Assessment of p-cresol and phenol antifungal interactions in an arthropod defensive secretion: the case of an endemic Balkan millipede, *Apfelbeckia insculpta* (L. Koch, 1867) (Diplopoda: Callipodida)

Оценка фунгицидных взаимодействий p-крезола и фенола в защитном секрете членистоного: пример эндемичной балканской диплоподы *Apfelbeckia insculpta* (L. Koch, 1867) (Diplopoda: Callipodida)

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КЛЮЧЕВЫЕ СЛОВА: алломоны, антимикотики, шахматный метод, двупарноногие, фенолики.

ABSTRACT. Millipedes (Diplopoda) are a group of arthropods that produce and deploy chemically diverse exudates from defensive glands in the event of predator attack. These exocrine secretions are also potent antimicrobials. In view of the fact that the defensive secretion of the endemic Balkan millipede, *Apfelbeckia insculpta* (L. Koch, 1867) consists of only two compounds with known antifungal properties (p-cresol and phenol), it represents an ideal model for studying the contribution of individual compounds to the overall antifungal activity of this natural product, thereby enabling us to define the nature and type of interactions between the main compound (p-cresol) and the trace compound (phenol). Twenty-five combinations of concentrations, ranging from 0.1 to 1.0 mg mL⁻¹, were tested on 14 filamentous fungi belonging to the genera *Aspergillus, Cladosporium, Fusarium, Penicillium* and *Trichoderma*; two yeasts (*Sporobolomyces roseus* and *Meyerozyma guilliermondii*); and one yeast-like filamentous fungus (*Aureobasidium pullulans* var. *melanogenum*) using the checkerboard method. Among the 29 interactions observed, the tested combinations of concentrations showed mainly additive (16 instances) and, to a lesser extent, indifferent (12 instances) properties, with notably 2–8 times lower concentrations of compounds needed to suppress fungal growth than those recorded for the individual compounds. A synergistic effect was observed only for *Aspergillus niger* when 0.1 mg mL⁻¹ of p-cresol was supplemented with 0.2 mg mL⁻¹ of phenol. Furthermore, *A. niger* was the only fungus where all three types of documented antifungal interactions, i.e., synergism, additivism and indifference, were observed. No antagonism between compounds was documented in any of the tested combinations. *Meyerozyma guilliermondii* was the only tested fungus where no interactions could be determined (MIC >1.0 mg mL⁻¹).

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РЕЗЮМЕ. Двупарноногие многоножки (Diplopoda) — это группа членистоногих, произрастающая и выделяющая разнообразные химические экскудаты из защитных желез в случае атаки хищника. Эти экзокринные выделения одновременно являются и потенциальными антимикробными агентами. Поскольку защитный секрет эндемичного балканского вида *Apfelbeckia insculpta* (L. Koch, 1867) состоит лишь из двух компонент с известными противогрибковыми свойствами (p-крезол и фенол), он представляет собой идеальную модель для изучения вклада отдельных компонент в общую фунгицидную активность этого натурального продукта, тем самым позволяя нам определить природу и тип взаимодействий между главной (p-крезол) и следовой компонентами (фенол). В 25 комбинаци-
я их концентраций, от 0,1 до 1,0 мг/мл, шахматным методом было проверено действие секрета на 14 видов мицелиальных грибков родов Aspergillus, Cladosporium, Fusarium, Penicillium и Trichoderma, два вида дрожжей (Sporobolomyces roseus и Meyerozyma guilliermondii) и один вид похожего на дрожжи мицелиального грибка (Aureobasidium pullulans var. melanogenum). Среди 29 наблюдавшихся взаимодействий проверяемые комбинации концентраций, в основном, оказывали аддитивное действие (16 случаев) и в меньшей мере были нейтральны (12 случаев) в концентрациях даже в 2–8 раз ниже требуемых, чтобы подавить рост грибков, чем для отдельных компонент. Синергетический эффект наблюдался только для Aspergillus niger при концентрации 0,1 мг/мл p-крезола с добавкой 0,2 мг/мл фенола. Более того, A. niger был единственным из грибков, где проявились все три типа документированных фунгицидных взаимодействий, т.е. синергизм, аддитивизм и нейтральность. Антагонизм не был выявлен ни в одной из тестируемых комбинаций компонент. Вид Meyerozyma guilliermondii был единственным тестируемым грибком, где не выявлено никаких взаимодействий (MIC >1,0 мг/мл).

Introduction

In contrast to faster moving and much more aggressive predators, organisms with a limited range of movement or limited control over their movements are usually well defended chemically [Berenbaum, 1995]. Many arthropod groups are included in the ranks of these organisms. Among arthropods that rely on chemical defence, millipedes (Diplopoda) are regarded as particularly “accomplished chemists” [Makarov, 2015; Shear, 2015; Ilić et al., 2018].

Besides the thick cuticle hardened by deposits of calcium [Hopkin, Read, 1992; Makarov, 2015], the majority of millipedes possess a serially arranged system of defensive glands (ozadenes), starting from the third or fifth trunk segment [Sierwald, Bond, 2007; Koch, 2015; Makarov, 2015; Shear, 2015]. Millipede defensive glands are places of production and storage of a wide range of chemical compounds — heterocyclic nitrogen-containing compounds, terpenes, benzoquinones and hydroquinones, fatty acid esters, various aliphatic compounds, phenolics and cyanogenic compounds [Makarov, 2015; Shear, 2015]. Among the Helminthomorpha, it is presumed that the phenolic defensive system is the oldest one [Rodriguez et al., 2018]. Although members of the order Callipodida are regarded as “phenolic” millipedes (sensu Eisner et al., 1978) because these millipedes rely exclusively on phenolic allomones [Ilić et al., 2019 and references therein], phenolics can also be found in the defensive secretions of members of other millipede orders, e.g., Polydesmida and Julida [Makarov, 2015; Shear, 2015].

Three phenolic compounds have been so far recorded in callipodidan defensive secretions: p-cresol, phenol and p-ethylphenol [Čurčić et al., 2009; Shear et al., 2010; Makarov et al., 2011; Ilić et al., 2019]. A common feature of all screened callipodidan defensive secretions is that p-cresol is the only or dominant compound. For example, p-cresol constitutes 99% of the defensive secretion in Apfelbeckia insculpta (L. Koch, 1867) (Figure) [Ilić et al., 2019], while phenol and p-ethylphenol can be present either as minor or trace...
compounds in other callipodidan defensive gland exudates [Curčić et al., 2009; Makarov et al., 2011; Ilić et al., 2019]. Millipede defensive secretions are in most cases bi-, oligo- or multicomponent mixtures. This fact, i.e., the existence of at least two chemical compounds in such gland exudates, is a precondition for different interactions (e.g. synergism or antagonism) between these chemicals. Both compounds that are recorded in the defensive secretion of A. insculpta (p-cresol and phenol) are known to possess high bactericidal and fungicidal activity [Murakami et al., 2014; Sabbineni, 2016] and are responsible for the antimicrobial potency of this secretion, which was proven for the first time by Ilić et al. [2019] on a number of pathogenic bacteria, yeasts and filamentous fungi. As this defensive secretion consists of only two compounds, it represents an ideal model on which to study the contribution of individual compounds to the overall antifungal activity of this potent natural product. Here we report for the first time the nature and type of antifungal interactions between the main and trace compounds of the defensive secretion of a callipodidan millipede.

Material and methods

**Chemicals**

Components of the defensive secretion of A. insculpta, viz., p-cresol (≥99% purity) and phenol (≥99% purity), were obtained from Sigma-Aldrich (Germany). Prior to the experiment, both compounds were dissolved in 30% ethanol (Zorka Pharma, Serbia).

**Tested fungi**

To study the type of interaction between p-cresol and phenol in the defensive secretion of A. insculpta, we used 14 filamentous fungi (Aspergillus creber BEOFB 3250m, A. flavipes BEOFB 391m, A. flavus BEOFB 313m, A. fumigatus BEOFB 321m, A. niger BEOFB 343m, Cladosporium cladosporioides BEOFB 1821m, C. uredinicola BEOFB 1841m, Fusarium verticillioides BEOBF 802m, Gibberella zeae BEOBF 820m, Penicillium digitatum BEOBF 1112m, P. griseofulvum BEOBF 1151m, P. lanosum BEOBF 1162m, P. rubens BEOBF 1181m and Trichoderma citrinoviride BEOBF 1220m), two yeasts (Sporobolomyces roseus BEOBF 4100m and Meyerozyma guilliermondii BEOBF 3001m) and one yeast-like filamentous fungus (Aureobasidium pullulans var. melanogenum BEOBF 4200m). All fungi used in the study came from the surface of 7-day-old Malt Extract Agar – MEA (Lab M Limited, UK) slants with sterile saline solution (0.9% NaCl, HemofarmhospitalicaLogica, Serbia) supplemented with 0.1% Tween 20 (v/v). The final concentration of suspensions was adjusted to 1.0 × 10^6 CFU mL⁻¹. Before use, a measured volume (10 µL) of suspensions was cultured on MEA plates to check validity of the inocula and verify the absence of contamination.

**Determination of interaction type**

The type of interaction between compounds of the defensive secretion of A. insculpta was assessed by the checkerboard method [Schwalbe et al., 2007]. Malt extract broth medium (of the following composition, per litre of deionized water: malt extract, 40 g; pH 6.8) was distributed into each well of 96-well microplates (F-bottom, Ratiolab). Two-fold serial dilutions of p-cresol and phenol were made individually, on two separate plates, with concentrations in the range of from 0.1 to 1.0 mg mL⁻¹. Next, well contents were mixed on a single plate and 25 combinations of concentrations were tested. Suspensions of tested fungi (10 µL) were added to each well reaching a final volume of 100 µL per well. After 72 h of incubation at 25 ± 2°C (UE 500, Memmert, Germany), the lowest concentrations without apparent growth observed under a Zeiss Stemi DV4 binocular microscope (Germany) were defined as minimum inhibitory concentrations (MICs) for certain combinations of compounds. The type of interaction (synergism, additivism, indifference or antagonism) of all tested combinations was then determined. The MICs of individual compounds and various tested combinations were transformed into fractional inhibitory concentrations (FIC) according to the following formulas:

\[
\text{FIC}_A = \frac{\text{MIC of compound } A}{\text{MIC of compound } A \text{ in the presence of compound } B} \\
\text{FIC}_B = \frac{\text{MIC of compound } B}{\text{MIC of compound } B \text{ in the presence of compound } A}
\]

The fractional inhibitory concentration index (FICI) was calculated from the FIC values for each compound as follows:

\[
\text{FICI} = \text{FIC}_A + \text{FIC}_B
\]

where A represents p-cresol and B represents phenol in combinations. The combination was considered synergistic when FICI was ≤0.5, additive when FICI was >0.5 – 1, indifferent when FICI was >1 – ≤4 and antagonistic when FICI was >4 [van Vuuren, Viljoen, 2011].

**Results**

The obtained MIC values, transformed into FIC and FICI, were used to define the nature and type of interactions. Individual MICs for p-cresol and phenol, MICs of tested combinations with the type of interaction determined and MIC reduction folds are summarized in Table.

With MICs ranging from 0.1 to 0.8 mg mL⁻¹, p-cresol was more potent in inhibiting fungal growth compared to phenol (MICs: 0.1 to >1.0 mg mL⁻¹). In both instances, P. digitatum was the most susceptible
fungus (MIC 0.1 mg mL\(^{-1}\)), while \(p\)-cresol also demonstrated the same range of activity against \(P.\ griseofulvum\) and \(T.\ citrinoviride\). With MICs of 0.8 and >1.0 mg mL\(^{-1}\) for \(p\)-cresol and phenol, respectively, \(M.\ guilliermondii\) was the most resistant tested fungus, followed by \(A.\ flavus, A.\ niger, F.\ verticillioides\) and \(F.\ graminearum\) (MICs of 0.4 and 0.8 mg mL\(^{-1}\) for \(p\)-cresol and phenol, respectively).

Due to the fact that the sensitivity of \(M.\ guilliermondii\) to phenol was above the tested range of concentrations (MIC >1.0 mg mL\(^{-1}\)), no FIC and FICI values could be calculated, and hence no interactions between compounds could be analysed. Among 29 determined interactions, the tested combinations of concentrations showed mainly additive (16 instances: FICI 0.625–1.000) and to a lesser extent indifferent (12 instances: FICI 1.125–2.000) properties, with notably lower concentrations (two to eight times lower) of compounds needed to suppress fungal growth than those recorded for individual MIC values (Table). A syner-

| Tested microfungi          | MIC of individual compounds | MIC of compounds in a mixture | MIC reduction fold | FICI | Interaction type |
|----------------------------|-----------------------------|-------------------------------|--------------------|------|------------------|
|                            | \(p\)-Cresol | Phenol | \(p\)-Cresol | Phenol | \(p\)-Cresol | Phenol |                   |
| \(A.\ creber\)             | 0.2          | 0.4    | 0.1          | 0.1    | 2              | 4      | 0.750             | Ad   |
| \(A.\ flavipes\)          | 0.4          | 0.4    | 0.2          | 0.2    | 2              | 4      | 0.750             | Ad   |
| \(A.\ flavus\)            | 0.4          | 0.8    | 0.2          | 0.2    | 2              | 4      | 0.750             | Ad   |
| \(A.\ fumigatus\)         | 0.2          | 0.4    | 0.2          | 0.1    | 2              | 8      | 0.625             | Ad   |
| \(A.\ niger\)             | 0.4          | 0.8    | 0.2          | 0.1    | 4              | 2      | 0.750             | Ad   |
| \(A.\ pullulans var. melanogenum\) | 0.2          | 0.4    | 0.2          | 0.2    | 4              | 2      | 1.500             | I    |
| \(C.\ cladosporioides\)   | 0.2          | 0.2    | 0.1          | 0.1    | 2              | 2      | 1.000             | Ad   |
| \(C.\ urdeuciola\)        | 0.2          | 0.2    | 0.2          | 0.1    | 2              | 2      | 1.500             | I    |
| \(F.\ verticillioides\)   | 0.4          | 0.8    | 0.2          | 0.2    | 4              | 2      | 0.750             | Ad   |
| \(G.\ zeae\)              | 0.4          | 0.8    | 0.4          | 0.1    | 8              | 2      | 1.125             | I    |
| \(M.\ guilliermondii\)    | 0.8          | > 1.0  | –            | –      | –              | –      | –                 | –    |
| \(P.\ digitatum\)         | 0.1          | 0.1    | 0.1          | 0.1    | 4              | 4      | 2.000             | I    |
| \(P.\ griseofulvum\)      | 0.1          | 0.2    | 0.1          | 0.1    | 2              | 2      | 1.500             | I    |
| \(P.\ lanosum\)           | 0.2          | 0.2    | 0.1          | 0.1    | 2              | 2      | 1.000             | Ad   |
| \(P.\ rubens\)            | 0.2          | 0.4    | 0.2          | 0.1    | 4              | 2      | 1.250             | I    |
| \(S.\ roseus\)            | 0.4          | 0.2    | 0.2          | 0.2    | 2              | 2      | 1.500             | I    |
| \(T.\ citrinoviride\)     | 0.1          | 0.4    | 0.1          | 0.1    | 4              | 2      | 1.500             | I    |

Ad — additive effect; FICI — fractional inhibitory concentration index; I — indifferent effect; S — synergistic effect; wr — without reduction of MIC value; — combinations and interactions not determined.

Table. Interaction type in a mixture of \(p\)-cresol and phenol from the defensive secretion of \(Apfelbeckia insculpta\). Minimum inhibitory concentrations (MICs) are expressed in mg mL\(^{-1}\).
The antifungal potential of the defensive secretion of *A. insculpta* and its individual compounds (*p*-cresol and phenol) was evaluated in several studies to date on a number of pathogenic yeasts (*Candida albicans, C. krusei, C. tropicalis* and *C. dubliniensis*) and filamentous fungi (*Aspergillus fumigatus, A. niger, Cladosporium cladosporioides, C. uredincola, Fusarium verticilloides, F. graminearum, Penicillium griseofulvum, *P. lanosum* and *P. rubens*) [Gallucci et al., 2014; Ilić et al., 2019]. Phenolic compounds (*p*-cresol and phenol among them) are generally known as potent antimicrobial agents [Gallucci et al., 2014]. Their molecular structure is optimal to permeate cell membranes and alter cellular permeability [Brocca et al., 2013]. Specifically, several molecular properties of phenolics have been linked with their antifungal potential – properties such as lipophilicity, reactivity and ability to form specific interactions between the phenolic compounds and their target receptor in cellular lipoprotein membranes [Dambolena et al., 2012; Gallucci et al., 2014; Pizolitto et al., 2015]. The last mentioned characteristic of phenolic compounds is mirrored by their ability to inactive microbial adhesins which lead to the leakage of cytoplasmic contents [Velásquez et al., 2019].

Every phenolic compound has different bioactive properties depending on its structure, number of aromatic and hydroxyl groups and their distribution in the structure [Pizolitto et al., 2015]. Both phenolics that are present in the defensive secretion of *A. insculpta* are monohydroxyl compounds, with the difference being that *p*-cresol has an additional methyl group on the fourth carbon atom of the benzene ring. This difference in structure can be seen as the reason for the difference in the level of antifungal activity between phenol and *p*-cresol which was observed in the present study. The general trend observed in our experiment was that the tested fungi are more susceptible to *p*-cresol than to phenol. Alexieva et al. [2008] tested the effects of mixtures of phenol and methyl-substituted phenols (*o*-m-, *p*-cresol) on the growth and degradation capacity of *Trichosporon cutaneum* and showed that *p*-cresol exhibited a stronger toxic effect on this fungal species than did phenol. Our data further corroborate this finding.

However, interactions between these compounds that result in potentially stronger or weaker overall antifungal activity of the secretion were never studied before. The widely accepted way of measuring interaction, i.e., the checkerboard method, which is based on calculation of the fractional inhibitory concentration index, was used for this purpose. This index is expressed as the interaction of two agents where the concentration of each tested agent in combination is expressed as a fraction of the concentration that would produce the same effect when employed independently, and it is used to define the nature and type of interactions [Berenaun, 1978; van Vuuren, Viljoen, 2011]. Both compounds when combined in various ratios required notably lower concentrations to suppress fungal growth than those recorded for individual MIC values. Most frequently, regardless of the tested fungal species, *p*-cresol and phenol produced a total effect that was equal to the sum of individual effects of the compounds, i.e., an additive effect, while a much greater effect of combination than the sum of effects of the individual compounds, i.e., a synergistic effect, was documented in only one instance in the case of *A. niger*. The rest of the tested combinations resulted in an indifferent effect of compounds.

Although our data point to the existence of synergism or additivism between *p*-cresol and phenol, our study also shows that indifference is usually present in mixtures with a higher proportion of *p*-cresol. Data obtained from such combinations are of interest because the defensive secretion of *A. insculpta* is characterized by the dominance of *p*-cresol. Thus, this type of interaction is present in the native defensive secretion of *A. insculpta*. However, we cannot generalize that only indifference exists between the two phenolics present in this callipodidan defensive secretion. Data from a previous study of ours [Ilić et al., 2019] indicate that the defensive secretion of *A. insculpta* has a stronger antifungal effect against two *Candida* species (*C. krusei* and *C. tropicalis*) than *p*-cresol and phenol. The minimum fungicidal concentrations of the tested diploid defensive secretion had three and six time lower values than either of the phenolics that constitute this secretion. In view of this result, it is apparent that a synergistic effect can also be present between *p*-cresol and phenol in the defensive secretion of *A. insculpta*.

Despite the fact that phenolic compounds are effective against various microorganisms, there are bacterial and fungal species that have the metabolic capability of using them as growth substrates [Alexieva et al., 2008; Gérecová et al., 2015 and references therein]. For example, some yeasts of the genera *Rhodotorula, Trichosporon* and *Candida* can metabolize phenolic compounds as a source for growth [Piakong et al., 2009; Karimi, Hasanshahian, 2016]. Utilization of phenolics as a substrate for growth can be achieved through several metabolic pathways. Specifically, phenol is degraded to catechol and further through a series of steps to succinate and acetyl coenzyme A via the catechol
branch of the 3-oxoadipate pathway [Gerecová et al., 2015]. Degradation of p-cresol by several fungal species has also been recorded [Kennes, Lema, 1994; Alexieva et al., 2008]. Our data show that survival of the yeast M. guillermondii was not affected by p-cresol and phenol, or by different combinations of these two phenolics. This result can be explained by the fact that the fungal species in question has the ability to degrade phenol and use it as a source of carbon and energy [Kari, Hassanshahian, 2016]. To our knowledge, there are no data indicating the ability of M. guillermondii to metabolize p-cresol, but we presume that this yeast can also survive and use p-cresol as a source for growth.

Both compounds of the defensive gland secretion of A. insculpta are structurally simple, but their biological activities can be diverse. In light of the knowledge that arthropod defensive secretions usually act parsimoniously [Blum et al., 1996], it would be interesting to test whether and how each compound (or class of chemical compounds) of the defensive secretion of A. insculpta (and other millipedes) contributes to biologically interactions between millipedes and other organisms, especially in acting against pathogenic bacteria and various invertebrate and vertebrate predators.

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