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

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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

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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

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 idaeus* 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.techneleysium.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 $\geq 98\%$ 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	<i>Acremonium sclerotigenum</i>	—	+	MH859618	99.8	99	MT573463
2	<i>A. alternata</i>	—	+	KT345696	100	100	MT573464
3	<i>Alternaria infectoria</i>	—	+	MK461063	99.50	100	MT573465
4	<i>A. tenuissima</i>	—	+	MK675103	100	99	MT573466
5	<i>Apiotrichum porosum</i>	+	—	KY558352	99.1	100	MT573467
6	<i>Aureobasidium pullulans</i>	—	+	EF690466	100	99	MT573468
7	<i>Bjerkandera adusta</i>	—	+	MH857085	99.8	99	MT573469
8	<i>Botrytis cinerea</i>	+	+	KU992700	99.50	100	MT573470
9	<i>Cladosporium allicinum</i>	—	+	MH857286	100	100	MT573471
10	<i>Cladosporium cladosporioides</i>	—	+	MH863979	99.6	100	MT573472
11	<i>Colletotrichum dematium</i>	—	+	MG978337	100	100	MT573473
12	<i>Colletotrichum salicis</i>	—	+	MT068551	99	99	MT573474
13	<i>Colletotrichum truncatum</i>	—	+	MH248046	100	99	MT573475
14	<i>Coniochaeta velutina</i>	—	+	MN341294	99.3	99	MT573476
15	<i>Cytospora cedri</i>	—	+	MN764316	98.3	94	MT573477
16	<i>Diaporthe eres</i>	—	+	MK352454	99.8	99	MT573478
17	<i>Epicoccum layuense</i>	—	+	MN396392	100	99	MT573479
18	<i>Epicoccum nigrum</i>	—	+	MF509753	99.6	100	MT573480
19	<i>Hypoxyton fragiforme</i>	—	+	MG098276	99.8	100	MT573481
20	<i>Ilyonectria crassa</i>	+	—	MT294410	99.6	100	MT573482
21	<i>Jackrogersella multiformis</i>	—	+	MK351664	99.8	98	MT573483
22	<i>Melanconis stilbostoma</i>	—	+	AY577811	99.7	99	MT573484
23	<i>Mucor hiemalis</i>	+	—	MF615076	99.7	99	MT573485
24	<i>Nemania serpens</i>	—	+	EF155504	99.8	99	MT573486
25	<i>Paraphaeosphaeria neglecta</i>	—	+	MG098298	99.5	97	MT573487
26	<i>Penicillium chrysogenum</i>	—	+	KT963794	100	99	MT573488
27	<i>Penicillium cosmopolitanum</i>	+	—	JN617682	99.8	100	MT573489
28	<i>Preussia minima</i>	—	+	KU713051	99.5	99	MT573490
29	<i>Pseudogymnoascus pannorum</i>	+	—	MH864434	98.6	99	MT573491
30	<i>Schizophyllum commune</i>	—	+	KP326577	99.5	99	MT573492
31	<i>Umbelopsis isabellina</i>	+	—	MF417265	98.7	98	MT573493
32	<i>U. maydis</i>	—	+	MH855355	100	99	MT573494
33	<i>Varicosporium elodeae</i>	+	—	JX981463	99.3	100	MT573495
34	<i>Xenodidymella applanata</i>	—	+	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 *Colletotrichum 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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