of this species in Poland. This meadow is protected as a Natura 2000 habitat. **A.** Clusters of Siberian iris ramets, **B.** single blue-purple flower. Phot. K. Świtalski

simple. Ramet tops with fruits representing individual clusters were placed in separate paper bags. In the laboratory, 100 seeds were collected from each flowering shoot situated at a ramet top. Next, the selected seeds from every cluster occurring in a given locality were combined. Seeds were spilled on a paper sheet, and 30 seeds per locality were selected.

Fungal isolation

The presence of fungal endophytes was checked in 180 seeds from six localities (30 seeds per locality) (Tab. 1). The seeds from each locality were subjected to surface sterilization (75 % ethanol 30 s, 5 % NaOCl 3.5 min, 75 % ethanol 15 s, distilled water rinsing). Next, they were placed in Petri dishes (3 seeds per dish) with Potato Dextrose Agar (PDA) medium containing antibiotics (chloramphenicol, 100 mg/l). To confirm the efficiency of the sterilisation process, 50 µl of rinse water was spread onto Petri dishes with PDA. The plates were placed in an incubator at 25 °C and kept there for 60 days. They were observed every day, and emerging fungi were successively transferred to new, fresh plates. For the identification of endophytes, the fungal isolates were grouped into morphotypes based on macroscopic characteristics, such as the appearance and colour of the mycelium. Then, isolates representative of each morphotype were analysed using molecular genetic methods.

Molecular genetic identification

The DNA was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocol and stored at –20 °C. A pair of primers, ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990), was used to amplify the ribosomal cassette, which consisted of partial SSU, ITS1, 5.8S, ITS2 and partial LSU rDNA. The PCR was conducted in a 25 µl volume containing 2.5 µl of 10× buffer, 2.5 µl of 2.5 mM dNTP mix, 0.5 µl of each primer at 10 µM, 0.5 µl of DNA Taq polymerase, 13.5 µl of nuclease-free water and 5 µl of DNA template. Amplification was conducted in a thermocycler using a programme with the following parameters: 2 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C; and 5 min at 72 °C. The PCR products were purified using alkaline phosphatase and exonuclease I and directly cycle sequenced with ABI BigDye Terminator ver. 3.1 (Applied Biosystems, USA). The obtained sequences were edited using Chromas (www.technelysium.com.au) software and submitted to GenBank. Finally, the sequences were compared to those published in the European Molecular Biology Laboratory (EMBL) nucleotide databases and in the NCBI (www.ncbi.nlm.nih.gov) databases using BLAST (Altschul et al. 1990). A positive identification of a species was confirmed if they shared ≥98 % ITS region sequence identity with the reference sequence from the databases.

Tab. 2. Endophytic fungal species identified in *Iris sibirica* seeds with molecular method.

No.	Fungal taxa	BLAST match sequence			GenBank no.
		Accession no.	Similarity (%)	Coverage (%)	
1	*Alternaria alternata*	MH879772	99.8	99	MK562060
2	*Alternaria infectoria*	JX454532	98.7	99	MK562061
3	*Botrytis cinerea*	KF859918	100	96	MK562062
4	*Cladosporium herbarum*	KX611004	100	100	MK562063
5	*Diaporthe rudis*	MG281140	99.7	98	MK562064
6	*Epicoccum nigrum*	MF782764.1	100	99	MK562065
7	*Fusarium armeniacum*	MG978342	99.8	99	MK562066
8	*Fusarium avenaceum*	MF509747	100	100	MK562067
9	*Fusarium equiseti*	MG664737	99.6	98	MK562068
10	*Fusarium poae*	KC989103	98.5	99	MK562069
11	*Fusarium sporotrichioides*	KC989104	98.9	97	MK562070
12	*Nigrospora oryzae*	MH855300	99.6	100	MK562071

Analysis

The infection rates and incidence of individual endophyte taxa for all seeds and for seeds from each habitat were calculated. The infection rate was expressed as the percentage of seeds with fungal infection, whereas the incidence of a fungal taxon was expressed as the percentage of seeds infected with a given taxon.

Results

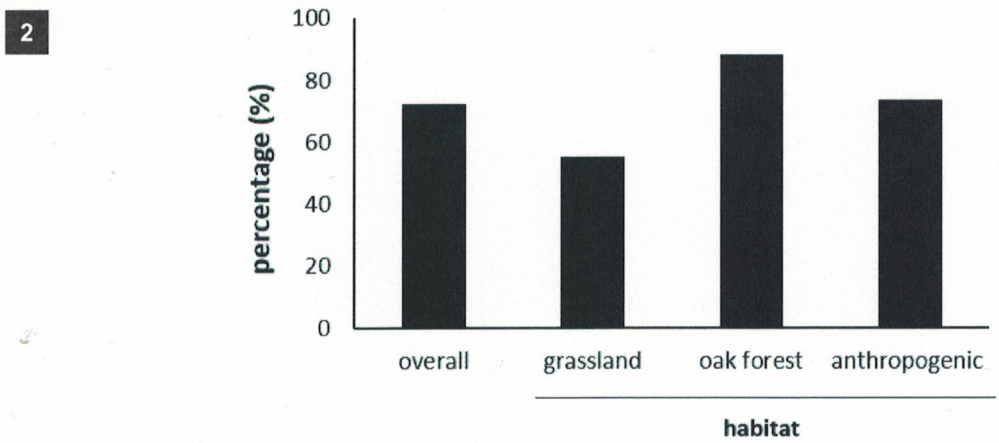
Among the seeds collected in the field, 130 of 180 seeds were infected with a fungus. All analysed seeds were infected with a single endophyte species. The obtained isolates were grouped into 12 morphotypes that turned out to be different species (Tab. 2).

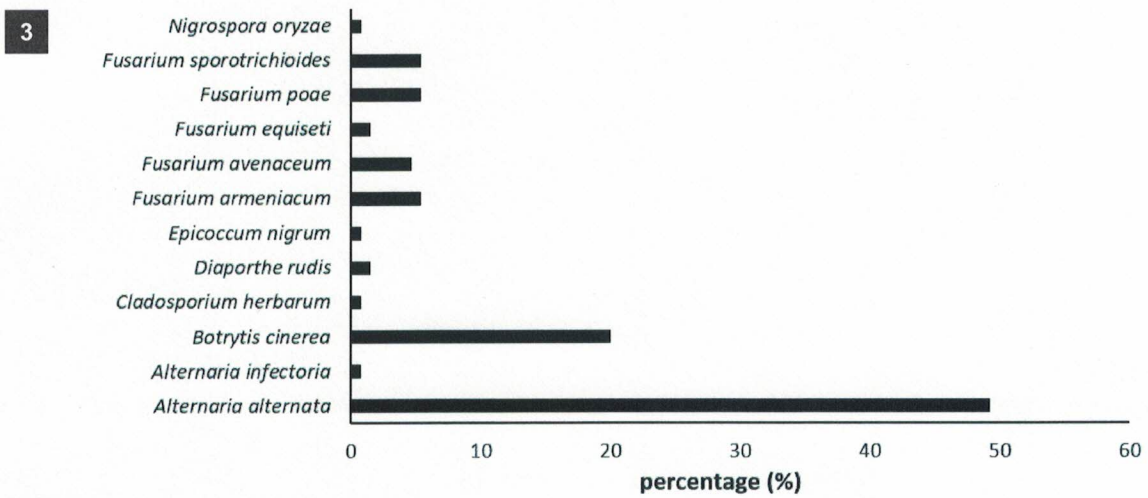
The overall percentage of seed infection among the seeds collected in the field was 72.2 %. The largest number of infected seeds was found in oak forest habitats –88.3 % – while the smallest number was found in grassland habitats –55 % (Fig. 2). The most abundant species was *Alternaria alternata*, which was present in 49.2 % of the infected seeds (Fig. 3). This fungus, like *Fusarium armeniacum*, occurred in all three types of habitat (Fig. 3). Other species occurred only in single localities, except for *Botrytis cinerea*, which was observed in the seeds from oak forest and anthropogenic habitats. The largest number of fungal species (8) was found in the seeds collected in anthropogenic habitats, while the smallest (4) was found in oak forest (Fig. 4).

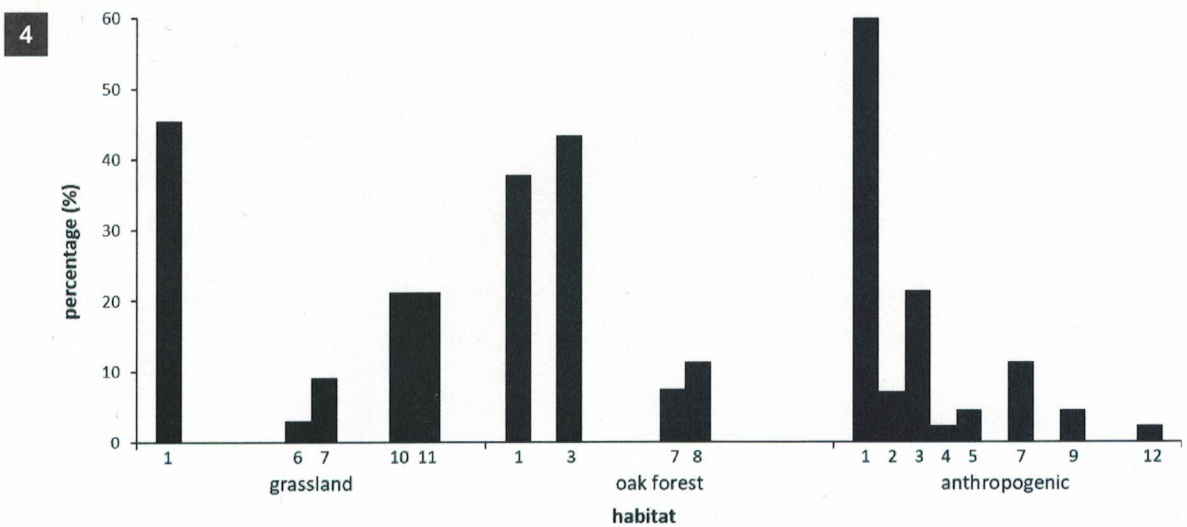
Analysis of the percentage of incidence of individual fungal species in the infected seeds depending on the type of habitat showed that *A. alternata* dominated in grassland and anthropogenic habitats, 45.5 and 65.9 %, respectively. In oak forest, the dominant species was *Botrytis cinerea* (43.4 %), followed by *Alternaria alternata* (37.7 %). *Botrytis cinerea* was also found in anthropogenic habitats, where it occurred in 21.4 % of seeds. Neither fungi nor bacteria were found in rinse water spread onto potato dextrose agar (PDA), which confirmed the efficiency of the sterilisation process and endophytic origin of the isolates.

Discussion

Plant seeds are colonized by fungal endophytes representing different taxonomic groups (e.g., Abe et al. 2015). Our study showed that the seeds of *I. sibirica* were inhabited by endophytic ascomycetes. The highest taxonomic diversity of fungal endophytes was found in the seeds from iris ramets occurring in anthropogenic habitats. In oak forest populations, the number of fungal taxa in the seeds was 50 % lower; however, there were more seeds with endophytes. Both habitats are typical for the studied species. In anthropogenic habitats, the seeds were probably initially inhabited by endophytes derived from the original habitat of the plant and afterwards, by new endophytes acquired during the colonization of a new habitat.







Figs. 2–4. *Iris sibirica* fungal endophytes. **2.** Percentage of seeds of *Iris sibirica* infected with endophytic fungi – overall and on each habitat type. **3.** Percentage of seeds with individual fungal endophyte species in *Iris sibirica*. **4.** Percentage of individual fungal endophyte species in *Iris sibirica* seeds in different habitats. 1 *Alternaria alternata*; 2 *A. infectoria*; 3 *Botrytis cinerea*; 4 *Cladosporium herbarum*; 5 *Diaporthe rudis*; 6 *Epicoccum nigrum*; 7 *Fusarium armenacium*; 8 *F. avenaceum*; 9 *F. equiseti*; 10 *F. poae*; 11 *F. sporotrichioides*; 12 *Nigrospora oryzae*.

Although Siberian iris colonized a new habitat type, it does not behave like an invasive plant, which, according to Enemy Release Hypothesis, ERH, loses its pathogens, and thus, the size of its populations may increase (Keane & Crawley 2002). We observed the reverse situation in the case of *I. sibirica* not only in anthropogenic habitats but also in oak forest populations. In these habitats, the largest number of seeds with fungal endophytes was found, and these endophytes showed the highest taxonomic diversity.

The endophytes identified in the seeds of Siberian iris belong to a group of plant pathogens. The two most common taxa were *Alternaria alternata* and *Botrytis cinerea*, which are considered plant pathogens (e.g., Hatta et al. 2002, Staats et al. 2005). Both species dominate in anthropogenic habitats. With 43 % *Botrytis cinerea* was the most abundant species in the seeds of iris plants growing in oak forest populations. *Botrytis cinerea* causes grey mould disease, which was found in over 200 plant species (Staats et al. 2005). Asymptomatic infection with *B. cinerea* was also observed; the fungus was usually present on the seed surface but sometimes also inside the seeds (e.g., Shipunov et al. 2008, Sowley et al. 2010). It can also occur asymptomatically in whole plants and cause symptoms only during the flowering stage (Barnes & Shaw 2003).

As many as five *Fusarium* species were identified in the Siberian iris seeds. These pathogens prefer moist habitats (Burgess 1981). Two species, *Fusarium poae* and *Fusarium sporotrichioides,* were the most abundant in the seeds of plants growing in a wet, periodically flooded grassland. *Fusarium* fungi may destroy developing seeds (e.g., Rosewich Gale et al. 2002, Ellis et al. 2012). Another commonly occurring plant pathogen, *Nigrospora oryzae*, was identified in a single anthropogenic habitat. This fungus was also observed on *Poa pratensis* leaves, and its presence manifested in black spots on the leaf surface (Zheng et al. 2012). In the case of the iris leaves, we did not observe this effect of *N. oryzae*.

In a single locality in the grassland habitat, we found some iris seeds infected with *Epicoccum nigrum*. This fungus may sometimes behave as a pathogen, but it can also be used to control other fungal pathogens, such as *Botrytis cinerea* (e.g., Biggs & Alm 1991, Peng & Sutton 1991, Larena 2004, Guerra-Guimarães et al. 2007). *Epicoccum nigrum* produces several well-known antibiotics, such as epicorazine A (Deffieux et al. 1976), epicorazine B (Baute et al. 1978) and flavipin (Bamford et al. 1961).

The absence of sexually produced individuals within a population may result in the accumulation of harmful mutations that may gradually or abruptly worsen the clone condition and, as a consequence, restrict its adaptive abilities to environmental changes caused, e.g., by drought (Dong et al. 2014). Such an accumulation may lead to the observed decrease in the size of this species' populations in Poland (Kaźmierczakowa et al. 2016). The variability in the ability of iris populations and individuals to cope with parasites, including fungal endophytes, also decreases. Because *I. sibirica* is a clonal plant, endophytes may be transmitted to seeds produced by ramets both vertically and horizontally, i.e., from ramet to ramet by rhizomes and stolons (Vannier et al. 2016). These two methods of the transmission of fungal endophytes (and other pathogens) may lead to their rapid accumulation in a clonal plant body.

Presently, we cannot prove and explain how the fungal endophytes infected the iris seeds. The concept formulated by Vannier et al. (2019), which considers clonal plants as meta-holobionts in which the microbiota of seeds and ramets are transmitted in different directions, is attractive, but it does not answer this question. Thus, the problem requires further studies.

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Oświadczenie

Informuję, że w pracy - Węgrzyn E., Dominiak-Świgoń M., Górzyńska K., Chmiel J., Świtalski K., Lembicz M. (2020). Fungal microbiota in the seeds of the clonal plant Iris sibirica – a threatened species in Europe. *Sydowia, DOI 10.12905/0380.sydowia72-2020-0107,* 72: 107-114, brałam udział we współtworzeniu koncepcji badań, identyfikacji molekularnej grzybów endofitycznych, przygotowaniu i wysłaniu sekwencji do GenBank, interpretacji uzyskanych wyników, pisaniu manuskryptu oraz w redakcyjnym przygotowaniu manuskryptu zgodnie z wymogami czasopisma.

Ewa Węgrzyn

(mgr Ewa Węgrzyn)

Poznań, 17.04.2023 r.

Oświadczenie

Informuję, że w pracy - Węgrzyn E., Dominiak-Świgoń M., Górzyńska K., Chmiel J., Świtalski K., Lembicz M. (2020). Fungal microbiota in the seeds of the clonal plant Iris sibirica – a threatened species in Europe. *Sydowia, DOI 10.12905/0380.sydowia72-2020-0107,* 72:107-114, brałam udział w prowadzeniu hodowli *in vitro* grzybów endofitycznych występujących w *Iris sibirica* oraz w przygotowaniu opisu hodowli w części metody badań.

Dominiak-Świgoń M.

(mgr Martyna Dominiak-Świgoń)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - *Węgrzyn E., Dominiak-Świgoń M., Górzyńska K., Chmiel J., Świtalski K., Lembicz M. 2020. Fungal microbiota in the seeds of the clonal plant Iris sibirica – a threatened species in Europe. Sydowia, DOI 10.12905/0380.sydowia72-2020-0107, pkt.40 72: 107-114* brałam udział w obróbce sekwencji wyizolowanych grzybów endofitycznych występujących w *Iris sibirica*.

K. Górzyńska

(dr Karolina Górzyńska)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Węgrzyn E., Dominiak-Świgoń M., Górzyńska K., Chmiel J., Świtalski K., Lembicz M. (2020). Fungal microbiota in the seeds of the clonal plant Iris sibirica – a threatened species in Europe. *Sydowia, DOI 10.12905/0380.sydowia72-2020-0107,* 72: 107-114, brałem udział w diagnozie taksonomicznej badanego gatunku – *Iris sibirica* i zbiorze materiału w terenie.

(dr hab. Julian Chmiel, prof. UAM)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Węgrzyn E., Dominiak-Świgoń M., Górzyńska K., Chmiel J., Świtalski K., Lembicz M. (2020). Fungal microbiota in the seeds of the clonal plant Iris sibirica – a threatened species in Europe. *Sydowia, DOI 10.12905/0380.sydowia72-2020-0107,* 72: 107-114, brałem udział w zbiorze materiału - liści i nasion *Iris sibirica* w terenie.

(dr Konrad Świtalski)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Węgrzyn E., Dominiak-Swigoń M., Górzyńska K., Chmiel J., Switalski K., Lembicz M. (2020). Fungal microbiota in the seeds of the clonal plant Iris sibirica – a threatened species in Europe. *Sydowia, DOI 10.12905/0380.sydowia72-2020-0107*, 72: 107-114, brałam udział we współtworzeniu koncepcji badań razem z doktorantką Panią mgr Ewą Węgrzyn oraz w czytaniu i w poprawianiu manuskryptu oraz wysyłaniu artykułu do czasopisma.

Mariana Lembicz

(prof. dr hab. Mariena Lembicz)

Publikacja nr 2

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Fungal microbiota in seeds, seedlings and mature plants of raspberry (*Rubus ideaus* L.)

Wojciech Wysoczański · Ewa Węgrzyn · Marlena Lembicz · Bogdan Jaroszewicz

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Abstract Presently, there is an intensive search for fungal endophytes to be used in agriculture for the protection and condition improvement of plants and in medicine. We screened for the presence of endophytes in raspberry, which occurs naturally in the Białowieża Forest. The fungal isolates representative of each morphotype were analysed using the molecular markers ITS1 and ITS2. In total, we found 34 taxa of endophytic fungi. The majority were potential pathogens. As many as 27 taxa were found in the leaves of mature plants. No fungi could be isolated from the surface sterilized seeds obtained from these plants. Seedlings were grown from the seeds deposited in the soil seed bank in the Białowieża Geobotanical Station of the University of Warsaw in Białowieża. 8 taxa of endophytic fungi were found in seedlings. It could be due to a possibility of seed infection with these endophytes in soil conditions.

Keywords Białowieża Forest · Fungal endophytes · Molecular detection · Raspberry · Vertical transfer

W. Wysoczański · E. Węgrzyn · M. Lembicz (✉)
Department of Systematic and Environmental Botany, Faculty of Biology, Adam Mickiewicz University, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland
e-mail: lembicz@amu.edu.pl

B. Jaroszewicz
Białowieża Geobotanical Station, Faculty of Biology, University of Warsaw, Sportowa 19, 17-230, Białowieża, Poland

Introduction

Fungal endophytes are endosymbionts that occur in almost every plant (Petrini, 1986). Their number and taxonomical diversity depend on many factors, including the species, genotype, developmental stage and living environment of the host plant (Cheplick & Faeth, 2009). Effects of interactions between plants and endophytic fungi are also diverse. There is a spectrum of physiological and ecological effects, from positive to adverse, even for the same fungal taxon (Saikkonen et al., 1998; Faeth & Fagan, 2002).

Currently, there is a search for fungal endosymbionts that could improve condition of plants through increasing their biomass and seed production and protect them against pathogenic parasites that generate losses in agriculture (e.g., Rodriguez et al., 2009; Turner et al., 2013). Such endosymbionts may be an alternative for chemical agents used against plant parasites. Endophytic bioinoculants have been already developed that increase the biomass of plants and protect them against pathogens (Kauppinen et al., 2016). Furthermore, there is a search for fungal endophytes that produce chemical substances useful in medicine (e.g., Strobel & Daisy, 2003; Egan et al., 2016; Pelo et al., 2020).

However, the application of symbiotically modified organisms in agriculture may have some downsides and should be treated with caution. Before an agricultural plant is subjected to inoculation, it must be ensured that the effects of such an interaction are safe in the long-term. From the evolutionary point of view, achieving such certainty is difficult. Symbiosis is the type of

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interaction in which the compatibility of both partners may change. The strains of endophytes we currently consider safe and introduce into plants may become pathogenic over time. We do not know the effects of mutation accumulation in the genetic material of these organisms. However, plants consumed by humans, such as crop plants and plants with confirmed medicinal properties, should be decidedly checked for endophyte presence.

The aim of this study was to check for the presence and taxonomical identification of fungal endophytes in raspberry (*R. idaeus* L.). We were interested to determine whether the number of species and taxonomical composition of fungal microbiota depend on the developmental stage of raspberry. Thus, we decided to conduct the endophyte detection not only in leaves but also in seeds originating from the same individuals. This is the first report of the occurrence of endophytic fungi in the different developmental stages of raspberry — in seeds and mature plants from forest and in seedlings obtained from seeds deposited in the soil seed bank. Raspberry is a fairly common plant in Poland that is cultivated as a fruit shrub. Poland is a leading producer of raspberry fruits both in Europe and in the world (Baranowska et al., 2015). Vegetative (leaves) and generative (seeds) organs of raspberry are widely used for medical and nutritional purposes and in cosmetology (Kalinowska et al., 2017).

Materials and methods

Material origin

Fungal microbiota of raspberry (*Rubus ideaus* L.) were studied in six localities in the Białowieża Forest (Fig. 1). The presence of fungi was checked in the juvenile stages of plants (seeds) and in the mature stage (leaves). Leaves and fruits with seeds were collected directly in the field. Seedlings were grown from the seeds deposited in the soil seed bank in the Białowieża Geobotanical Station of the University of Warsaw in Białowieża. Seedlings were grown directly from the soil collected in the forest. Seeds were not extracted from the soil sample but allowed to germinate in the greenhouse together with seedlings of all other species present there. All seedlings for analysis were collected at the same date, and their age was between 5 and 14 days. The collected leaves and fruit, as well as the grown seedlings were transported to the laboratory of the Department of Systematic and Environmental Botany of the Adam Mickiewicz University in Poznań. The material was healthy and prepared in the same year in 2019.

Cultivation and passage of fungi

Before the establishment of the in vitro culture of fungi, the plant material (seeds and both seedlings and mature plant leaves) was subjected to sterilization to exclude externally occurring fungi (Górzyńska et al., 2019). The presence of fungal endophytes was checked in 180 seeds (30 seeds per site), 24 seedlings and 60 leaves (10 leaves per site). This number of seedlings was successfully grown from seeds obtained from a seed bank. The seeds were subjected to surface sterilization with 4.5% NaOCl for 60 min, with distilled water for rinsing. The seedlings and leaf fragments were subjected also to surface sterilization (75% ethanol 30 s, 5% NaOCl 3.5 min, 75% ethanol 15 s, with distilled water for rinsing). Then, seedlings and leaves were cut into small pieces. Next, the seeds and plant fragments were placed in Petri dishes with Potato Dextrose Agar (PDA) medium containing antibiotics (chloramphenicol, 100 mg/L). In total, 18 dishes for seeds (10 seeds per dish), 24 for seedlings (1 seedling per dish) and 60 for mature plants (1 leaf per dish) were prepared. The plates were placed in dark in an incubator at 25 °C. They were observed every day, and emerging fungi were successively transplanted to new, fresh plates. For the identification of endophytes, the fungal isolates were grouped into morphotypes based on macroscopic characteristics, such as the appearance and colour of the mycelium. Then, isolates representative of each morphotype were analysed using molecular methods.

Molecular identification

The DNA was isolated using a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocol and was stored at −20 °C. A pair of primers, ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990), was used to amplify the ribosomal cassette, which consisted of SSU, ITS1, 5.8S, ITS2 and LSU rDNA. The PCR was conducted according to the protocol used in another research (Węgrzyn et al., 2020). The PCR products were purified using alkaline phosphatase and exonuclease I and directly cycle-sequenced with ABI BigDye Terminator ver. 3.1 (Applied Biosystems, USA). The obtained sequences were edited using Chromas (www.technelysium.com.au) software and were submitted to

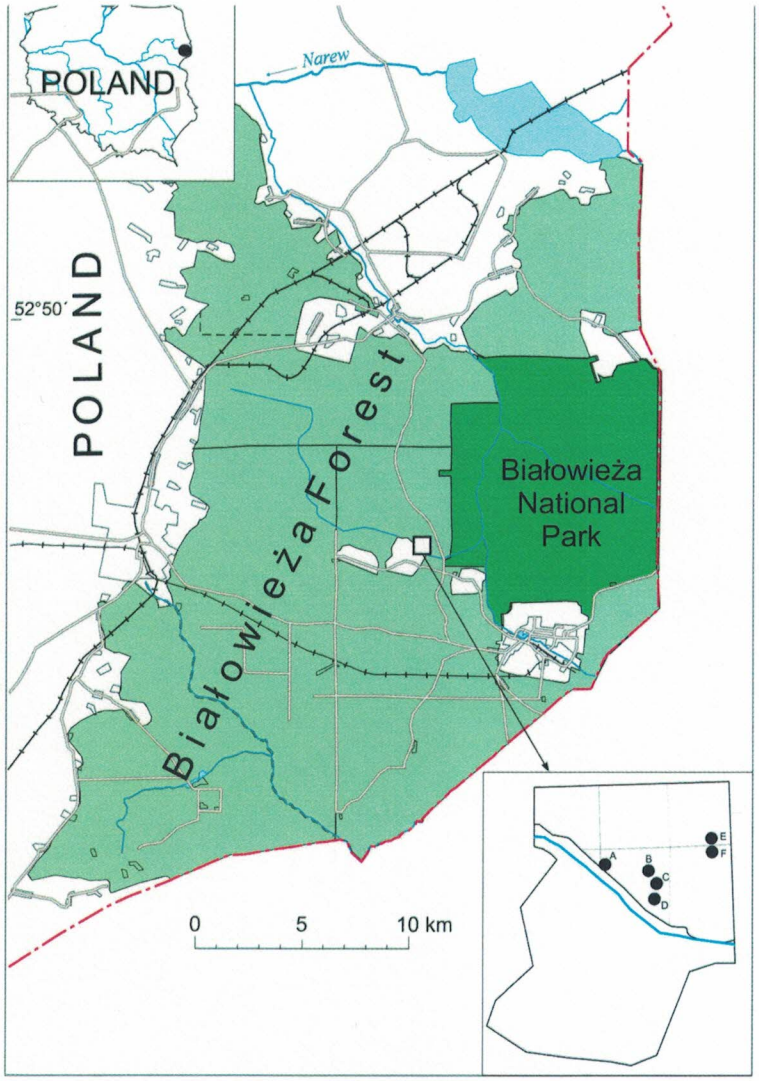

Fig. 1 Localities of material collection (leaves and fruits with seeds) of raspberry (*Rubus ideaus*) in the Białowieża Forest, Poland. Seedlings were raised in the experimental greenhouse of the Białowieża Geobotanical Station (University of Warsaw) from seeds contained in soil samples collected at sampling sites

GenBank (Table 1). Finally, the sequences were compared to those published in the European Molecular Biology Laboratory (EMBL) nucleotide databases and in the NCBI (www.ncbi.nlm.nih.gov) databases using BLAST (Altschul et al., 1990). A positive identification of a species was confirmed if ≥98% of the ITS region sequence identity was shared with the reference sequence from the databases.

Results and discussion

There were no endophytic fungi found in 180 seeds extracted from fresh fruits of raspberry (*Rubus ideaus*). The highest number of fungi (27 taxa) was identified in the leaves of mature plants, while 8 taxa were found in the seedlings (Table 1). In total, 34 endophytic fungi were recorded in the seedlings and leaves of mature plants. The only species that was present in both these developmental stages was pathogen species *Botrytis cinerea*. In both the mature plants and seedlings, there were also representatives of the genus *Penicillium*: in the former, *Penicillium chrysogenum* was identified, while *Penicillium cosmopolitanum* was found in the latter. In the mature plants, three species of *Alternaria* were identified, *A. alternata*, *Alternaria infectoria* and *A. tenuissima*, three species of *Colletotrichum*, and two each from the genera *Cladosporium* and *Epicoccum*. Other genera were represented by single species.

Table 1 Endophytic fungal species identified in *Rubus ideaus* seedlings and mature plants

No.	Fungal taxa	seedlings	mature plants	BLAST match sequence			GenBank no.
				Accession no.	Similarity (%)	Coverage (%)	
1	*Acremonium sclerotigenum*	–	+	MH859618	99.8	99	MT573463
2	*A. alternata*	–	+	KT345696	100	100	MT573464
3	*Alternaria infectoria*	–	+	MK461063	99.50	100	MT573465
4	*A. tenuissima*	–	+	MK675103	100	99	MT573466
5	*Apiotrichum porosum*	+	–	KY558352	99.1	100	MT573467
6	*Aureobasidium pullulans*	–	+	EF690466	100	99	MT573468
7	*Bjerkandera adusta*	–	+	MH857085	99.8	99	MT573469
8	*Botrytis cinerea*	+	+	KU992700	99.50	100	MT573470
9	*Cladosporium allicinum*	–	+	MH857286	100	100	MT573471
10	*Cladosporium cladosporioides*	–	+	MH863979	99.6	100	MT573472
11	*Colletotrichum dematium*	–	+	MG978337	100	100	MT573473
12	*Colletotrichum salicis*	–	+	MT068551	99	99	MT573474
13	*Colletotrichum truncatum*	–	+	MH248046	100	99	MT573475
14	*Coniochaeta velutina*	–	+	MN341294	99.3	99	MT573476
15	*Cytospora cedri*	–	+	MN764316	98.3	94	MT573477
16	*Diaporthe eres*	–	+	MK352454	99.8	99	MT573478
17	*Epicoccum layuense*	–	+	MN396392	100	99	MT573479
18	*Epicoccum nigrum*	–	+	MF509753	99.6	100	MT573480
19	*Hypoxylon fragiforme*	–	+	MG098276	99.8	100	MT573481
20	*Ilyonectria crassa*	+	–	MT294410	99.6	100	MT573482
21	*Jackrogersella multiformis*	–	+	MK351664	99.8	98	MT573483
22	*Melanconis stilbostoma*	–	+	AY577811	99.7	99	MT573484
23	*Mucor hiemalis*	+	–	MF615076	99.7	99	MT573485
24	*Nemania serpens*	–	+	EF155504	99.8	99	MT573486
25	*Paraphaeosphaeria neglecta*	–	+	MG098298	99.5	97	MT573487
26	*Penicillium chrysogenum*	–	+	KT963794	100	99	MT573488
27	*Penicillium cosmopolitanum*	+	–	JN617682	99.8	100	MT573489
28	*Preussia minima*	–	+	KU713051	99.5	99	MT573490
29	*Pseudogymnoascus pannorum*	+	–	MH864434	98.6	99	MT573491
30	*Schizophyllum commune*	–	+	KP326577	99.5	99	MT573492
31	*Umbelopsis isabellina*	+	–	MF417265	98.7	98	MT573493
32	*U. maydis*	–	+	MH855355	100	99	MT573494
33	*Varicosporium elodeae*	+	–	JX981463	99.3	100	MT573495
34	*Xenodidymella applanata*	–	+	MH855770	100	99	MT573496

A plant may be inhabited by a consortium of fungal microbiota (Shade et al., 2017; Vannier et al., 2018). We revealed that the number of species and taxonomical diversity of such microbiota in raspberry are different in seeds, seedlings, and mature plants. The highest number of fungi – 27 taxa of 34 in total, was present in the mature plants, followed by seedlings (8 taxa), while the seeds were free of endophytes. It is possible that the low number of identified fungi in seedlings is caused by the fact that detection was performed on a smaller sample. The identified taxa represent mainly non-systemic fungi of Ascomycota.

Non-systemic endophytes occur in different groups of plants (e.g., Ruotsalainen et al., 2002; Rodriguez et al.,

2009; Loro et al., 2012). Their activity in plants is poorly known, particularly their effect on plant defence mechanisms (Saikkonen et al., 1998; Rodriguez & Redman, 2008; Cook et al., 2009). The majority of endophytes identified by us in raspberry are regarded as plant pathogens, particularly such taxa as *Alternaria* spp. or *Botrytis cinerea*. *A. alternata* is mainly known as a parasite. It contains seven pathogenic variants (pathotypes) that produce the host-specific toxins and, as a result, may cause disease in different hosts (Hatta et al., 2002). *A. infectoria* had been already previously regarded as an endophyte (Martin & Dombrowski, 2015). This species also occurs in the tissues of apple tree buds. During flower and fruit development, it metamorphoses into a pathogenic form that causes core rot (Serdani et al., 1998). Moslemi et al. (2017) found a new pathogen in *T. cinerariifolium* that causes double-sided leaf necrosis and was identified as *A. infectoria*. *B. cinerea* promotes grey mould disease and may attack over 200 species of host plants (Staats et al., 2005). Species of the genus *Colletotrichum* are also plant pathogens that cause anthracnose diseases (Freeman et al., 1998).

The activity of fungal microbiota in plants is an effect of interactions between individual organisms. It is possible to evaluate the activity of a single fungal taxon in laboratory conditions in plants experimentally devoid of their microbiota. However, the activity of the same fungus involved in interactions with other fungi that inhabit a plant in natural conditions may be different. A single taxon may have a negative effect on its host, but in the presence of another taxon, this effect may be reduced or eliminated. Some endophytes may also stimulate other endophytes to produce chemical compounds that affect the plant defence system (e.g., Markert et al., 2008).

We found that the taxonomical composition of endophytic fungal microbiota changes throughout the plant's life. Some taxa, present in raspberry in the seedling stage (e.g., *Apiotrichum porosum* or *Mucor hiemalis*), are absent from the leaves of mature plants and vice versa. However, the mature plants were not cultivated from the cohort of analysed seedlings, thus, the observed differences may be effect of high spatial diversity of soil fungal communities. Particularly interesting is the fact that there were no fungal endophytes in the seeds sampled directly from fruits of these mature plants. Vaughan et al. (1993) reported the presence of many chemical substances in mature raspberry fruits that inhibit the development of fungi, including such species as *A. alternata*, *B. cinerea* and *Coletotrichum gloeosporioides*. These substances may be the cause of fungal endophyte absence from the seeds collected from raspberry fruits, which breaks vertical transmission of endophytes from mature plants to seedlings of the next generation via seeds. Vertical transmission through host seeds is a mechanism that enables fungal spread within the plant population; thus, it ensures the continuance of endophytes in populations of a given species (Hodgson et al., 2014; Wiewióra et al., 2015). Our results confirm previous studies reporting that the majority of non-systemic fungal endophytes are not transmitted through seeds (Márquez et al., 2012). Thus, the question arises: how did the identified fungal endophytes infect the studied raspberry seedlings? We presume that seed infection took place when the seeds were deposited in the soil. We think this is the case because in the analysis of fungal microbiota, we used seedlings that originated from the seeds deposited in the soil seed bank. We will test this hypothesis in our future research.

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Declarations

Informed consent Not applicable.

Human participants and/or animals Not applicable.

Conflict of interest All authors declare that they have no conflict of interest.

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Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Wysoczański W., Węgrzyn E., Jaroszewicz B., Lembicz M. (2021). Fungal microbiota in seeds, seedlings and mature plants of raspberry (*Rubus ideaus* L.). *European Journal of Plant Pathology*, 1-6, przygotowałem hodowle *in vitro* grzybów endofitycznych, pomagałem w identyfikacji molekularnej grzybów endofitycznych, przygotowałem sekwencje grzybowe do GenBank oraz brałem udział w dyskusji uzyskanych wyników.

Wysoczański

(mgr Wojciech Wysoczański)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Wysoczański W., Węgrzyn E., Jaroszewicz B., Lembicz M. (2021). Fungal microbiota in seeds, seedlings and mature plants of raspberry (*Rubus ideaus* L.). *European Journal of Plant Pathology*, 1-6, brałam udział we współtworzeniu koncepcji badań, identyfikacji molekularnej grzybów endofitycznych, wysłaniu sekwencji do GenBank, interpretacji uzyskanych wyników, pisaniu manuskryptu oraz w redakcyjnym przygotowaniu manuskryptu zgodnie z wymogami czasopisma.

Ewa Węgrzyn

(mgr Ewa Węgrzyn)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Wysoczański W., Węgrzyn E., Jaroszewicz B., Lembicz M. (2021). Fungal microbiota in seeds, seedlings and mature plants of raspberry (*Rubus ideaus* L.). *European Journal of Plant Pathology*, 1-6, brałam udział we współtworzeniu koncepcji badań razem z doktorantką Panią mgr Ewą Węgrzyn, w interpretacji wyników, pisaniu dyskusji oraz wysyłaniu artykułu do czasopisma.

Marlena Lembicz

(prof. dr hab. Marlena Lembicz)

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Oświadczenie

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Mycobiota diversity and its vertical transmission in plants along an elevation gradient in mountains

Wojciech Wysoczański [a], Ewa Węgrzyn [a,*], Paweł Olejniczak [b], Marlena Lembicz [a]

[a] Faculty of Biology, Adam Mickiewicz University, Uniwersytetu Poznańskiego 6, 61-614, Poznań, Poland
[b] Institute of Nature Conservation, Polish Academy of Sciences, al. Adama Mickiewicza 33, 31-120, Kraków, Poland

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ABSTRACT

Plants are colonized by fungal endophytes. In this study we tested the hypothesis that endophyte communities in mountain plants changes along the elevation gradient. We identified fungal endophytes in aboveground parts and seeds of five plant species at altitudes of 1000–1750 m in the Tatra National Park. Endophytes isolated from them were grouped into morphotypes on the basis of macroscopic features, such as mycelium shape and colour. Isolates representing individual morphotypes were identified using molecular markers ITS1 and ITS2. When comparing species composition, we used Bray-Curtis distance matrices, calculated on the basis of frequency of the given fungal species. We identified 16 species of fungal endophytes. Five taxa were absent from seeds in spite of their occurrence in mother plant leaves. Differences in altitude were not significantly correlated with fungal species composition observed at a given sampling site. There was also no significant correlation between the species composition of leaf and seed mycobiota. This suggests imperfect vertical transmission in the studied plant species.

1. Introduction

A consortium of endophytic fungi is present in nearly every plant. Most taxa are Ascomycota and Basidiomycota (Fierer et al., 2009; Toju et al., 2013). Many experiments show that endophytes stimulate plant growth, as a result of increased access to macro- and micronutrients (e.g., Rana et al., 2020). Fungal symbionts can transport mineral nutrients to plants (Card et al., 2016; Baron et al., 2018), plant adaptation to environmental stress improves, as does resistance to herbivore grazing and pathogens causing various diseases. Moreover, the symbiosis between plants and fungal endophytes effectively improves soil quality and nutrient cycling (Hassan 2017). Plants provide fungal endophytes with shelter and resources necessary for survival, while new metabolic pathways are initiated, which lead to adaptation of the endosymbionts and facilitate their spread by way of so-called vertical transmission (e.g., Hassani et al., 2018).

Vertical transmission of endophytes occurs by hyphal growth into flowers. Next, fungal hyphae enter the ovule, by gradual penetration of its tissues. After fertilization, the ovule develops into a seed, containing a network of fungal hyphae in the aleurone layer (Philipson and Christey, 1986). At the beginning of seed germination, hyphae in the apical meristem of the shoot start to colonize leaf primordia and move vertically to the developing aboveground parts of the plant host (Christensen et al., 2008).

The composition of plant endophyte communities varies between individuals of the same species in the same area, and can change during a plants lifetime (Jousset et al., 2017; Toju et al., 2018; Wysoczański et al., 2021). Altitudinal gradient may be one of the factors that affects endophyte diversity in the plant. Increasing altitude is linked with changes in temperature, soil moisture content, species composition, duration of the growing season, while the soil is shallower and less fertile, all of which can affect the endophyte species composition in adult plants. As a result, this can also influence the diversity in seeds and the potential offspring – seedlings. So far, only a few publications describe the endophyte communities of mountain plants (e.g., Zubek et al., 2009; Li et al., 2012; Kang et al., 2021), and there are no reports on endophyte transmission from the mother plant to seeds along the altitudinal gradient. However, it is known that not all seeds of infected plants (and, consequently, the future offspring of the host plant – seedlings) contain hyphae of fungal endophytes (Afkhami and Rudgers 2008).

In this study, we identified fungal endophytes in mountain plants of

* Corresponding author.
E-mail address: e.wegrzyn@amu.edu.pl (E. Węgrzyn).

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the same species growing at various altitudes. We expected changes in taxonomic composition of endophytic fungi with the elevation gradient, both in the mother plants and in their offspring – seeds.

2. Methods

2.1. Sampling

Plant material was composed of leaves and seeds of 5 plant species in the Tatra National Park, collected in various vegetation zones (1000–1750 m a.s.l.). They included Carpathian snowbell (*Soldanella carpatica*), alpine coltsfoot (*Homogyne alpina*), pink alpine lovage (*Mutellina purpurea*), subalpine groundsel (*Senecio subalpinus*), and a Eurasian groundsel (*Senecio nemorensis*). For each species, seeds and leaves were collected from 10 individuals at each sampling site where it was present. For each individual, we examined 3 leaves and 10 seeds (Table 1, in total 390 leaves and 1300 seeds). The collected material was placed in plastic self-seal bags and transported to the laboratory of the Department of Systematic and Environmental Botany.

2.2. Fungal culture

Seeds and leaves were surface-sterilized in a laminar air flow cabinet, before placing on a medium for detection of endophytes. Seeds were first rinsed with distilled water, next sterilized with 4.5% sodium hypochlorite for 1 h, and then rinsed in sterile water. On each Petri dish, 10 seeds were placed, so the total number of Petri dishes was 130. The leaves were surface-sterilized in 75% ethanol (30 s), 4.5% sodium hypochlorite (3 min 30 s), 75% ethanol (15 s), and then rinsed in sterile water. After sterilization, the leaves were cut into fragments and placed on Petri dishes with PDA medium and chloramphenicol (an antibiotic). Three leaves were placed on each of 130 dishes. The dishes were protected with a thin plastic film, and kept in a growth chamber at 25 °C. After about 2 weeks, the cultured mycelium was transferred to new Petri dishes, to separate pure colonies. To identify endophytes, the fungal isolates were grouped into morphotypes based on macroscopic characteristics, such as the appearance and colour of the mycelium. Then, isolates representative of each morphotype were analysed using molecular methods. To confirm the efficiency of the sterilization process, 50 μl of rinse water (for seeds and leaves) was spread onto potato dextrose agar (PDA) and incubated at room temperature for 14 days.

2.3. Molecular identification

The DNA was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA), according to the manufacturer's protocol, and stored at −20 °C. A pair of primers, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al., 1990), was used to amplify the ribosomal cassette, which consisted of SSU, ITS1, 5.8S, ITS2, and LSU rDNA. The PCR was conducted in a 25 μl volume containing 2.5 μl of 10X buffer, 2.5 μl of 2.5 mM dNTP mix, 0.5 μl of each primer at 10 μM, 0.5 μl of DNA Taq polymerase, 13.5 μl of nuclease-free water, and 5 μl of DNA template. Amplification was conducted in a thermocycler using a programme with the following parameters: 2 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C; and finally 5 min at 72 °C. The PCR products were purified using alkaline phosphatase and exonuclease I, and directly cycle-sequenced with ABI BigDye Terminator ver. 3.1 (Applied Biosystems, USA). The sequences obtained were edited using Chromas (www.technelysium.com.au) software and submitted to the GenBank. Finally, the sequences were compared to those published in the European Molecular Biology Laboratory (EMBL) nucleotide databases and in the NCBI (www.ncbi.nlm.nih.gov) databases, using BLAST (Altschul et al., 1990). A positive identification of a species was confirmed if they shared ≥98% ITS region sequence identity with the reference sequence from the databases (Canals et al., 2014).

2.4. Statistical analysis

The relationship between endophyte community composition and elevation was compared using relevant distance matrices. For each fungal species its relative abundance was assessed: the total number of occurrences was divided by the number of localities at which at least one occurrence of the species was detected. The values obtained were assigned to given localities to quantify the species prevalence at each elevation; zero was assigned when the species was not present. A 5 × 16 table (5 localities x 16 fungal species abundances) was used to calculate Bray-Curtis distances between the localities. Additionally, a Bray-Curtis distance matrix was created on the basis of presence (1) or absence (0) of all the fungal species. These 2 distance matrices were then correlated, with the use of the Mantel test, to the matrix containing Euclidean distances between elevations of the localities.

Analogous fungal abundance estimates were used to analyse the correlation between the endophyte community composition of leaves and seeds of host plants. In this case, each plant species within each locality was treated separately, generating two 12-row matrices with 16 species abundance variables for leaf and seed microbiota. For both matrices, Bray-Curtis distances were calculated and the significance of their correlation was tested using the Mantel test. To complement the analyses, an equivalent Mantel test based on the presence or absence of fungal species in seeds and leaves was performed. All calculations and analyses were performed in R (version 4.1.3, package *vegan*).

3. Results

In total, we identified 16 taxa of fungal endophytes in 5 plant species (Table 2). The most frequently recorded fungi were *Alternaria* species (*A. alternata* and *A. tenuissima*) as well as *Fusarium avenaceum*. The rarest taxa were *Paraphoma fimeti, Lapadostoma amoneum, Rosellinia corticium* and *Phomopsis lactucae*. Out of the 16 recorded taxa, 5 were absent from seeds – *Colletotrichum utrechtense, L. amoneum, P. fimeti, P. lactucae* and *R. corticium,* in spite of their presence in mother plant leaves, while 2 of those found in seeds were absent from leaves – *Alternaria quercicola* and *Epicoccum tritici* (Fig. 1). Differences in elevation (expressed by a matrix of Euclidean distances between localities at specified altitudes) were not

Table 1
Location of sampling sites in the Tatra National Park (Consent of the Minister of Climate and Environment, concerning plant collection in the Tatras along an elevation gradient, issued on 30 August 2021, number DOP-WPN.61.141.2021.DW).

Altitude (m a.s.l.)	Location	Species				
		S. subalpinus	*S. nemorensis*	*S. carpatica*	*H. alpina*	*M. purpurea*
1000	Kuźnice	x	x	x		
1250	Kondrackie Rówienki	x	x			
1500	Murowaniec	x				
1500	Dolina Kondratowa		x	x	x	x
1750	Żółta Turnia			x	x	x
Analysed material						
Leaves		90	90	90	60	60
Seeds		300	300	300	200	200

Table 2
Mycobiota identified in seeds and leaves of five plant species: *Soldanella carpatica*, *Homogyne alpina*, *Mutellina purpurea*, *Senecio subalpinus*, and *Senecio nemorensis*.

No.	Fungal taxa	BLAST match sequence			GenBank accession No.
		accession No.	coverage (%)	similarity (%)	
1	*Alternaria alternata*	MH368103	99	99.5	OM980199
2	*Alternaria infectoria*	MT883449	100	99.2	OM980200
3	*Alternaria quercicola*	MK460940	98	98.4	OM980201
4	*Alternaria tenuissima*	MW720805	99	99.8	OM980202
5	*Boeremia exigua*	MF599109	98	98.2	OM980203
6	*Botrytis cinerea*	KU992697	100	99.3	OM980204
7	*Colletotrichum utrechtense*	MW774940	99	99.5	OM980205
8	*Epicoccum nigrum*	MW580410	99	99.6	OM980206
9	*Epicoccum tritici*	KX965725	100	99.1	OM980207
10	*Fusarium acuminatum*	MT514385	99	99.1	OM980208
11	*Fusarium avenaceum*	KT963799	98	99.3	OM980209
12	*Fusarium oxysporum*	MT530238	99	98.9	OM980210
13	*Lopadostoma amoenum*	KC774569	99	98.0	OM980211
14	*Paraphoma fimeti*	MF494612	98	98.4	OM980212
15	*Rosellinia corticium*	MZ493068	98	98.8	OM980213
16	*Phomopsis lactucae*	KR870839	98	98.0	OM980214

linked with fungal species composition observed at a given site. The correlations were tested with a matrix based on abundances of fungal species (Mantel test, $r = 0.074$, $p = 0.408$) and with a matrix based on their presence or absence (Mantel test, $r = 0.014$, $p = 0.458$). Similarly, there was no significant correlation between fungal species composition in leaves and in seeds both for fungal abundances (Mantel test, $r = 0.057$, $p = 0.288$) and for presence-absence data (Mantel test, $r = -0.046$, $p = 0.633$).

4. Discussion

Probably all plants have endophytic fungi (Petrini 1986). The presence of endophytes in plant species found in various natural ecosystems and in those modified by human impact are continually being reported (e.g. Hamzah et al., 2018; Hiruma and Saijo, 2018; Rim et al., 2021; Franić et al., 2022). Those studies confirm the earlier reported significant influence of genotype and abiotic factors – like temperature, soil moisture content and composition – on mycobiota composition in the host plant (Saikkonen et al., 1998; Clay and Holah 1999).

In this study, we expected that the elevation gradient affects the taxonomic composition of the endophyte community both in the mother plant and in its seeds. We expected also differences between plants of various species. Our results, however, suggest that composition in the studied mountain plants is similar irrespective of species and altitude where they grow. This may also indicate that the identified fungal species do not show any preferences in respect of altitude. Zubek et al. (2009) showed an increase in the number of species of fungal root endophytes when root colonization by AMF declined. We studied endophytes in aboveground parts of plants. Our results suggest that in mountain conditions, which are increasingly difficult for plants with increasing elevation, species richness of fungal root endophytes is more beneficial than leaf endophytes.

There was no significant correlation between the species composition of endophytes in leaves and seeds. The seeds could receive fungal endophytes from the mother plant by way of vertical transmission, which is a frequent process of transmission of fungal endophytes in plants (e.g. Liu et al., 2017). Endophytes could also penetrate seeds in the process of pollination, using pollen as a vector (Obersteiner et al., 2016). Nevertheless, our results show that seeds were significantly less colonized by fungal endophytes than aboveground parts of the mother plant.

In the scientific literature, three classes of endophytes are distinguished (Rodriguez and Redman, 2008). In our study, the identified fungal endophytes represent mostly classes 2 and 3. This classification reflects the great diversity of fungal endophytes present in plants. The diversity of endophytes in nature results in a broad spectrum of influence on the colonized plants (e.g., Brundrett 2006), plant communities (e.g., Clay and Holah 1999), and on other microbial communities inhabiting the plants (e.g., Omacini et al., 2001). The general role of endophytes forming the so-called core mycobiota (transmitted from the mother to offspring) and satellite mycobiota (acquired in the place where the plant lives) is well known (Rodriguez and Redman, 2008). Thanks to the core mycobiota, plants grow better and produce more seeds, which germinate better, than plants devoid of endophytes (Rodriguez et al., 2009; Turner et al., 2013). In other words, endophytes change resource allocation during the plant's life history (e.g. Olejniczak and Lembicz 2007). In the case of so-called satellite endophytes, it appears that the plant copes better with pathogens acquired in the course of life and with environmental stress factors present in the place where it lives (Hol et al., 2015; Mallon et al., 2015). In the studied plants, the transmission of the core mycobiota from the mother plant to seeds was poor. Most of the mycobiota found in the mother plant are acquired during its life and they are so-called satellite endophytes. Life of these endophytes is limited only to vegetative parts of plants and are not further transmitted to seeds.

The impact of mycobiota on functioning of plant communities is being studied (e.g. Matthews and Clay 2001; Rudgers et al., 2010). So far researchers have reported that the influence of mycobiota on communities is reflected by their survival in places with abiotic stress factors (e.g. during drought), increasing plant biomass, and persistence and dominance of some species in the community (Rodriguez and Redman, 2008). Relations between plants and endophytic fungi have been evidenced in fossil records for at least 400 million years (Krings et al., 2007). In plant bodies, fungal endophytes initiate metabolic processes, thanks to which the plants cope with drought better. That is why Pirozynski and Malloch (1975) suggest that endophytes participated in the colonization of land by plants very much like mycorrhizal symbioses (Redecker et al., 2000). Thanks to endophytes of class 2 (such as *Fusarium oxysporum*), plant hormones are induced, or they are synthesized by fungi, and consequently shoot and root biomass increases (Tudzynski and Sharon 2002). This group of endophytes also protects host plants against fungal pathogens (Narisawa et al., 2002; Campanile et al., 2007), through production of secondary metabolites (Schulz et al., 1999) or induction of systemic resistance (Vu et al., 2006). It cannot be excluded, however, that symbiotically provided protection against diseases can be due to an inability of pathogens to compete with endophytes for resources or living space in the plant. Undoubtedly, however, endophytes of this class are characterized by a high frequency of infection (90–100%) of plants growing in habitats with a high stress level. In the case of endophytes of class 3 (such as *Colletotrichum utrechtense*), found in individuals of the studied plant species, their role for the plant hosts is ambiguous and poorly studied. This group of endophytes is very diverse and abundant in plants. Each plant leaf represents a densely packed mosaic of various endophyte species (Lodge et al., 1996; Arnold et al., 2000; Gamboa-Angulo et al., 2008). Single plants can be microhabitats for hundreds of species, and plant species within their native ranges can be colonized by thousands of species. Individual taxa can interact with others. It is difficult, even in laboratory conditions, to separate the influence of one taxon on the plant (Rodriguez and Redman, 2008).

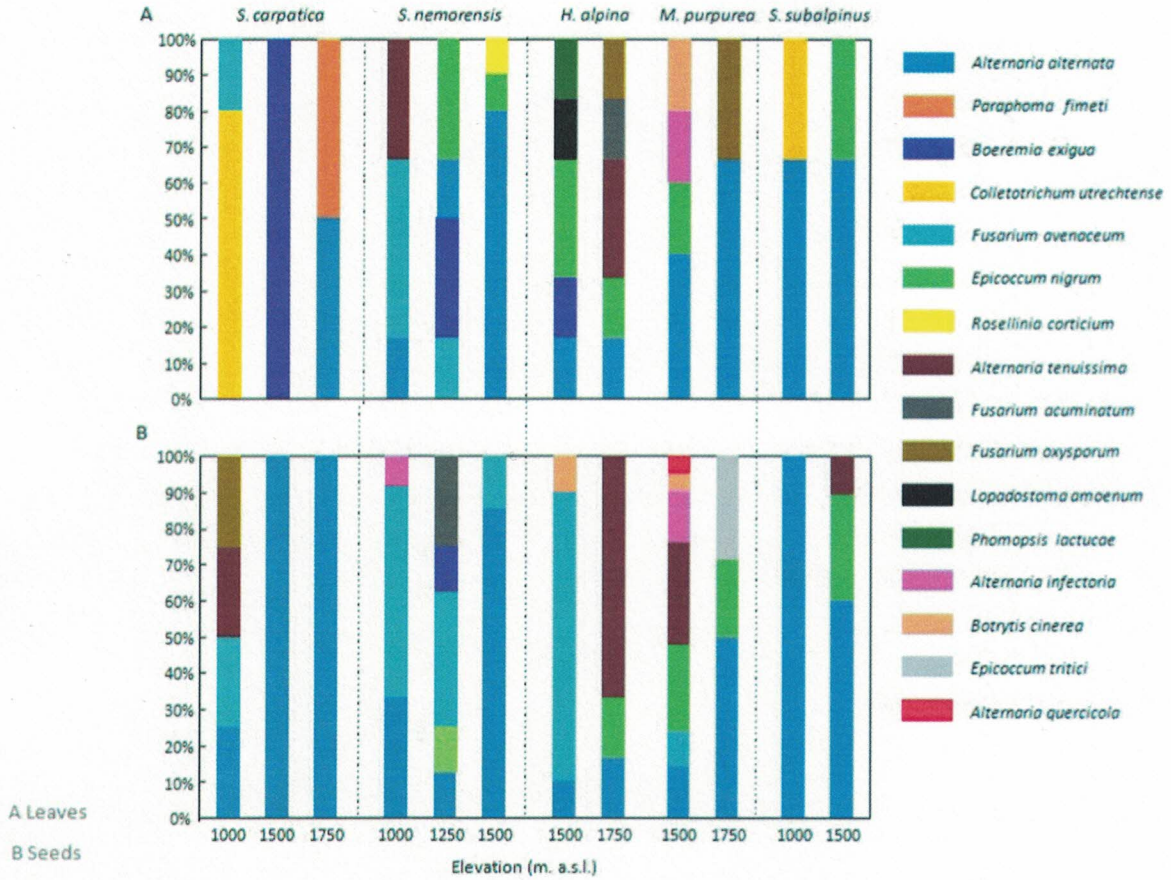

Fig. 1. Mycobiota composition along an elevation gradient in leaves and seeds of five plant species: *Soldanella carpatica*, *Homogyne alpina*, *Mutellina purpurea*, *Senecio subalpinus*, and *Senecio nemorensis*.

Additionally, fungal endophytes of this class are characterized by horizontal transmission. In that case, endophytes become plant parasites. This also means that endophytes of class 3 rarely are transmitted vertically to enter seeds (Arnold et al., 2003; Gallery et al., 2007). In the present study, the efficiency of vertical transmission of the recorded fungal endophytes of this class was low in the studied plant species.

In conclusion, our results show that endophyte community composition in plants is similar regardless of altitude. The identified fungal species do not show any preferences in respect of altitude and/or do not influence plant function more favourably at any specific range of altitudes than at others. The observed endophyte transmission from the mother plant to its seeds in the field was low. This could be for two reasons: (1) the identified endophytic taxa, especially those of class 3, are often not transmitted vertically within the plant body from leaves to seeds and/or (2) the life strategy of endophytes in mountain plants is different from those in plants that do not live along a gradient of altitudes. In the second case, the endophyte could colonize the seeds, but as a result of predominantly vegetative reproduction of the plants, and difficult conditions for germination at high altitudes, it would have low chances of spread by way of vertical transmission. This could be an evolutionarily retained life strategy of these endophytes. Research conducted by Kang et al. (2021) was one of the first studies showing an association between mycobiota composition and the mode of reproduction of mountain plants. An increased share of individuals deriving from vegetative reproduction of plants causes changes in endophyte communities. A more detailed description of the influence of endophytes on plants requires further experimental research. Nonetheless, the identification of endophyte communities of five plant species along an elevation gradient is the first step to understanding their effects on plant survival and condition in mountain ecosystems as well as creation of a bank of fungal endophytes isolated from plants growing still in their natural environment. Knowledge of the endophytes of mountain plants is necessary to develop effective strategies for their protection. Currently we know that plants without endophytic fungi are not able to survive in the intensively changing environment.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Wysoczański W., Węgrzyn E., Olejniczak P., Lembicz M. (2023). Mycobiota diversity and its vertical transmission in plants along an elevation gradient in mountains. *Fungal Ecology,* 63: 101244, brałem udział w pracach dotyczących hodowli *in vitro* grzybów endofitycznych, ich identyfikacji molekularnej, przygotowaniu sekwencji do GenBank oraz w przygotowaniu opisu hodowli w części metody badań.

(mgr Wojciech Wysoczański)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Wysoczański W., Węgrzyn E., Olejniczak P., Lembicz M. (2023). Mycobiota diversity and its vertical transmission in plants along an elevation gradient in mountains. *Fungal Ecology,* 63: 101244, brałam udział na wszystkich etapach powstawania wyników oraz pisania i przygotowania manuskryptu do druku – współtworzyłam koncepcję badań, zajmowałam się przygotowaniem hodowli *in vitro* grzybów, identyfikacją molekularną grzybów endofitycznych, wysłaniem sekwencji do GenBank, interpretacją uzyskanych wyników, brałam udział w pisaniu manuskryptu oraz w redakcyjnym przygotowaniu manuskryptu zgodnie z wymogami czasopisma. Jestem autorem korespondencyjnym w/w artykułu.

Ewa Węgrzyn

(mgr Ewa Węgrzyn)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Wysoczański W., Węgrzyn E., Olejniczak P., Lembicz M. (2023). Mycobiota diversity and its vertical transmission in plants along an elevation gradient in mountains. *Fungal Ecology*, 63: 101244, brałem udział we współtworzeniu koncepcji badań, zebraniu materiału, w analizach statystycznych uzyskanych wyników, czytaniu i poprawianiu manuskryptu.

(dr hab. Paweł Olejniczak, prof. IOP PAN)

Poznań, 17.04.2023

Oświadczenie

Informuję, że w pracy - Wysoczański W., Węgrzyn E., Olejniczak P., Lembicz M. (2023). Mycobiota diversity and its vertical transmission in plants along an elevation gradient in mountains. *Fungal Ecology,* 63: 101244, brałam udział we współtworzeniu koncepcji badań wraz z doktorantką Panią mgr Ewą Węgrzyn oraz w dyskusji interpretacji uzyskanych wyników, w czytaniu, i poprawianiu manuskryptu.

Marlena Lembicz

(prof. dr hab. Marlena Lembicz)

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