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ORIGINAL RESEARCH PAPER in PLANT PATHOLOGY

Epicoccum nigrum Link as a Potential Biocontrol Agent Against Selected Dermatophytes

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Abstract

Epicoccum nigrum Link is well known for producing biologically-active substances with activities against prokaryotic and eukaryotic cells. The major goal of this study was to assess *E. nigrum* as a potential in vitro agent against selected species of dermatophytes. The effects of the types of media used in this study on the interactions between the microscopic fungi were also examined. *Epicoccum nigrum*'s bioactive metabolites exhibited a strong growth inhibitory effect against the dermatophytes, suggesting its potential as a biocontrol agent. Notably, the strength of these interactions was dependent on the type of the medium. These secondary metabolites are not toxic against the higher eukaryotic organisms, which was further demonstrated by using the *Galleria mellonella* model.

Keywords

secondary metabolites; fungi; endophyte; toxicity

1. Introduction

Epicoccum nigrum Link (syn. *E. purpurascens* Ehrenb. ex Schlecht) is an endophytic fungal species, which is widely distributed as it is found on plant surfaces and in water, soil, and air. This species is particularly known for producing a variety of biologically-active substances. *Epicoccum nigrum* isolated from the marine environment produces extracellular polysaccharides with free radical scavenging activity and is potentially useful in the prevention of oxidative damage in higher organisms (Sun et al., 2011). Somjaipeng et al. (2016) showed that *E. nigrum* could also produce taxol, which is a diterpenoid anticancer drug, and is induced by the elicitors, like water activity or pH. Colored secondary metabolites, such as prodigiosins, which are also excreted by *E. nigrum,* may have a potential role as antimicrobial or antitumor compounds (Perveen et al., 2017). *Epicoccum nigrum* extract, which was isolated from the *Ferula sumbul* leaves, was also found to contain prodiginine and was shown to exhibit strong antimicrobial activity against the microscopic fungi and bacteria (e.g., *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*). It was also shown to exhibit anticancer activities against melanoma cell lines (Perveen et al., 2017). This fungus, when isolated from the cambium of *Phellodendron amurense,* has also been used for the extracellular synthesis of silver nanoparticles with a wide variety of biological activities (Qian et al., 2013). Epicorazines A and B, isolated from *E. nigrum*, exhibited antibacterial activity. Cultured *E. nigrum* hyphae were also shown to excrete several dyes, including β- and ɣ-carotene, rhodoxantin, and epicocconone, in the medium (Baute et al., 1978).

The variety of biologically-active secondary metabolites produced by *E. nigrum* makes it a potential organism for the biocontrol of phytopathogens. Although there is one report describing the pathogenic interaction of *E. nigrum* with *Lotus*

corniculatus (Colavolpe et al., 2018), in general, this fungus is considered to be a facultative saprotroph, exhibiting an important role in plant protection against pathogens (de Cal et al., 2009). It has previously been shown that *E. nigrum* isolated from sugarcane inhibits several phytopathogens, such as *Cyanophora paradoxa* and *Fusarium verticilloides*, and is involved in enhancing the root growth (Fávaro et al., 2012).

Although there are other reports describing the antimicrobial action of *E. nigrum* metabolites against yeast-like fungal human pathogens, the data on their effects against other classes of mycoses-causing fungi, such as dermatophytes, are rather limited. Only one example of growth inhibition of *Trichophyton mentagrophytes* has been described previously (Mallea et al., 1991). Dermatophytes are a cause of communicative diseases that are acquired from infected animals and humans. The clinical manifestations of the infections that are caused by dermatophytes includepedis and tinea capitis. The most common etiological agents causing dermatophytoses are fungal anamorphs, such as *Trichophyton* sp. and *Paraphyton* sp. (Weitzman & Summerbell, 1995).

The genus *Trichophyton* causes the infections among farm animals, mainly in calves and horses, but also in rabbits, sheep, rats, monkeys, cats, and dogs. Human infections might occur after coming in contact with an infected animal. People with impaired immunological system are particularly vulnerable to these pathogens. In contrast, the genus *Paraphyton* is comprised of anthropophilic, zoophilic, and geophilic species. Among the latter, there are fungi that cause diseases in humans but are not yet reported as pathogenic fungal strains (Weitzman & Summerbell, 1995).

It is thus important to search for new antagonists and/or biologically active substances against the dermatophytes. Since higher eukaryotes are the potential hosts of these dermatophytes (Achterman et al., 2011), it is crucial that the biological agents are not toxic against them, and thus in vitro and in vivo toxicity assays should be performed, preferably on mammals, prior to their application (Jorjão et al., 2018). Since the tests on mammals might raise several ethical issues, therefore researchers should develop other eukaryotic models, such as insects for the same. Insect systems have now been extensively used to assess the virulence of fungal pathogens and for in vivo drug toxicity assays due to their low cost, easy culture, and lack of ethical restrictions. Their innate immune system shares many similarities with that of mammals. Among many different insect models, the greater wax moth, *Galleria mellonella* has been frequently used by the scientific community (Kavanagh & Sheehan, 2018).

The major goal of our study was to assess the potential of *Epicoccum nigrum* as a biocontrol agent against dermatophytes by determining whether it shows in vitro biotic interactions with selected species of dermatophytes, as well as by assessing the effects of its secondary metabolites on the survival and growth of *Galleria mellonella* larvae.

2. Material and Methods

The in vitro antagonism between *E. nigrum* and dermatophytes were studied using the biotic series method described by Mańka and Mańka (1992) and Ogórek and Pląskowska (2011) on PDA (potato dextrose agar; Biocorp) and YPG (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar) media plates, and described as an individual biotic effect. All the strains used in this study are deposited in the Department of Mycology and Genetics, Institute of Genetics and Microbiology, University of Wrocław, Poland. Two rye isolates of *E. nigrum*, UP_EPC_31 and UP_EPC_49 (accession numbers KM434173.1 and KM434171.1, respectively), and four species of dermatophytes, including *Trichophyton tonsurans* Malmsten, isolated from abdominal skin, *T. terrestre* Durie & D. Frey, isolated from soil, *T. mentagrophytes* (C. P. Robin) Sabour, isolated from an ambulatory patient, and *Paraphyton cookei* Ajello KU687323.1, isolated from cave soil, were tested for the interspecies interactions. The fungal inoculates of ca. 4 mm diameter

were taken from the ten days old cultures on PDA, and then the mycelium was placed downwards and 2 cm apart in the center of the PDA and YPG plates. Each combination was prepared in four replicates.

Additionally, plates with mycelium of a single fungal species were used as a reference. After inoculation, the plates were incubated in the dark at 24 ± 0.5 °C. Biotic effects of the fungi in the combined cultures were evaluated after 10 days of growth. While evaluating the biotic effects, the surrounding area of one colony that was captured by another fungal species was observed, and then the occurrence of inhibition zone between the two colonies, as well as the reduction in the colony size were considered. The appearance of each effect was scored, and points were summarized according to the scale described by Mańka (1974), and the results are presented in Table 1. The biotic effect induced by a particular fungal species was evaluated as an individual biotic effect (IBE). The positive effect indicates the suppression of pathogen growth, and the negative effect indicates the lack of growth suppression. The effect might be scored with the value of 0, which indicates neutral influence (Mańka, 1974; Ogórek & Pląskowska, 2011). The size of the zone formed by *E. nigrum*'s colored metabolites and the size of the inhibition zones created by different *E. nigrum* isolates were measured. Each measurement was performed in four replicates.

Table 1 The scale of scoring the biotic effects of the *Epicoccum nigrum* colony on the dermatophyte colony.

Petri dish appearance		Points
0	Both fungal colonies straightly abut to each other	0
I	*Epicoccum nigrum* colony abut to the dermatophyte colony in a slightly curved manner, surrounding less than 1/3 of the tested[1] colony	+1
II	*E. nigrum* colony abut to the dermatophyte colony in a slightly curved manner, surrounding at least 1/3 but no more than 1/2 of the tested colony	+2
III	*E. nigrum* colony abut to the dermatophyte colony in a slightly curved manner, surrounding less than 1/2 but no more than 2/3 of the tested colony	+3
IV	*E. nigrum* colony abut to the dermatophyte colony in a slightly curved manner, surrounding at least 2/3 of the tested colony	+4
V	Every mm of the inhibition zone between both colonies, caused by the *E. nigrum* colony	+1
VI	Dermatophyte colony smaller by at least 1/3 but no more than 1/2 than the control colony grown on separate plate	+1
VII	Dermatophyte colony smaller by at least 1/2 but no more than 2/3 than the control colony grown on separate plate	+2
VIII	Dermatophyte colony smaller by at least 2/3 than the control colony grown on separate plate	+3
IX	Undeveloped dermatophyte colony	+4

The points were given based on the appearance of both the colonies in a Petri dish, as described by Mańka (1974).
[1] Tested colony refers to the dermatophyte colony.

Additionally, the toxicity effect of *E. nigrum* filtrates was examined by using the *Galleria mellonella* larvae model. Sterile fungal filtrates derived from 14-day-old cultures were incubated at 25 ± 0.5 °C in Sabouraud dextrose broth (peptone 10 g/L and glucose 40 g/L). Thereafter, the caterpillars were treated with 40 µL of the sterile fungal filtrates. Inoculations were performed directly into the hemocoel via the prolegs, by injections using the insulin syringes with 26G needles (Fuchs et al., 2010). Injections were preceded by the disinfection of the puncture sites with 70% ethanol. The inoculations with phosphate-buffered saline (PBS) and Sabouraud dextrose broth were used as the experimental controls. After the injection, the larvae were incubated at 37 ± 0.5 °C, and the viability of the caterpillars was

monitored every 24 hr consecutively for 7 days. Caterpillars that were selected for the experiment were in the final instar larval stage (330 ± 30 mg in body weight). Each filtrate was tested on a group of 60 individual caterpillars.

The results were analyzed by one-way analysis of variance (ANOVA) using the software package Statistica version 12.0 (StatSoft Polska, Kraków, Poland). Means of different test conditions were compared using the Tukey's HSD (honestly significant difference) at $\alpha \leq 0.01$.

3. Results

Overall, both the *E. nigrum* isolates showed a positive biotic effect towards the tested dermatophytes, with an exception of *E. nigrum* UP_EPC_31 in the coculture with *T. terrestre* on YPG (Table 2). The strongest biotic effect was observed for *E. nigrum* UP_EPC_31 against *T. tonsurans* on PDA ($p_{T.\ tonsurans,\ P.\ cookei} = 0.003661$). The same trend was observed in the case of YPG ($p_{T.\ tonsurans,\ T.\ mentagrophytes} = 0.009396$). In the case of *E. nigrum* UP_EPC_49, all the interactions were positive, but were not significantly different on both the media plates.

Table 2 The individual biotic effect (IBE) between the strains of *Epicoccum nigrum* and dermatophytes after 10 days of combined growth on PDA and YPG media plates. The same experiment was performed in four independent replicates.

Dermatophyte species	*E. nigrum* UP_EPC_31				*E. nigrum* UP_EPC_49			
	PDA[1]		YPG		PDA		YPG	
Paraphyton cookei	5.00	bA[2]	1.50	abB	5.00	aA	2.50	aA
Trichophyton mentagrophytes	4.25	bA	0.75	bB	4.20	aA	1.25	aB
Trichophyton terrestre	4.00	bA	−1.00	cB	2.75	aA	1.25	aA
Trichophyton tonsurans	7.00	aA	2.50	aB	3.75	aA	3.00	aA

[1] PDA (potato dextrose agar), YPG (yeast extractpeptone dextrose).
[2] For each variant of the experiment, means followed by the same letter are not statistically different at $\alpha \leq 0.01$ according to Tukey's HSD test. Small letters mark differences in the interaction between a particular *E. nigrum* isolate and the individual dermatophytes species; they refer to column means. Capital letters mark the effect of media on these biotic effects within a given *E. nigrum* isolate and a given species of dermatophytes; they refer to row means.

The biotic effects were significantly stronger on the PDA plates in comparison to the YPG plates (Table 2). The effect of a culture medium was specifically observed for *E. nigrum* UP_EPC_31, for which all the interactions varied in a highly significant manner ($p_{PDA,\ YPG} = 0.000327$ for *T. tonsurans*, $p_{PDA,\ YPG} = 0.000349$ for *T. terrestre*, $p_{PDA,\ YPG} = 0.000850$ for *T. mentagrophytes*, and $p_{PDA,\ YPG} = 0.000643$ for *P. cookei*). In the case of *E. nigrum* UP_EPC_49, statistically significant differences between media were recorded only for *T. mentagrophytes* ($p_{PDA,\ YPG} = 0.004612$) (Table 2).

The results of this study showed that the coculturing of one species with another species, as well as the culture medium, have an effect on the amount of pigments produced by *E. nigrum* and consequently, the appearance of inhibition zones (Table 3). All the tested dermatophytes stimulated *E. nigrum* UP_EPC_31 to secrete the colored substances on the PDA plates, with the strongest effect observed for its coculture with *T. tonsurans* ($p_{T.\ mentagrophytes,\ T.\ terrestre} = 0.002184$). In contrast, on the YPG plates, this isolate produced colored substances only when it was cocultured with *P. cookei*. Surprisingly, *E. nigrum* UP_EPC_49 always synthesized the pigments during the coculture with different dermatophytes, regardless of the medium. Moreover, there was no significant effect of the dermatophyte species on the synthesis of these colored substances by *E. nigrum* UP_EPC_49 on the YPG media, whereas on the PDA media, this isolate was highly stimulated by *T. terrestre* ($p_{T.\ terrestre,\ T.\ tonsurans} = 0.000670$) to produce the pigments. There was also a significant impact of the media on the amount of secreted pigments by *E. nigrum* UP_EPC_31 ($p_{PDA,\ YPG} = 0.002048$ for *P. cookei*, $p_{PDA,\ YPG} = 0.000291$ for *T. mentagrophytes*, $p_{PDA,\ YPG} = 0.002488$ for *T. terrestre*, and $p_{PDA,\ YPG} = 0.000385$ for *T. tonsurans*), as well as by *E. nigrum* UP_EPC_49 ($p_{PDA,\ YPG} = 0.000291$ for *P. cookei*, $p_{PDA,\ YPG} = 0.000292$ for *T. mentagrophytes*, and $p_{PDA,\ YPG} = 0.006348$ for *T. tonsurans*), with an exception in the coculture of *E. nigrum* UP_EPC_49 with *T. terrestre*. However, in the case of *E. nigrum* UP_EPC_31, the inhibition zones were only formed on PDA plates in the cocultures with *P. cookei* (Figure 1),

Table 3 The ability of *Epicoccum nigrum* isolates to synthesize colored metabolites and create inhibition zones after 10 days of combined growth with the dermatophytes. A (+) indicates the formation of an inhibition zone, and a (–) indicates the lack of such zones. The indicated values are the average of the values from four independent experiments.

Dermatophyte species	*E. nigrum* UP_EPC_31						*E. nigrum* UP_EPC_49					
	Colored metabolite zone (mm)				Inhibition zone		Colored metabolite zone (mm)				Inhibition zone	
	PDA[1]		YPG		PDA	YPG	PDA		YPG		PDA	YPG
Paraphyton cookei	7.00	abA[2]	1.00	aB	+	–	4.10	bB	7.50	aA	+	+
Trichophyton mentagrophytes	8.03	aA	0.00	aB	–	–	3.25	bB	7.00	aA	+	–
Trichophyton terrestre	4.00	bA	0.00	aB	–	–	9.00	aA	7.45	aA	+	–
Trichophyton tonsurans	8.18	aA	0.00	aB	–	–	5.11	bB	7.18	aA	–	–

[1] PDA (potato dextrose agar), YPG (yeast extract peptone dextrose).
[2] For each variant of the experiment, means followed by the same letter are not statistically different at $\alpha \leq 0.01$ according to Tukey's HSD test. Small letters mark the effect of a given species of dermatophytes on the synthesis of color metabolites by a given *E. nigrum* within a particular medium; they refer to column means. Capital letters mark the effect of media on the synthesis of color metabolites by a given *E. nigrum* isolate in the combined growth with a given species of dermatophytes; they refer to row means.

Figure 1 An example of the inhibition zone created by the secondary metabolites secreted by *Epicoccum nigrum* UP_EPC_31 (left side of the plate) to the PDA medium after 10 days in the paired growth with *Paraphyton cookei* (right side of the plate); IBE = 5.00.

whereas *E. nigrum* UP_EPC_49 formed the inhibition zones in a coculture with all the dermatophytes on PDA plates (besides *T. tonsurans*), and only with *P. cookei* on the YPG plates (Table 3).

The experiments performed to test the safety of *G. mellonella* larvae against the *E. nigrum* filtrates showed that the survival of larvae after 168 hr of incubation with the medium and the *E. nigrum* UP_EPC_31 filtrate decreased up to 96.7%, and in the case of *E. nigrum* UP_EPC_49, it decreased up to 95% (Figure 2). Since both, the medium and the secondary metabolites secreted by *E. nigrum*, reduced the survival rate of *G. mellonella* larvae to a similarly extent, this reduction in the viability is attributed to the medium, and not to the fungal filtrates.

4. Discussion

Epicoccum nigrum Link is a cosmopolitan fungus frequently isolated from plants, soil, or water, and is a well-known producer of various secondary metabolites (Sun et al., 2011). The results obtained in our studies confirm the potential application of this species as a biocontrol agent with antimicrobial properties. The antibacterial activity of the secondary metabolites produced by *E. nigrum* is well documented (Baute et al., 1978; Perveen et al., 2017). Moreover, antifungal properties of this

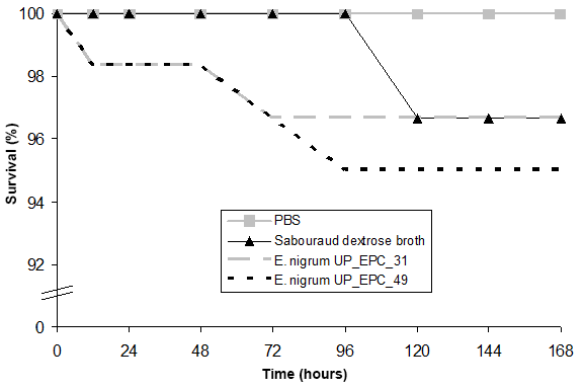

Figure 2 The effect of *Epicoccum nigrum* UP_EPC_31 and UP_EPC_49 secondary metabolites on the survival of *Galleria mellonella* larvae. PBS and SDB medium were used as the controls.

species were also proved against numerous plant, animal, and human pathogens (Mallea et al., 1991). In the present study, we demonstrated the antifungal potential of *E. nigrum* against dermatophytes, since inhibitory biotic interactions were observed between the *E. nigrum* isolates and the dermatophytes, including *P. cookei*, *T. terrestre*, *T. tonsurans*, and *T. mentagrophytes*.

As shown in this study, the type of culture medium is an important factor in estimating fungal interspecies interactions (Ogórek et al., 2016). PDA is a medium preferable for *E. nigrum*, whereas dermatophytes exhibit better growth on YPG, and thus there were some differences between the strength of the interactions that were dependent on the medium. *Epicoccum nigrum* is also a well-known producer of colored metabolites and some of them, such as prodiginine, exhibits antimicrobial properties (Perveen et al., 2017). In addition, there is a correlation reported between the secretion of pigments and epicorazine A and B, by this species (Baute et al., 1978). Since the zones created by colored substances and biotic effects were stronger on the PDA medium, we can speculate that medium composition plays an important role in stimulating interactions between *E. nigrum* and the dermatophytes. PDA is a carbon-rich medium but is deficient in other nutrients (unlike YPG). Such stress conditions might also stimulate the pigment production by *E. nigrum* (Pradeep et al., 2013), and thus these colored substances might enhance the inhibitory biotic effects towards the dermatophytes (Fatima et al., 2016). As previously reported in the literature, fungi of the genus *Epicoccum* can also secrete bioactive metabolites with cytotoxic properties, e.g., some terpene metabolites and epicoccamide D (Palacio-Barrera et al., 2019). However, in this research study, we could show that *E. nigrum* species probably does not produce any cytotoxic substances, since the culture filtrates from *E. nigrum* strains did not reduce the viability of *G. mellonella* larvae at a significant level.

In conclusion, this study is the first report describing about the antagonistic interactions between *E. nigrum* and dermatophytes (*P. cookei*, *T. terrestre*, *T. mentagrophytes*, and *T. tonsurans*), as well as the effects of its secondary metabolites on *G. mellonella*, which is an eukaryotic model organism. The results indicate towards the possible application of *E. nigrum* secondary metabolites for the treatment of skin dermatophytoses. Therefore, in the near future, further studies will help to isolate and identify different secondary metabolites and determine their fungicidal properties.

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