

Epicoccum nigrum – isolation, characterization and potential for biological control of *Botrytis cinerea*

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Abstract

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Twelve *Epicoccum nigrum* isolates were obtained from ash and elm tree species located at different forest areas in the country. The species identification was performed by sequencing of the internal transcribed spacer (ITS) region of fungal rDNA using primers ITS5 and ITS4. *E. nigrum* isolates formed typical for the species colonies, colored from yellow to orange and decorated in different shades. The mycelial growth rate in a temperature range between 5 and 30°C for all isolates was determined and the optimum for most of them was calculated to be at 20°C. Two isolates, A 7-2 and E 1-1, demonstrated maximum growth at 25°C, whereas the isolate A 1-6 has the lowest temperature optima at 15°C. The ability of selected *E. nigrum* to inhibit the mycelium growth of phytopathogenic fungus *Botrytis cinerea* was proved by dual culture test. All twelve *E. nigrum* isolates were capable to inhibit the growth of the pathogen to various degrees. The most perspective *E. nigrum* isolates (A 3-1, E 1-1 and E 5-1) with possible application for biological control of *B. cinerea* were selected. The potential of the species as biocontrol agent for a plant protection in the agriculture was discussed.

Keywords: *Epicoccum nigrum*; gray mold; antagonist; plant protection

Introduction

Epicoccum nigrum (syn. *E. purpurascens* Ehrenb. ex Schlecht.) is an anamorphic ascomycete, determined as a saprotrophic fungus. It is worldwide distributed and is commonly associated with various host plants. It was reported as endophyte that was isolated from inner tissue of several plant hosts (Schulz & Boyle, 2005; Arnold, 2007; Stuart et al., 2010; Fávares et al., 2012; Perveen et al., 2017). The species is characterized by a highly variability and occurrence of different morphological and physiological types (Kilpatrick & Chilvers, 1981; Fávares et al., 2011).

A lot of investigations demonstrated ability of *E. nigrum* to inhibit the growth of different plant pathogens and therefore its potential as an agent for biological control of some of most dangerous plant diseases (Elmer et al., 2001). *E. nigrum* is known as an antagonist of several *Fusarium* species

as *F. oxysporum*, *F. avenaceum*, *F. graminearum* and *F. verticillioides* (Park et al. 2002, Ogórek & Płaskowska, 2011; Fávares et al., 2012; Jensen et al., 2016). It also can suppress mycelia growth of *Rhizoctonia solani* (Lahlali & Hijri, 2010), *Venturia inaequalis* (Tshomo et al., 2003), *Sclerotinia sclerotiorum* (Hoyte et al., 2007; Huang & Erickson, 2008), *Diplodia corticola* (Campanile et al., 2007) and *Monilinia laxa* (Larena et al., 2005; De Cal et al., 2009; Larena & Melgarejo, 2009). Several reports represented also the potential of *E. nigrum* to control *Botrytis cinerea* (Szandala & Backhouse, 2001; Elmer & Reglinski, 2006; Walter et al., 2006; Card et al., 2009; Alcock et al., 2015).

B. cinerea is ranked as the second most important for the science and the economic fungal pathogen according to a survey of the Molecular Plant Pathology journal (Dean et al., 2012). The fungus is necrotrophic plant pathogen that infects over 200 plant species (Boddy, 2016). It caused grey

mould on a variety of field crops, grape, small fruits, orchards and ornamentals; therefore the pathogen is responsible for annual losses of \$10 billion to \$100 billion worldwide (Boddy, 2016). The control of *B. cinerea* is difficult because of its broad host range, various attack modes and both asexual and sexual stages to survive in favorable or unfavorable conditions (Fillinger & Elad, 2016). The main strategy of *B. cinerea* control is fungicide application, but this is cost expensive and does not respond to current food safety requirements and trends. An alternative method to control *B. cinerea* is biocontrol by using of effective antagonists like *E. nigrum*. Several investigations demonstrated the ability of *E. nigrum* isolates to produce antimicrobial agents as epicorazin, epicoccin, epicoccarine, epipyridone, flavipin and epirodins (Bamford et al., 1961; Baute et al., 1978; Ikawa et al., 1978; Zhang et al., 2007; Wangun & Hertweck, 2007; Alcock et al., 2015).

In this study isolation and characterization of *E. nigrum* are presented. Twelve isolates originating from ash (*Fraxinus* sp.) and elm (*Ulmus* sp.) trees from eight different locations were collected. Their inhibition effect on *B. cinerea* was tested and the potential of *E. nigrum* as an agent for biological control is discussed.

Material and Methods

Sample collection and isolation of *E. nigrum*

E. nigrum isolates were obtained from leaves of ash (*Fraxinus* sp.) and elm (*Ulmus* sp.) trees located in the different parts of the country (Table 1) during the health status survey of the two forest species. Leaf samples were collected and were surface sterilized with 70% ethyl alcohol and rinsed twice in sterile water. Leaf segments were incubated on PDA (Potato Dextrose Agar, Difco) at room temperature for 3-5 days. A mycelium plug from each colony with different morphological type was transferred on water agar and single mycelium colonies were obtained by transferring of hyphal tips 3-4 days later on fresh PDA media.

Table 1. Collection of *E. nigrum* isolates from ash (*Fraxinus* sp.) and elm (*Ulmus* sp.)

Isolate	Host	Location	GPS coordinates
A 1-6; A 1-7	ash	Sofia	42.683621, 23.332755
A 3-1; A 3-4	ash	Isrec village	42.977344, 23.286208
A 4-2	ash	borderland of Topolnica River	42.399681, 24.008503
A 7-2	ash	Anton village	42.42933, 24.12404
E 1-1	elm	Pancharevo district	42.588560, 23.421960
E 2-1; E 2-6	elm	Pancharevo district	42.588560, 23.421960
E 4-1	elm	borderland of Topolnica River	42.401257, 24.006652
E 5-1	elm	borderland of Topolnica River	42.399176, 24.008565
E 9-2	elm	Tulovo	42.559094, 25.551421

DNA extraction, amplification and sequencing

DNA was isolated from fresh mycelium using DNeasy Plant Mini Kit (QIAGEN GmbH). The PCR amplification with primers ITS5 (5'-GGAAGTAAAGTCGTAA-CAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') was performed using PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare Life Sciences), according to the manufacturer's instructions under the following PCR program: 96°C – 2 min, followed by 35 cycles of 96°C – 1 min, 55°C – 1 min, 72°C – 2 min and final elongation at 72°C – 10 min. The PCR products were purified using Sephadex and send for sequencing to GATC Biotech AG (Germany). A data analyses was performed using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database.

Morphological characterization and mycelial growth rate

E. nigrum isolates were cultivated on PDA at 20°C in the dark for morphological characterization. They were monitored daily for a period of one month.

The mycelial growth rate of *E. nigrum* isolates was determined by cultivation on PDA at 5, 10, 15, 20, 25 and 30°C. Colonies were incubated for 24 h at 20°C to synchronize onset of the hyphal growth. Then mycelial growth was marked along two lines intersecting the center of the each colony and two replicate plates were incubated at testing temperatures. An enlargement of the colonies was marked again after inoculation for 5-7 days and the radial growth rate per day (mm/d) was calculated.

Inhibition effect of *E. nigrum* against *B. cinerea*

The inhibition effect of *E. nigrum* against *B. cinerea* was analyzed by a dual culture test. *B. cinerea* is a part of the fungal collection of AgroBioInstitute and originated from a strawberry fruit.

In the experiment, 10 day-old cultures of each *E. nigrum* isolate and 5 day-old culture of *B. cinerea* were used. Myce-

lial plugs (5/5 mm) of both fungi were placed approximately 2 cm from each other in a 9-cm Petri dish containing PDA medium. Two replicates for each combination of *E. nigrum* isolate and *B. cinerea* were prepared. The dual cultures were incubated at 20°C in the dark. The effect of the joint cultivation of the two fungal species was daily monitored.

Results and Discussion

Isolation and identification of *E. nigrum*

Twelve *E. nigrum* isolates from leaf samples of ash and elm trees originated from different forest habitats in Bulgaria were collected. They were identified as *E. nigrum* in a result of the sequence analyses of the ITS region of the fungal rDNA. The BLAST search showed a high homology of the obtained isolates with sequences of *E. nigrum* deposited in the GenBank database at the NCBI.

Morphological characteristics

Most of collected *E. nigrum* isolates have a typical for the species yellow to orange mycelium, as follow A 1-6, A 1-7, A 4-2, E 1-1, E 2-6, E 4-1 and E 9-2 (Figure 1a, b). Some isolates have gray (A 3-1, A 7-2) or pink (A 3-4) mycelium (Figure 1a), whereas other demonstrated variable morphology of the colony with gray and pink (E 2-1) or gray and yellow colors (E 5-1) (Figure 1b). The majority of *E. nigrum* cultures were characterized by vigorous aerial mycelial growth, except for dark gray colored colonies of A 3-1 and A 7-2 that were with thick mycelia and irregular shape (Figure 1a).

Collected *E. nigrum* isolates showed typical for the species variety of colorful colonies that is standard characteristic used for a classical morphological identification. No morphological differentiation of isolates according to their origin from ash or elm was found during this study. The created collection of *E. nigrum* cultures has enriched the variety of isolated and characterized fungi associated with plants at AgroBioInstitute.

Mycelial growth rate

Collected *E. nigrum* isolates express a variety of growth rate at the temperature range from 5 to 30°C (Figure 2). Some isolates like A 1-6, A 3-1 and A 7-2 were consistently slower growing at all temperatures, whereas most other isolates have a distinct growth peak at their optimum temperature. Most of isolated *E. nigrum* have temperature optima at 20°C, with an exception of A 7-2 and E 1-1, that demonstrated maximum growth rate at 25°C, and the isolate A 1-6 with the optimum at 15°C (Figure 2).

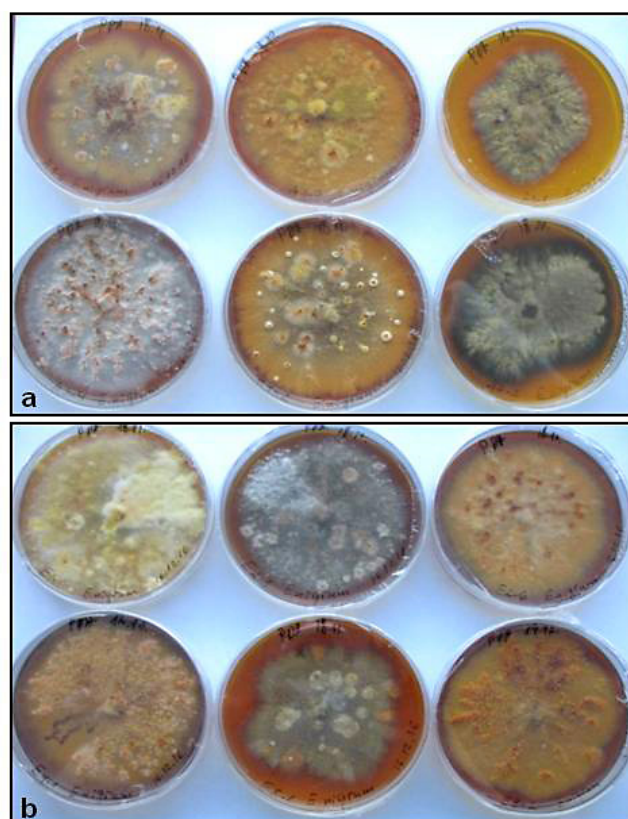


Fig. 1. Colony of *E. nigrum* isolates derived from ash (a) and elm (b) tree species, cultivated on PDA:

**a – first row: A 1-6, A 1-7 and A 3-1;
second row: A 3-4, A 4-2 and A 7-2 (from left to right);
b – first row: E 1-1, E 2-1 and E 2-6;
second row: E 4-1, E 5-1 and E 9-2 (from left to right)**

Some of the *E. nigrum* isolates exhibit specific characteristics at the tested temperatures. The isolate A 7-2 is the only one among the collected fungi that do not growth at 5°C. No mycelial growth of this isolate was also observed at 10°C, as well as 30°C. Therefore, it was determined as the isolate with the most limited temperature range among the investigated *E. nigrum* collection. The isolate A 7-2 was characterized by slow radial growth rate that was calculated to be 1.8 mm/d at the optimum temperature. Two other isolates, A 1-6 and A 3-1, also have relatively slower growth with calculated rate of 1.4 mm/d at their temperature optimum. It is interesting to note that A 1-6 demonstrated relatively constant growth rate at temperature range from 10 to 25°C. In addition to A 7-2, the isolate A 3-1 was also not able to grow at 30°C. However, the growth inhibition of the both isolates was reversible after transferring the colonies at 20°C.

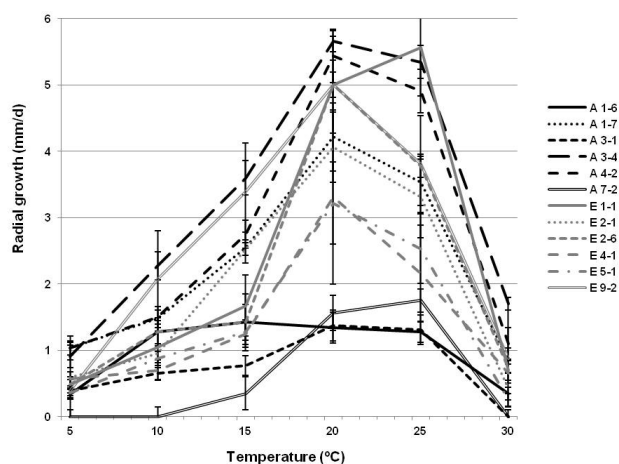


Fig. 2 Mycelial growth rate of *E. nigrum* isolates in a temperature range from 5°C to 30°C at PDA

In contrast to slow-growing isolates, some other like A 3-4, A 4-2 and E 1-1 demonstrated fastest mycelial growth with the rate of 5.6, 5.6 and 5.4 mm/d respectively at the optimum temperature (Figure 2). While isolates A 3-4 and A 4-2 have the same temperature optimum (20°C) and similar growth rates with relatively high values at the investigated temperatures, the isolate E 1-1 has an optimum at 25°C, but at temperatures below 20°C and above 25°C shows a rather slow development. Relatively fast mycelium growth was also calculated for *E. nigrum* isolates E 2-6 and E 9-2 (5 mm/d) at temperature optima, but both are quite differ at 10 and 15°C, in which the isolate E 9-2 grows twice as fast as the isolate E 2-6.

Two other isolates, A 1-7 and E 2-1, showed similar characteristics in the testing temperature range with the mycelial growth of 4.2 and 4.1 mm/d at 20°C, respectively. Such comparable pattern of the growth rate was calculated for isolates A 1-7 and E 2-1 as well (3.3 and 3.2 mm/d at the temperature optimum, respectively).

Inhibition of B. cinerea* mycelium growth by *E. nigrum

The results of dual growth test showed that all analyzed *E. nigrum* isolates are capable to restrict the mycelium growth of *B. cinerea* (Figure 3). Incompatible reaction between both fungal species was observed after 3 days of mutual cultivation. The release of pigments by *E. nigrum* isolates in the medium was also monitored at that time. The isolates A 3-4, E 2-6 and E 4-1 demonstrated less impact on the development of *B. cinerea* with an inhibition zone of only 1 mm. The inhibition effect of the majority of the tested isolates (A 1-6,

A 1-7, A 4-2, E 2-1 and E 9-2) was characterized by an suppression area of 2-3 mm. The inhibition zone of isolate A 7-2 was 4 mm. The strongest inhibition effect was monitored for the isolates A 3-1, E 1-1 and E 5-1 with the suppression area of about 5 mm and these isolates are considered as the most perspective ones that can be applied for biological control of *B. cinerea* (Figure 3). Interestingly, all three isolates pointed here developed relatively small mycelium colonies comparing to the isolates that demonstrated less inhibition effect of the mycelium growth of *B. cinerea*. The possible reason for this effect is the production of more effective antimicrobial compounds by A 3-1, E 1-1 and E 5-1 compared to the other analyzed isolates. *Epicoccum* sp. is known to produce variety of secondary metabolites such as polyketides, polyketide hybrids, diketopiperazines, siderophores, carotenoid and others. Among them polyketides flavipin, epicolactone, epicocolide A and B were shown to express antifungal and an-

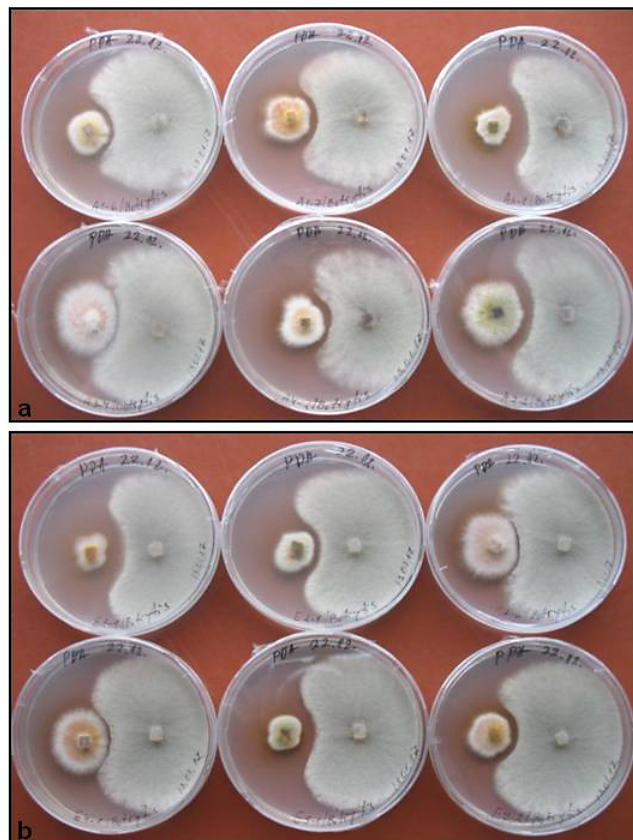


Fig. 3 Dual culture assay of *E. nigrum* isolates from ash (a) and elm (b) trees and *B. cinerea* after 5 days at 20°C
 a – first row: A 1-6, A 1-7 and A 3-1;
 second row: A 3-4, A 4-2 and A 7-2 (from left to right);
 b – first row: E 1-1, E 2-1 and E 2-6;
 second row: E 4-1, E 5-1 and E 9-2 (from left to right)

tiomycete effect (Elkhateeb & Daba, 2019), which assumes the possible use of *Epicoccum* sp. for the control of some fungal and oomycete diseases in plants.

The ability of different *E. nigrum* isolates from several hosts to inhibit germination and development of *B. cinerea* based on the production of a yellow compounds have been reported (Elmer et al., 2001). Furthermore, it was found that *E. nigrum* isolates exuded several antimicrobial agents that diffuse into culture media (Alock et al., 2015). In addition to *in vitro* laboratory tests for the inhibition effect of *Epicoccum* sp. on *B. cinerea*, the antagonistic relationships between both species have been proved *in vivo* during harvesting of fruits (Szandala & Backhouse 2001; Walter et al., 2006; Card et al., 2009). The suppression of *B. cinerea* sporulation by application of *E. nigrum* has been observed in a leaf-disc bioassay and the results showed that timing of the *Epicoccum* application, temperature and water potential were the most important factors that influence on the antagonist effect (Szandala & Backhouse, 2001). The inhibition of *B. cinerea* sporulation by *E. nigrum* was also established by *in vitro* dual plate and necrotic grape leaf disc assays (Walter et al., 2006). Our results from the dual growth test also confirmed the potential of *E. nigrum* to be used for biological control of the gray mold pathogen *B. cinerea*.

Based on the antimicrobial effect of *E. nigrum*, different strategies for biological control of several plant pathogens have been developed. The efficacy of *E. nigrum* and its exudates was tested and effectively used to control the pre- and post-emergence damping-off and root-rot of Egyptian cotton *in vitro* and *in vivo* (Hashem & Ali, 2004). The application of *E. nigrum* into soil mixture not only led to protection of cotton seedlings against *Pythium* damping-off and root-rot, but also stimulated growth of the plants. The population dynamics of *E. nigrum* on peaches and nectarines was also investigated and the enhancement of fruit's colonization to improve its biocontrol efficacy against brown rot was estimated (De Cal et al., 2009). It was proved that application of *E. nigrum* conidial formulations decreased the number of conidia of *Monilinia* spp. on the fruit surface during the growing season to the same extent as fungicides and can be used as biocontrol agent against brown rot in stone fruits.

The accumulation of data for a variety of antagonists of plant pathogens as a result of laboratory analyzes will facilitate the determination of agents for biological control that can be used in the field. The application of alternative practices to control plant diseases is one of the main principals in sustainable agriculture at present. Among main options for biocontrol in a crop production is the treatment with microorganisms or their products. They can be used as direct antagonists of plant pathogens, by competing with them, but

also as biofertilizers (Heydari & Pessarakli, 2010; Rahman et al., 2017). Thereby, the applying of biocontrol to manage plant diseases will lead to increased crop production, as well as careful and effective protection of the environment.

Conclusions

A small collection of isolates from the saprotrophic fungus *E. nigrum* with a different origin was created. The morphological diversity of the collected isolates and the differences in their growth rate over a wide temperature range were described. All tested *E. nigrum* isolates demonstrated an inhibition effect on the growth of phytopathogenic fungus *B. cinerea*. These results show a potential for the application of selected *Epicoccum* isolates as biocontrol agent of grey mold disease in plants.

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