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Original Article

Strong antimicrobial and low insecticidal activity of mandibular gland reservoir content in Bornean "exploding ants" *Colobopsis explodens* Laciny & Zettel, 2018 (Hymenoptera: Formicidae)

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Abstract

Minor workers of some ant species belonging to the *Colobopsis cylindrica* (Fabricius, 1798) (COCY) species group can suicidally eject their sticky and potentially toxic mandibular gland reservoir content (MGRC) to ward off putative arthropod opponents. Since the MGRC can also be ejected non-suicidally as droplets at the mandible base, it was hypothesized that the secretion also serves other roles than just defense. In former studies, a range of potentially antimicrobial compounds has been identified in the MGRCs of different COCY species, which is why a function in shaping of the ant-associated microbiome has been proposed. Here, we aimed to assess the putative insecticidal and antimicrobial properties of the MGRC of the COCY species *Colobopsis explodens* Laciny & Zettel, 2018. For this, we conducted in-situ confrontation assays with *C. explodens* and sympatric insects. In in-vitro studies, individuals of *Acheta domesticus* (Linnaeus, 1758) and *Atta sexdens* (Linnaeus, 1758) were treated with the isolated MGRC as well as with its identified dominant phenolic constituents, that is, 1-(2,4,6-trihydroxyphenyl)ethanone (monoacetylphloroglucinol, MAPG) and 5,7-dihydroxy-2-methylchromen-4-one (noreugenin) in separate approaches. To determine a possible antimicrobial effect, the MGRC as well as MAPG and noreugenin were tested individually on *Trichoderma* spp., *Candida* sp., *Bacillus velezensis*, and *Escherichia coli*. We showed that neither the naturally expelled secretion nor the isolated MGRC or its dominant compounds caused acute lethality of the tested insects. In contrast, antimicrobial assays with the MGRC resulted in growth inhibition of some microorganisms. When the antimicrobial activity of the major constituents was further assessed, MAPG, but not noreugenin, induced profound growth inhibition. The results suggest that the MGRC of *C. explodens* does not primarily act via deadly toxins, but that rather its adhesive properties are mainly responsible for rendering an arthropod opponent innocuous. The demonstrated antimicrobial potential of the MGRC further supports the hypothesis about its role in influencing the microbe community associated with COCY ants.

Key words: Adhesive secretion in insects, ant microbiome, autothysis, exocrine defensive glands, 2,4,6-trihydroxyacetophenone (THAP), monoacetylphloroglucinol (MAPG), 2-methyl-5,7-dihydroxychromone (noreugenin).

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Introduction

Colobopsis explodens Laciny & Zettel, 2018 is a recently described ant species belonging to the *Colobopsis cylindrica* (COCY) species group (Hymenoptera: Formicidae) (Laciny & al. 2018) occupying mostly dipterocarp trees in the lowland rainforests of South-East Asia (Davidson & al. 2007, Cook 2008). When threatened by another ant, a *C. explodens* minor worker suicidally ruptures its gastral integument to eject the content of its greatly enlarged mandibular gland reservoirs (MGRs, for detailed anatomical description please refer to Davidson & al. 2012 and Fig. S1 in Appendix, as digital supplementary material to this article, at the journal's web pages). The targeted opponent is subsequently entangled by the sticky secretion and thus unable to move (Maschwitz & Maschwitz 1974, Davidson & al. 2016). This suicidal behavior is shown by several species belonging to the COCY species group in various intensities (e.g., *Colobopsis saundersi* (Emery, 1889), as well as the hitherto undescribed *Colobopsis* spp. "all red" and "red head yellow goo", as nick-named by Cook 2008) and leads to their common name "exploding ants". This phenomenon is described as autothysis, or suicidal altruism via secreting of a harmful substance onto a potential rival through rupturing of the body (Maschwitz & Maschwitz 1974). Autothysis is rare among other insects, with only some termite and one aphid species known to use it (reviewed by Shorter & Rueppell 2012).

Due to the observed suicidal release of the MGR content (MGRC) by threatened *Colobopsis explodens* minor workers, its main use has been assumed to be a defensive secretion against arthropod opponents (Maschwitz & Maschwitz 1974, Jones & al. 2004). It was further suggested that the adhesive properties of the secretion are substantial for making an opponent innocuous (Jones & al. 2004, Voegtle & al. 2008, Davidson & al. 2016). The sticky secretion entangles the opponent and thus heavily restricts its motility (Maschwitz & Maschwitz 1974). It is initially fluid and becomes more viscous shortly after being expelled, which results in the irreversible gluing of the exploded *Colobopsis cylindrica* (Fabricius, 1798) ant to the opponent (Maschwitz & Maschwitz 1974). Two valuable publications dealing with exocrine glands and the role of adhesion in predator defense in arthropods are provided by Betz & Kölsch (2004) and Betz (2010). Although insect defensive secretions are suggested to act mostly mechanically, irritating compounds might be mixed with the secretion and function chemically (Pasteels & al. 1983, Dettner 2010). These irritants are usually quite volatile and rather reactive compounds (Pasteels & al. 1983). They might comprise aromatic compounds, quinones, small aliphatic aldehydes, ketones, acids, or monoterpenes (Betz 2010). Terpenoids, which can be considered as toxicants, irritants and alarm pheromones, have been reported from only a few COCY species (Jones & al. 2004, Davidson & al. 2016, Hoenigsberger & al. 2019). In contrast, hydrocarbons with supposed slight toxicity have been found in the MGRCs of all COCY species investigated to date (Jones & al. 2004, Davidson & al. 2016, Hoenigsberger & al. 2019). They may act as surfactants, penetrators or evaporatory retardants, but may also interfere with chemoreception (Pasteels & al. 1983). A number of potentially irritant or corrosive phenolic compounds have been identified in high quantities in the MGRs of all COCY species analyzed to date (Jones & al. 2004, Davidson & al. 2016, Sakolrak & al. 2018, Hoenigsberger & al. 2019). When the MGRC of *C. explodens* was analyzed in a recent study by Hoenigsberger & al. (2019), the phenolics benzene-1,3,5-triol (phloroglucinol), 1-(2,4,6-trihydroxyphenyl)ethanone (monoacetylphloroglucinol, MAPG), 5,7-dihydroxy-2-methylchromen-4-one (noreugenin) and 1-(3-acetyl-2,4,6-trihydroxyphenyl)ethanone (2,4-diacetylphloroglucinol) were the dominating compounds. It was hypothesized that these compounds may contribute to the assumed toxicity of the secretion to arthropod opponents (Jones & al. 2004) and thus enhance the defensive effect of the sticky secretion.

In addition to the insecticidal activity, several other roles of the MGRC have been proposed, including roles in aposematism and shaping of the ant-associated microbiome (Jones & al. 2004, Davidson & al. 2009, Davidson & al. 2016). The suggested aposematic property might be due to the bright colors of the MGRC, ranging from white to yellow, orange and red in the different species of the *Colobopsis cylindrica* (COCY) group (Jones & al. 2004). When workers are disturbed, they can raise their gaster to display the MGRC, which is then visible through the intersegmental membranes (Jones & al. 2004). Defensive secretions of insects often contain antibacterial and antifungal agents used for colony- or nest hygiene (e.g., Cremer & al. 2007, Morgan 2008, Dossey 2011). Also for COCY ants, the use of their MGRCs as antiseptics, has been proposed (Davidson & al. 2009, Davidson & al. 2016, Hoenigsberger & al. 2019). It was observed, that at least some COCY species can eject the MGRC at their mandible base, but without eliciting a suicidal explosion (Maschwitz & Maschwitz 1974, Davidson & al. 2016 and Fig. S2). It was speculated, that the adhesive and potentially antimicrobial MGRC may be applied onto the ant's body via self-grooming (Fig. S5 in Davidson & al. 2016) and thus serve for hygienic purposes. Moreover, *Colobopsis explodens* was reported to deposit its MGRCs on floors and plastic nest chambers (Davidson & al. 2009), which may indicate a nest hygiene behavior. Indeed, the phloroglucinol compounds identified as main compounds in the MGRCs of diverse COCY species have reported antibacterial and antifungal properties (Guihen & al. 2004, Davidson & al. 2009, Hoenigsberger & al. 2019). The hypothesis that COCY ants accumulate antimicrobial compounds for nest- or colony hygiene in their MGRs is further supported by the findings that the MGRC of an ant species presumably belonging to the COCY species group (Sakolrak & al. 2018, ant species herein designated as *Colobopsis saundersi* Emery, 1889), was shown to inhibit spore germination of hypocrealean entomopathogenic fungi *Ophiocordyceps polyrhachis-furcata* and *Beauveria bassiana* (Sakolrak & al. 2018). Intriguingly, the

main constituents of the gland were reported to be four phenolic compounds, including MAPG and noreugenin (Sakolrak & al. 2018). Interestingly, the metapleural gland, generally assumed to contain the antiseptic constituents used for hygiene by Myrmicinae and Formicinae, is secondarily absent in most Camponotini (Maschwitz & al. 1970, Maschwitz 1974, Hölldobler & Engel-Siegel 1984, Fernández-Marín & al. 2006, Graystock & Hughes 2011, Yek & Mueller 2011). It can therefore be speculated that the hypertrophied MGRs have adopted the functions of the metapleural glands in COCY minor workers (Voegtle & al. 2008, Davidson & al. 2016). The antimicrobial compounds may not only be beneficial for warding off ant-pathogens, but may also support the establishment and conservation of symbiotic microorganisms associated with *Colobopsis cylindrica* ants (Davidson & al. 2009). Similar to other ant species (Mueller 2012, Kautz & al. 2013, Lucas & al. 2017, Moreau 2020), also COCY ants are associated with potentially beneficial microbes, as the mycoparasitic *Trichoderma* spp. fungi or *Burkholderia* spp. bacteria (Davidson & al. 2009, Davidson & al. 2016). The roles of these potentially symbiotic microorganisms for the lifestyle of COCY ants has yet to be understood. In general, many social organisms rely on associations with mutualistic microbes, which can aid in nest mate recognition, serve as nutritional symbionts, or provide protection through production of antimicrobial compounds (Lucas & al. 2017, Moreau 2020). Foraging COCY workers were frequently seen "grazing" on leaf or bark surfaces, thereby picking mosses, lichens and fungi, which has previously been hypothesized to contribute to their nutrition (Davidson & al. 2016, Laciny & al. 2018). It has even been speculated that the extreme territoriality of COCY ants could have evolved to protect foraging grounds from contamination by intruding ants bearing potentially pathogenic microbes (Davidson & al. 2016).

This study aimed to investigate the putative roles of the MGRC of *Colobopsis explodens* minor workers in the defense against arthropod opponents and potentially pathogenic microorganisms. In-situ and in-vitro assays were performed to test for the possible insecticidal and antimicrobial effects of the MGRC itself as well as of its two identified major phenolic compounds MAPG and noreugenin.

Materials and methods

Ants: Minor workers of one *Colobopsis explodens* colony (for type colony identification see Laciny & al. 2018) were collected in the lowland tropical rain forest surrounding the Kuala Belalong Field Studies Center (KBFSC, 4° 32' 48.2" N, 115° 09' 27.9" E) located in the Temburong District of Brunei Darussalam in April 2015. Ants used for MGRC isolation were preserved, transported and dissected as described in detail in Hoenigsberger & al. (2018). In short, these ants were collected and immediately preserved by rapid freezing on-site. Samples were transported on dry ice to the laboratory and dissected under cooled conditions to obtain the MGRCs.

Chemicals and reagents: Table 1 shows the chemicals and reagents used in the presented experiments.

Extraction of MGRC constituents: Twenty-five ants were dissected to obtain five analytical samples, each consisting of pooled MGRCs taken from five ants (range: 3.9 - 5.9 mg MGRC wet weight per sample). Samples were extracted with ice-cold EtOAc using 1 mg wet weight of MGRC and 15 µl EtOAc. The mixture was then vortexed for 5 minutes at 7 °C room temperature, followed by a 10 min centrifugation step (4400 rpm, 7 °C). For estimation of extraction efficiency, four successive extractions per sample were carried out.

Preparation of standards, retention index (RI)-calibrants and blanks: For GC-MS analysis, individual standard solutions of MAPG and noreugenin were prepared in EtOAc. The RI values were established on the basis of n-alkanes. Therefore, the RI calibrant solution contained the following compounds: n-alkane standard solution C_8-C_{20}, n-alkane standard solution C_{21} - C_{40} and hexane (1 + 1 + 6 v / v / v). Vials containing pure EtOAc were used to record background noise (blank) chromatograms with the aim to recognize substances originating from the solvent or the GC-MS system.

Gas chromatography coupled with mass spectrometry (GC-MS): A 1µl aliquot of each EtOAc extract was injected into the gas chromatograph (Agilent 7890 A, Agilent Technologies, California, USA) fitted with a 30 m x 0.25 mm x 0.25 µm cross-linked 5%-phenyl 95%-methyl polysiloxane capillary column (HP-5MS UI, Agilent Technologies, California, USA). The oven program started at 65 °C, followed by an increase of 10 °C min^{-1} to reach 270 °C, and was then followed by an increase of 20 °C min^{-1} to 300 °C with a subsequent hold for 5 min. The gas chromatograph was coupled to an Agilent 5975 C mass selective detector (Agilent Technologies, California, USA). The MS scan range was *m/z* 45-500 at a scan rate of approximately 3 scans sec^{-1}.

Identification of MAPG and noreugenin: Peak detection, spectrum deconvolution (computational separation of co-eluting components to create a pure spectrum for each component, see also Du & Zeisel 2013), RI calibration, and comparison of RIs and mass spectra against either an in-house database or the libraries of Wiley Registry 10th Edition / NIST 2014 Mass Spectral Library (Wiley) were carried out with the open source software MetaboliteDetector (Hiller & al. 2009) version 3.1.Lisa20170127Ra-Linux (Hiller & Koschnitzki 2019, current version available online). For this, the software parameters for appearance, deconvolution, identification and quantification were set according to Hoenigsberger & al. (2018). After GC-MS measurement of standard solutions containing MAPG or noreugenin, their RIs and mass spectra were added to the in-house library implemented in MetaboliteDetector. For compound identification, the overall similarity score (OSS, Hiller & al. 2009), considering a combination of spectrum similarity and RI similarity, was used. When the OSS was equal to or greater than 0.9 after comparison of the experimental data

Tab. 1: Chemicals and reagents used in the presented study.

Chemical / reagent	Trivial name / abbreviation	Purity [%]	Obtained from
Ethyl acetate	EtOAc	99.8	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Propan-2-one	Acetone	99.9	
Carboxymethylcellulose sodium salt	CMC	≥ 99.5	
2-[2-[3,4-Bis(2-hydroxyethoxy) oxolan-2-yl]-2-(2-hydroxyethoxy) ethoxy]ethyl- dodecanoate	Tween 20		
Ethanol	EtOH	≥ 99.5	
D(+)-glucose			
Mueller-Hinton Broth (MHB) medium			
Water		purified by reverse osmosis and an ELGA Purelab Ultra-AN-MK2 system	Veolia Water (Vienna, Austria)
1-(2,4,6-Trihydroxyphenyl)ethan-1-one	Monoacetylphloroglucinol, MAPG	> 98	TCI Europe N.V. (Eschborn, Germany)
n-Hexane		GC-grade	Merck (Darmstadt, Germany)
Potato Dextrose Agar	PDA		Sigma-Aldrich (Vienna, Austria)
Yeast Nitrogen Base Without Amino Acids	YNB		
(1R,3R)-3-(2,2-Dibromoethenyl)-2,2-dimethylcyclopropane-1-carboxylate	Deltamethrin	Analytical standard	
Dimethyl sulfoxide	DMSO	≥ 99.5	
5,7-Dihydroxy-2-methylchromen-4-one	Noreugenin	≥ 99	MolPort (Riga, Latvia)
n-Alkane standard solution C_8-C_{20}			Fluka (Vienna, Austria)
n-Alkane standard solution C_{21}-C_{40}			

with the library entries, the compound was accepted as identified.

Quantification of MAPG and noreugenin: Additionally, the amount of MAPG and noreugenin were quantified in the MGRC of *Colobopsis explodens*. For GC-MS instrument calibration, stock solutions of MAPG and noreugenin (each 1000 mg L^{-1} in EtOAc) were used to prepare mixed calibration standards, containing 100, 200, 300, 400, 500, 600 and 700 mg L^{-1}, and 50, 100, 150, 200 and 250 mg L^{-1} of MAPG and noreugenin, respectively. Measurement of the calibrants and construction of calibration curves (linear regression, sum of least squares) were carried out together with the pooled MGRC samples and resulted for MAPG in y = 5,828x – 278,600 (R^2 = 0.9992; method coefficient of variation V_{x0} = 1.7%) and for noreugenin in y = 13,923x – 391,902 (R^2 = 0.9992, V_{x0} = 1.8%). In order to prevent column overloading and distorted peak shapes, the raw MGRC extracts obtained after first sample extraction were further diluted with EtOAc (1 + 9 v / v) and analyzed with the same GC-MS parameters as described above, but at a GC split ratio of 1:10. All successive extracts were measured without dilution at a split ratio of 1:2 (second extract) or splitless (third and fourth extract), respectively. For peak area determination the following compound-defining fragments were used: *m / z* 168 for MAPG and *m / z* 192 for noreugenin. Sample files were processed with the batch quantification tool of MetaboliteDetector with the following parameter settings: Compound matching: ΔRI 5; pure / impure 0.5; identification: ΔRI 10; pure / impure 0.5; quantification: Redetect all quantification ions and extended single ion chromatogram (SIC) scan activated. From the four replicate extractions, the extraction efficiency of the first extraction stage was estimated to be 93% and 94% for MAPG and noreugenin, respectively (Tab. S1). Since extraction efficiencies ranged close to 100%, quantification was based on the extracts obtained after initial extractions and results were not further corrected for extraction losses.

Activity assays with MGRC, MAPG, and noreugenin: To investigate for the potential insecticidal and antimicrobial activities of the MGRC, as well as of MAPG and noreugenin, in-situ and in-vitro assays with arthropod

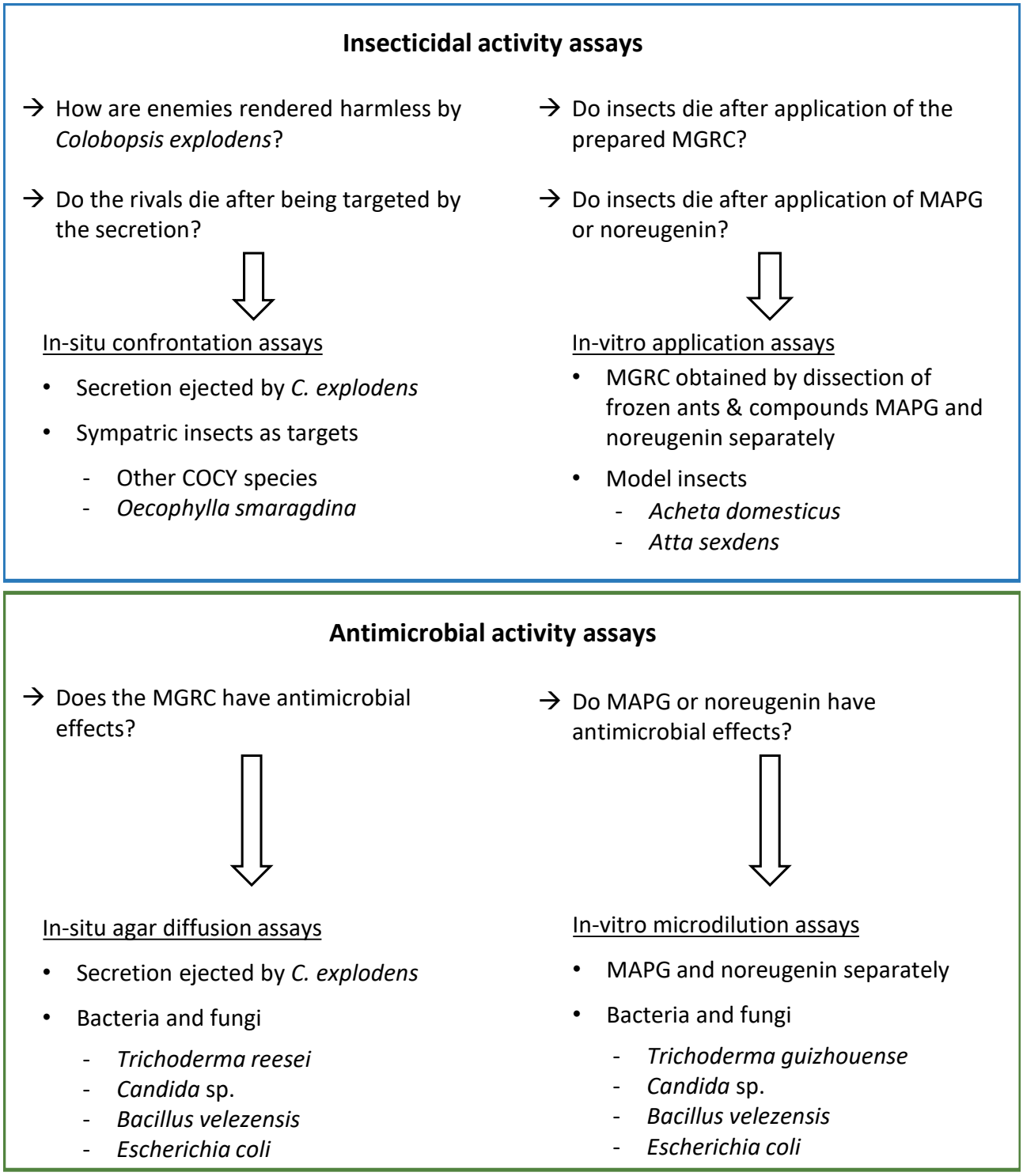

Fig. 1: Experimental questions and schematic overview of experiments. The in-situ assays were carried out with ejected defense secretion in the natural environment of *Colobopsis explodens*. The in-vitro assays were carried out either with the MGRC as obtained from dissected ants, or with artificial standard compounds MAPG and noreugenin.

insects and microbes were conducted. Figure 1 gives an overview about the activity assays and the underlying research questions. To investigate for the deadly potential of *Colobopsis explodens*' MGRC on insect opponents, confrontation assays with *C. explodens* minor workers and other ants, collected at the field site, were conducted. The prepared MGRC and the major constituents MAPG and noreugenin were then tested separately on surrogate insect opponents in vitro.

Insecticidal activity assays: To observe how arthropod rivals are rendered harmless by the MGRC, in-situ confrontation assays with *Colobopsis explodens* workers

and sympatric insects were performed. For this, the following ants were sampled around the field site: (i) minor *Colobopsis explodens* workers (n = 38), (ii) individuals belonging to four different species of the *Colobopsis cylindrica* (COCY) species complex, which were approximately of the same size as a *C. explodens* minor worker (n = 7 for species "all red", "*near saundersi*" and "barbecue" and n = 10 for "*saundersi*"; undescribed species sensu Cook 2008), as well as (iii) the much larger, non-COCY formicine ant species (*Oecophylla smaragdina* (Fabricus, 1775, n = 7)). Before conducting the confrontation experiments, the ants were kept separately in 50 mL tubes for 30 to 60 min to calm down from sampling- and transport-related stress. For each confrontation scenario, a *C. explodens* minor worker was placed in a Petri dish together with one ant individual from another species (different COCY species or *O. smaragdina*). To minimize accumulation of volatile compounds in the Petri dish, the lid was removed immediately after *C. explodens* had grabbed the opponent. In addition, confrontations were conducted between *O. smaragdina* individuals and individuals from another, unidentified non-COCY species (n = 7) under the same conditions. The number of dead ants and their time of death was evaluated within an observation period of 12 h. When an ant showed no reaction to being touched with forceps, it was considered dead. Due to the limited number of the hard-to-collect COCY ant samples, the confrontation assays were not repeated. These in-situ experiments should be considered as groundwork for the following in-vitro assays, in which the lethal effects of the pure phenolic compounds on insects were investigated.

To observe the potentially fatal effects of the isolated MGRC and the individual major phenolic MGRC compounds MAPG and noreugenin on insects, in-vitro tests were performed. Since the preparation and handling of the individual materials was not feasible in the field, these experiments were carried out in the laboratory in Austria. Due to governmental regulations prohibiting the export of living *Colobopsis cylindrica* individuals or sympatric insects from their natural habitat in Brunei, house crickets (*Acheta domesticus* (Linnaeus, 1758); purchased in a pet shop) and small individuals of leafcutter ants (*Atta sexdens* (Linnaeus, 1758)*;* generously provided by the Zoo Schönbrunn, Vienna, Austria) were used as surrogate target species. During the experiments the insects were kept in a terrarium conditioned to 28 °C and 95% relative humidity. The MGRC of one dissected *Colobopsis explodens* minor worker (MGRC has an average weight of 1 mg) was applied onto each insect, specifically on body regions, which had also been targeted by *C. explodens* MGRC in the in-situ confrontations. For the smaller *Atta sexdens* individuals (n = 10), the gland content was smeared onto the head, antennae and front thorax including the first pair of legs. For the larger *Acheta domesticus* (n = 10) the gland content was applied onto the head (including antennae) and parts of the front thorax only. Due to the limited number of frozen *C. explodens* samples, this assay could not be repeated. For testing MAPG and noreugenin, each compound was mixed with a viscous "surrogate matrix" (CMC + H_2O 1 + 34 w / v) to yield the determined concentrations of approximately 6% for MAPG and 1.5% for noreugenin (see Results). Then, 0.9 mg ± 0.2 mg of these CMC solutions containing the respective compound were applied separately onto *Acheta domesticus* (n = 10). CMC + H_2O alone served as negative controls. The insecticide deltamethrin was used as positive control. To this end, a 1 g L^{-1} solution of deltamethrin was prepared in acetone, then further diluted 1+9 (w/v) with CMC + H_2O (1 + 34 w / v). Thereof, 0.9 mg ± 0.2 mg of the CMC-deltamethrin mixture, which corresponds to 0.1 ng of deltamethrin, was applied onto the head, antennae and front thorax of A*cheta domesticus* (n = 10). The ants were observed for their death or survival within 12 h after treatment. The insects were evaluated as dead, when there was no movement after being touched with forceps.

Antimicrobial activity assays: The in-situ and in-vitro antimicrobial assays were performed with a set of model organisms stored in the TUCIM Collection of Industrial Microorganisms, TU Wien, Vienna, Austria. MGRCs isolated from *Colobopsis explodens* were tested by the agar diffusion method against *Trichoderma reesei* TUCIM 7201 (Hypocreales, Ascomycota, Fungi; *tef1* DNA, GenBank accession number: MT894432), *Candida* sp. TUCIM 7204 (Saccharomycetales, Ascomycota, Fungi; ITS1 and 2 rRNA, GenBank accession number: MG189904.1), *Bacillus velezensis* TUCIM 5485 (Gram positive, Bacillales, Firmicutes; 16S rRNA, GenBank accession number: CP006890.1) and *Escherichia coli* TUCIM 4420 (Gram-negative, Enterobacterales, Proteobacteria; 16S rRNA, GenBank accession number: NC_000913.2). To this end, suspensions of the tested microorganisms (OD_{600nm}: 0.1) were inoculated on the surface of a PDA plate by streaking the swab. Five µL of a MGRC suspension (containing MGRCs ejected by 20 *C. explodens* worker ants dissolved in 180 µL sterile water) were added to the middle of the plate which was subsequently incubated for 24 h (bacteria) and 48 h (fungi) at 28 - 30 °C. The ejection of the MGRC was triggered by the soaking of an ant in 70% ethanol for 3 seconds (Fig. S3). The assays were repeated three times with new cultures and freshly prepared MGRC.

The antimicrobial activity of MAPG and noreugenin was assessed by the broth microdilution method in 96-well polystyrene F-bottom plates. MAPG was dissolved to a stock concentration of 40 g L^{-1} in acetone. Mueller Hinton broth was used for the assessments of *Escherichia coli* MG1655, *Bacillus velezensis* TUCIM 5485, and *Trichoderma guizhouense* NJAU 4742 (Druzhinina & al. 2018). Yeast Nitrogen Base medium + 1.5% glucose was used for the tests with *Debaryomyces* sp. TUCIM 5826 (Saccharomycetales, Ascomycota, Fungi; ITS1 and 2 rRNA, GenBank accession number: MG189903.1). To 100 µl broth per well MAPG was added and serially diluted, resulting in initial concentrations ranging from 8-4096 µg mL^{-1}. Subsequent adding of 100 µl microbial suspension with a concentration of 10^5 CFU mL^{-1} resulted in the final concentration of $5x10^4$ CFU mL^{-1} per well and 4-2024 µg

mL^{-1} MAPG. A sterility control with MHB only, YNB + 1.5% glucose respectively, a growth control without any of the components, as well as a solvent control with acetone were done in the same microplate. The 96-well plates were then incubated at 28 °C for 24 h for *E. coli* and *B. velezensis* or for 66 h for *Debaryomyces* sp. and *T. guizhouense*, and the optical density at 600 nm (OD_{600nm}) was determined by using the microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA) after 12, 18 and 24 h of incubation for bacteria or after 18, 42 and 66 h of incubation for fungi, respectively. The same experiment was performed for noreugenin, with a concentration range from 0-169 mg L^{-1} (see chapter "in-vitro antimicrobial assays with noreugenin" in Appendix). The biomass of each type of microorganisms, cultured either with solvent, or with different concentrations of MAPG, was assessed in triplicates.

Results

Identification and quantification of MAPG and noreugenin in the MGRC of *Colobopsis explodens*: MAPG and noreugenin were identified in all MGRC samples analyzed for this study. As already reported in Hoenigsberger & al. (2019), those compounds clearly dominated the GC-MS chromatograms of all analyzed MGRC extracts. For MAPG, the arithmetic mean concentration was 396 ± 31 mg L^{-1} (n = 5, standard error of the mean). For noreugenin, the extracts contained 106 ± 6 mg L^{-1} (n = 5, standard error of the mean). This corresponds to 59 ± 5 µg MAPG and 16 ± 0.9 µg noreugenin per 1 mg of fresh MGRC. Thus, together these two compounds constituted 7.5 ± 1% of the freshly isolated MGRC.

Defense behavior and effect of MGRC on sympatric insects: In the 38 one-to-one combats between *Colobopsis explodens* minor workers and individual ants from other species, autothysis behavior was observed in all *C. explodens* individuals, except in five out of ten confrontations with "*saundersi*" workers. The *C. explodens* minor worker first attached itself to its opponent by grabbing either one of the antennae or a front leg with its mouthparts, before expelling the sticky defensive secretion onto the rival. In all cases, the first slightly viscous, yellow secretion hardened after being ejected, thereby gluing the *C. explodens* minor worker irreversibly to its opponent (Fig. 2 and video "Defense behavior of Cexplodens" in Appendix). None of the targeted ants was able to detach itself from the "exploded" *C. explodens* worker during the observation period of 12 h. All *C. explodens* workers died ≤ 5 min after suicidal ejection of the secretion. When in confrontations with ants of approximately the same size as a *C. explodens* minor worker, the MGRC targeted large areas of the opponent's body, such as head (including antennae), mesosoma and legs (Fig. 2A and video "Defense behavior of Cexplodens"). In one scenario, the MGRC targeted only one hind leg of an "all red" worker, which did

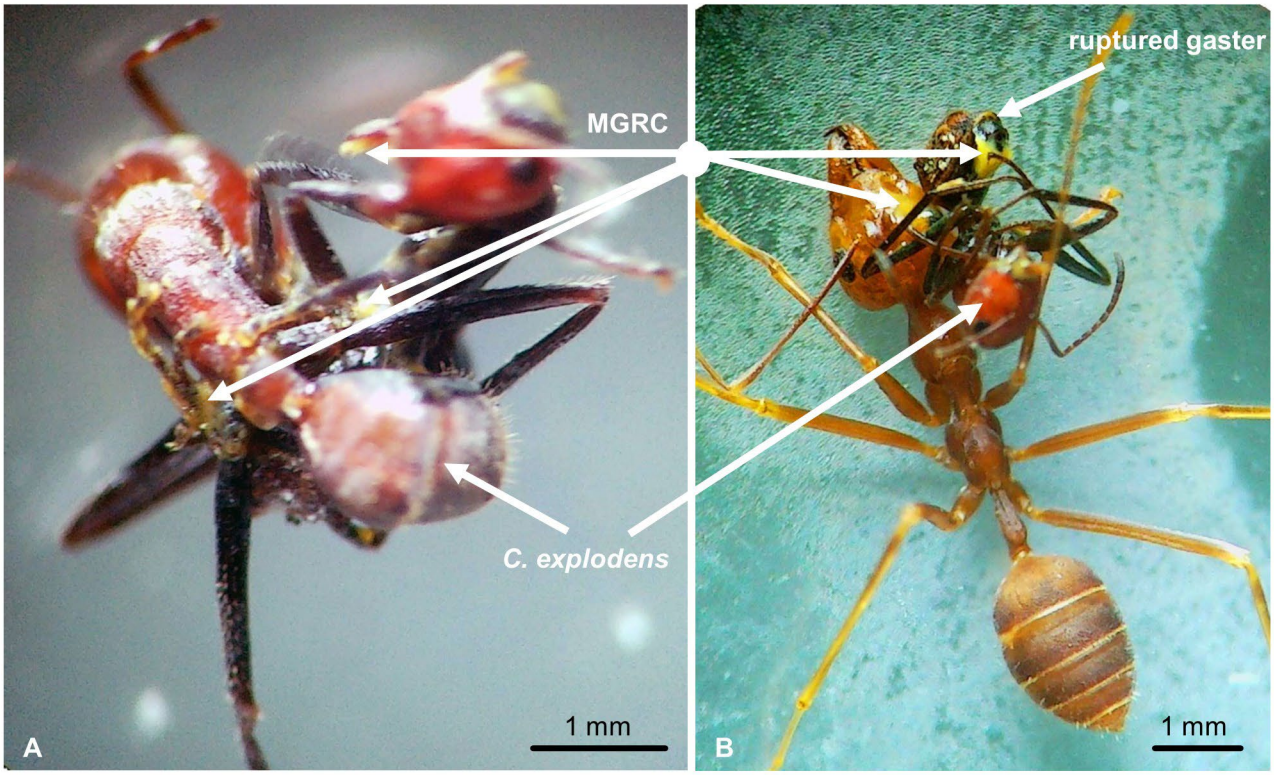

Fig. 2: Images of *Colobopsis explodens* minor workers rupturing their gasters to suicidally eject the yellow MGRCs onto the opponents. (A) The MGRC is dispersed over the head (including eyes, antennae and mandibles), the mesosoma and legs of an individual from another COCY species of approximately the same size as *C. explodens*. (B) *C. explodens* ejects its MGRC onto the head of the much larger *Oecophylla smaragdina* worker.

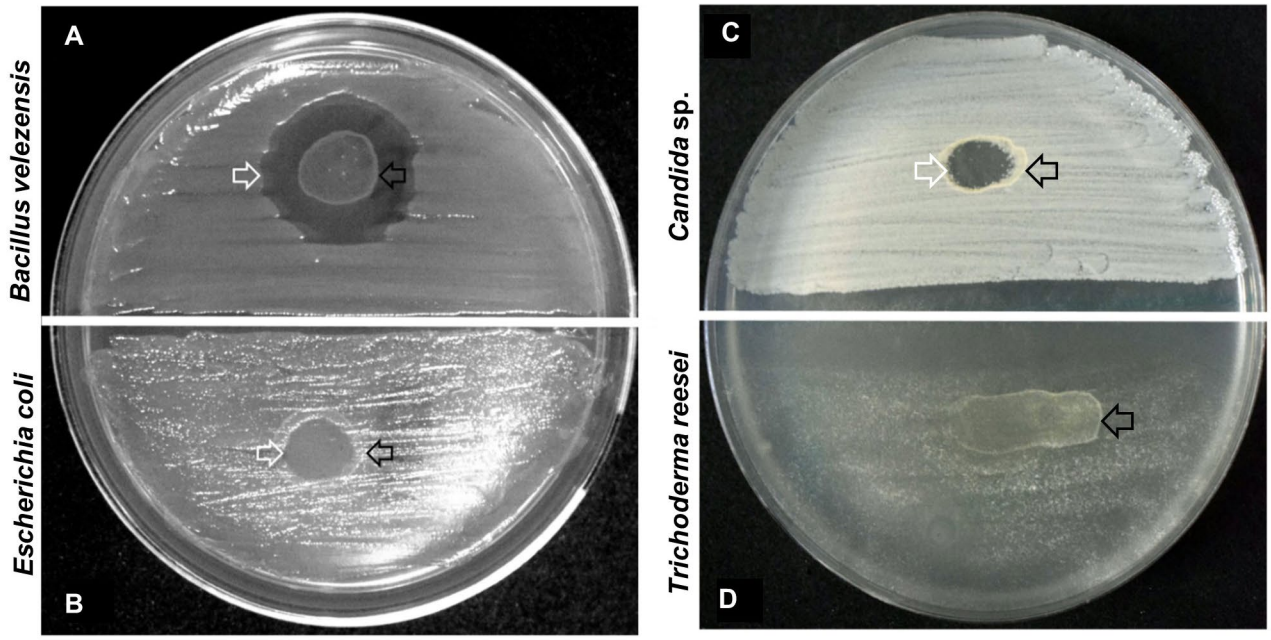

Fig. 3: Antimicrobial assays of the MGRC of *Colobopsis explodens*. The MGRC suspension was placed on the middle of the PDA plate inoculated with the respective microorganism and incubated for 24 h (A and B) and 48 h (C and D) at 28 - 30 °C. Black arrows show the spread of the MGRC suspension, white arrows indicate the edge of the clearance zone. (A) The growth of *B. velezensis* was inhibited by MGRC compounds that had diffused in the medium. The growth of *E. coli* (B) and *Candida* sp. (C) was only affected by MGRC compounds that remained on the application site. (D) The growth of *T. reesei* was not inhibited by the MGRC.

not result in the complete entanglement of the ant. Only the hind legs were affected (Video "Defense behavior of Cexplodens"). Due to the contact with the sticky secretion, the targeted ants were heavily restricted in their motility and in most cases were not able to use their mandibles for a counterattack. The latter was due to the positioning of the "exploded" ants' bodies, resulting in the opponent's inability to reach the *C. explodens* worker ant with its mandibles or because the mandibles were glued together by the MGRC (Video "Defense behavior of Cexplodens"). When in combat with much larger *Oecophylla smaragdina* workers (range of body lengths: 8 - 10 mm), the MGRC was dispersed on a limited area of the opponent's head only (Fig. 2B). Five of the seven *O. smaragdina* opponents died within the first 5 min after physical contact with the MGRC, but this was also the case in control confrontation experiments involving *O. smaragdina* and other (unidentified) non-*Colobopsis cylindrica* individuals. In 31 combat scenarios with ants belonging to other COCY species, only four opponents died within 5 min after physical contact with the MGRC. The remaining ants, although being glued to the dead *C. explodens* workers, reacted with movements of non-glued body parts upon touch by the experimenter, and had not died until 12 h after treatment. Interestingly, none of the COCY opponents with known ability to use autothysis (species "all red", "*near saundersi*" and "*saundersi*") ejected its MGRC by autothysis as counter reaction to the attack of *C. explodens*.

Lethality of the MGRC and MAPG and noreugenin on insects: When the MGRCs or the solutions containing either of the major phenolic compounds were applied onto the surrogate insects, neither *Acheta domesticus* individuals, nor *Atta sexdens* workers died within the observation period of 12 h. They were able to remove the MGRC as well as the compounds by self-grooming. In contrast, all deltamethrin-treated insects died within seven minutes of application of the insecticide.

Antimicrobial effect of the MGRC on model organisms: In this assay, we observed the inhibitory property of the MGRC that consisted of overlapping effects of different MGRC compounds. In case of *Bacillus velezensis* (Fig. 3A), the inhibitory effect was provided by at least two compounds: One diffused to a greater extent, but had less inhibitory activity, while the other diffused slightly less and either alone or in a mixture with the previous or other compounds, stopped the growth of this bacterium. Cells of *Escherichia coli* were not affected by any compounds diffusing in the medium, but were inhibited by those that remained on the application site (Fig. 3B). Similarly, a moderate fungicidal activity against *Candida* sp. was observed (Fig. 3C), while the growth of filamentous fungi was not influenced (Fig. 3D and Fig. S4). Since the results (double-halo, single-halo, or no halo around the application site) were comparable between the biological replicates of each species, only one representative picture per culture type was taken.

In-vitro antimicrobial assays: The tested concentration range covered the calculated biologically relevant concentration of 59 µg ± 5 µg of MAPG per 1 mg of fresh MGRC, correspondingly a concentration of 300 mg L^{-1}

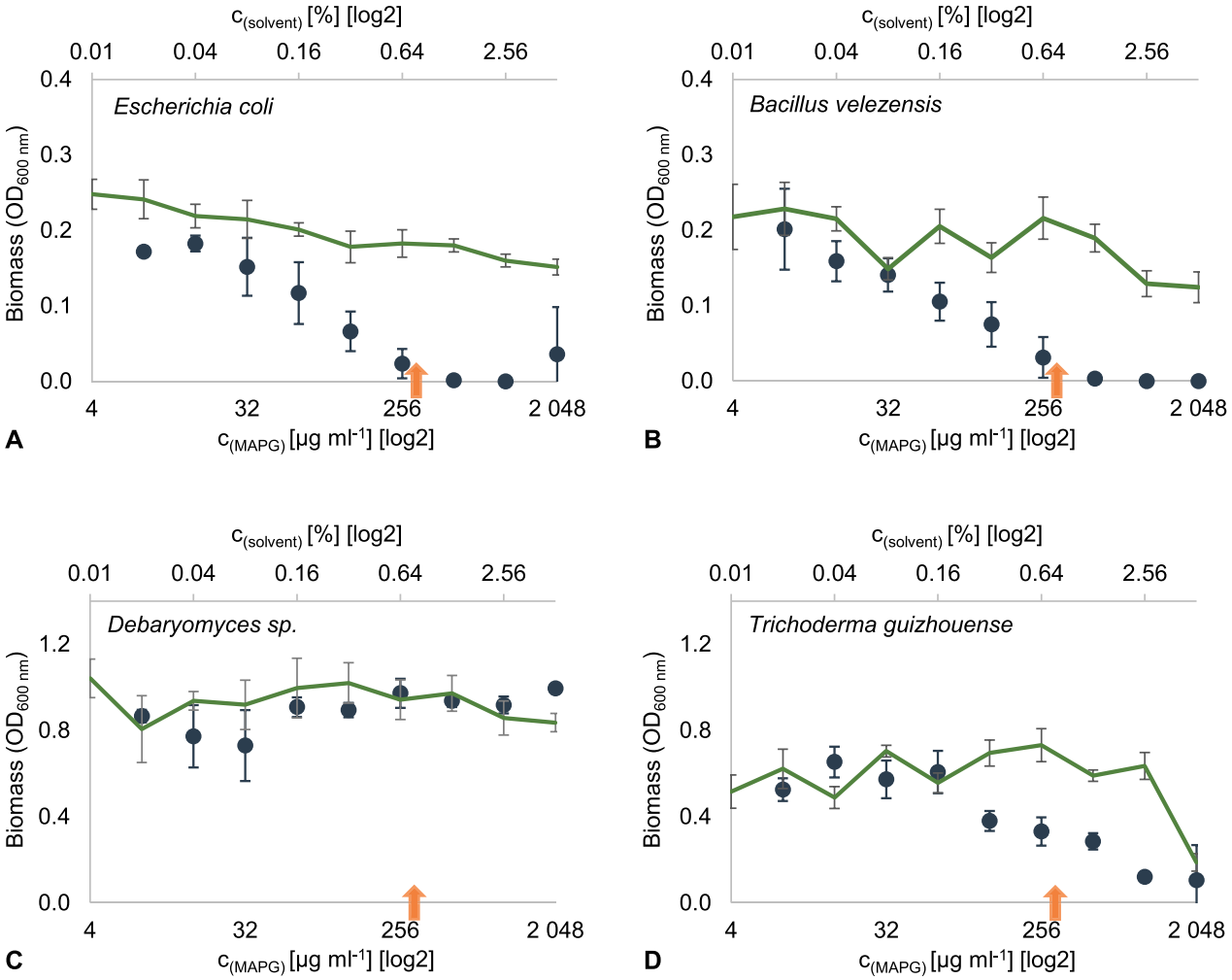

Fig. 4: Antimicrobial activity assay of MAPG against four model microorganisms: *E. coli* (A) and *B. velezensis* (B) after 24 h of incubation; *Debaryomyces* sp. (C) and *T. guizhouense* (D) after 66 h of incubation. Blue dots indicate growth of the respective microorganism at different concentrations of MAPG. Green line shows the growth of the respective microorganism in presence of the solvent only (control). The orange arrow indicates the estimated mean concentration of MAPG that would be present during the assay (300 µg mL^{-1}) based on the measured absolute concentration of this compound in the MGRC of *C. explodens*. Error bars depict standard deviations calculated from three replicates.

considering the assay volume of 200 µL. The in-vitro assays showed a strong inhibitory effect of MAPG on both types of bacteria tested (Fig. 4A and 4B), but no effect on *Debaryomyces* sp. (Fig. 4C). Effects on the growth of *Escherichia coli* were observed starting from a concentration of 32 mg L^{-1} of MAPG, being essentially below the estimated concentration of MAPG (Fig. 4A), whereas the latter inhibited bacterial growth almost completely (Fig. 4A). The growth of the filamentous fungus *Trichoderma guizhouense* was also reduced already starting at a concentration of 128 mg L^{-1} of MAPG (Fig. 4D). Similar assays with noreugenin showed that it had no antimicrobial activity on the tested microorganisms (Fig. S5).

Discussion

Multiple roles have been proposed for the MGRC of *Colobopsis explodens* minor workers. It may act (i) as glue that becomes sticky after being expelled (Maschwitz & Maschwitz 1974), (ii) as irritant causing repellence, deterrence or disorientation (Jones & al. 2004, Laciny & al. 2018), (iii) as pheromone blend (Jones & al. 2004, Voegtle & al. 2008), (iv) as antimicrobial agent (Davidson & al. 2009, Davidson & al. 2016), (vi) as warning coloration (Jones & al. 2004), and (vii) as food processing aid (Davidson & al. 2016). Since this study focused on the lethal effects of the MGR secretion on arthropods, as well as on its potentially antibacterial and antifungal activity, these two aspects will be discussed further.

MGRC in defense against arthropods: In the in-situ confrontations between *Colobopsis explodens* and other *Colobopsis cylindrica* (COCY) species group workers, acute lethality occurred in only a few rivals, suggesting that the secretion ejected by *C. explodens* does not necessarily kill a COCY enemy via deadly toxic compounds contained therein. Rather, the stickiness of the secretion seems to be responsible for rendering the opponents harmless.

Since for those confrontations, only COCY species were used, it cannot be excluded, that non-COCY species may be more susceptible to the MGRC compounds. The MGRCs of diverse COCY species show overlap of the same or similar chemical compounds (Jones & al. 2004, Davidson & al. 2016), which is why they may be less vulnerable to the, otherwise potentially lethal effects. In natural scenarios, the glued and thus immobilized rival, although still alive, would not be a threat to the *C. explodens* colony anymore and would eventually fall down to the forest floor, or be discarded by other *C. explodens* individuals (Davidson & al. 2016). Interestingly, none of the COCY opponents known to be able to use autothysis (species "all red", "*near saundersi*" and "*saundersi*") ejected their MGRC by autothysis as counter reaction to the attack of *C. explodens*. In contrast, the autothysis behavior for those species was observed, when the ants were grabbed with forceps (see figure 1 in Maschwitz & Maschwitz 1974 and Tab. S2). This indicates that the attack mechanism of *C. explodens* may contain a strategy that prevents the execution of autothysis in other, otherwise "explosive" ants. Since the proportion of *Oecophylla smaragdina* individuals, that died during the observation period was the same, when in combat with *C. explodens*, as in confrontations with non-COCY individuals, a lethal effect of the MGRC (based on both physical as well as chemical properties) against these large ants cannot be confirmed. Nevertheless, the experiments with the much larger *O. smaragdina* showed, that the effectiveness of the entangling mechanism may be dependent on the size of the rival. The amount of the ejected secretion is not sufficient to target larger areas of the rival's body and thus leaves it motile. Other effects, such as neurotoxicity or repellent effects of the secretion could not be assessed under the given experimental conditions. Therefore, it cannot be ruled out, that the secretion contains at least slightly toxic or repellent compounds. In contrast to the observed lasting stickiness of the naturally ejected secretion, the application of prepared MGRCs did not result in the prolonged entanglement of *Acheta domesticus* and *Atta sexdens* individuals in vitro. All surrogate insects were able to remove the MGRC, which is in accordance with reports by Maschwitz & Maschwitz (1974). For their studies, the cited authors applied prepared gland contents obtained from the COCY species "*saundersi*" onto individuals belonging to three different ant species, which were all able to remove it by grooming. Since the in-vitro experiments reported in the presented study were carried out at temperature and humidity similar to that in the natural habitat of *C. explodens*, we can exclude these ambient factors as cause for the observed difference in the glue characteristic. The sticky properties of the expelled secretion might be influenced by processes occurring during autothysis under natural conditions, such as enzymatic reactions or mixing of compounds originating from different glands as reported for the bombardier beetle (Carabidae) (Schildknecht & Holoubek 1961, Eisner & al. 1977). Such a multi-exocrine gland secretion cannot occur when isolated MGRCs are applied onto insects, which might be responsible for the contradictory results of the two experimental designs. To imitate the missing entangling effect during application of MAPG and noreugenin, the compounds were mixed with viscous CMC, before being applied onto *Acheta domesticus* individuals, but also in this setup, no lethal effects could be observed. In contrast, the deltamethrin treated insects died fast, which shows that toxic compounds can principally show their effects via the chosen mode of application. Due to the low available sample sizes, the experiments involving living COCY individuals or frozen *C. explodens* samples could not be repeated. The statements about the effect of the MGRC on arthropod rivals are thus based on observations and should be seen as basis for upcoming studies dealing with the role of the MGRC in COCY ants.

Antimicrobial activity of the MGRC: The in-situ antimicrobial assays with isolated MGRC indeed demonstrated the antimicrobial potential of the MGRC. The constituents obviously diffused into the medium, which resulted in a halo around the site of application lacking *Bacillus velezensis* culture. Cells of *Escherichia coli* and *Candida* sp. were affected in their growth only upon direct contact with the MGRC containing liquid, whereas growth of *Trichoderma reesei* was not affected. The choice for culturing *E. coli* and *B. velezensis* on a PDA medium, which is frequently used for fungi, was based on the conditions in the field, which did not allow the preparation of different media. Although the growth of the two bacterial strains on PDA medium was comparable to their growth on their specialized media, we cannot rule out that bacteria grown on this substrate may be more susceptible to antibiotics. Thus, we consider these results as complimentary to the results of the in-vitro assays, which demonstrated the selective antimicrobial activity of MAPG. For the conditions of antimicrobial assays, the differences in the *Trichoderma* strains used for in-situ (*T. reesei*) and in-vitro (*T. guizhouense*) assays can be neglected. Both strains are the established models for the genus *Trichoderma* investigated in our laboratory (Druzhinina & al. 2018, Kubicek & al. 2019) and have similar resistance to xenobiotic compounds. The choice of *T. guizhouense* for the in-vitro assay was determined by the more suitable optimal temperature and a reliable growth profile of this species. *Debaryomyces* sp. was tested as a representative of saccharomycetales yeasts and was only available for in-vitro assays. The antimicrobial activity of the MGRC might arise from the acidic and slightly acidic phenolic compounds reported from *Colobopsis cylindrica* (COCY) ants (Maschwitz & Maschwitz 1974, Jones & al. 2004, Davidson & al. 2016, Hoenigsberger & al. 2019). Acidic compounds such as carboxylic and phenolic acid moieties may be used to maintain colony health (Attygalle & al. 1989, Vander Meer 2012, Tragust & al. 2013, Guarda & Lutinski 2020), presumably by lowering of the pH value in pathogen-threatened locations as reviewed by Yek & Mueller (2011) for the metapleural gland secretions of fungus-growing ants. When nest walls obtained from different COCY species were analyzed for their pH, a value

of 4 was determined in all samples (Davidson & al. 2009), which suggests an acidic milieu in the nests. Interestingly, the pH values of the MGR secretions ejected by different COCY species ranged from about 2.5 to about 4, pointing to an acidic property of the secretions (Tab. S2 and S3). Since some COCY species can eject their MGRC via their mandibles and without eliciting autothysis (Maschwitz & Maschwitz 1974, Davidson & al. 2016 and Tab. S2) these acidic mixtures may be dispersed on the ants' bodies via self- or allogrooming or applied onto the nest walls to fight off potential pathogens (Yek & Mueller 2011). When the identified dominant phenolic constituents were applied singularly onto microbe cultures, MAPG exhibited profound antimicrobial activity on some of the cultures, indicating that this compound may be an active antimicrobial ingredient in the defensive secretion of *Colobopsis explodens* and thus may serve to modulate the ants' microbiome. In the antimicrobial assays performed in this study, no effect was observed for noreugenin, thus the role of the highly abundant phenolic pentaketide in the MGRC of *C. explodens* remains unclear and needs further evaluation.

With respect to defense against potential opponents, our confrontation experiments demonstrated that the stickiness of the secretion rather than deadly chemicals contained therein is likely to be responsible for warding off arthropod opponents. Since glandular hypertrophy and the unique chemistry of the MGRCs occur even in taxa that do not use the MGR products in autothysis, the role of the secretion in (territorial) defense is probably a secondary one (Davidson & al. 2016). Our results point to a selective antimicrobial activity of the secretion, which is beyond the currently supposed role of polyphenolic constituents in autothysis. Whether modulation of the microbe community constitutes a primary role of the MGRC may be revealed in future studies dealing with *Colobopsis cylindrica* ants and their associated microbiomes.

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