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Ant-produced chemicals are not responsible for the specificity of their *Ophiocordyceps* fungal pathogens

Baramee Sakolrak [a, b], Rumsaïs Blatrix [d, **], Uthaiwan Sangwanit [a, b], Nuntanat Arnamnart [c], Wasana Noisripoom [c], Donnaya Thanakitpipattana [c], Bruno Buatois [d], Martine Hossaert-McKey [d], Noppol Kobmoo [c, *]

[a] Department of Forest Biology, Faculty of Forestry, Kasetsart University, 50 Ngam Wong Wan Road, Lat Yao, Chatuchak, Bangkok 10900, Thailand
[b] Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Bangkok 10900, Thailand
[c] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Thailand Science Park, Phahonyothin Road, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand
[d] CEFE UMR 5175, CNRS, Université de Montpellier, Université Paul Valéry Montpellier, EPHE, 1919 route de Mende, 34293 Montpellier Cedex 5, France

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ABSTRACT

In this study, we investigated the effects of exocrine glandular extracts (mandibular glands, post-pharygeal glands) and cuticular extracts of two species of formicine ant *Polyrhachis furcata* and *Colobopsis saundersi* on spore germination of two species of host-specific, entomopathogenic fungi *Ophiocordyceps unilateralis* (*Ophiocordyceps polyrhachis-furcata* and *Ophiocordyceps camponoti-saundersi*). Extracts from the two ant species inhibited the germination of *O. polyrhachis-furcata*, but did not affect the germination of *O. camponoti-saundersi*, invalidating the hypothesis of specificity mediated by exocrine glands. We also showed that metapleural gland extracts—well known for their anti-microbial activities—from ants not targeted by *O. unilateralis*, inhibited spore germination. Finally, we tested the effect of the glands of the two formicine ants on *Beauveria bassiana*, a widespread pathogen of insects. Germination of *B. bassiana* was stimulated by most extracts, suggesting that insect chemicals are used as signals for germination by this fungus. The chemical characterisation of ant glandular extracts was also performed.

1. Introduction

The species complex *Ophiocordyceps unilateralis sensu lato* is a group of entomopathogenic fungi that is specialized on ants of the tribe Camponotini (Hymenoptera, Formicidae), in particular of the genera *Camponotus*, *Colobopsis*, *Dinomyrmex* and *Polyrhachis*, and constitutes the most common fungal pathogen of these insects. Infected ants leave their colony a few days after infection and fix themselves to the vegetation by biting a leaf or a twig, hanging upside down until death. The fungus then secures the ant's grip on the plant by growing mycelium at the tip of the legs that attaches to the plant. The fungus then produces a stalk from the back of the ant's head from which the reproductive structure (perithecium) develops and spores are then released and fall to the floor. Such manipulation of the host behaviour is believed to increase the developmental success of the stalk and the dispersal capacity of the spores (Andersen et al., 2009; Pontoppidan et al., 2009).

Recent studies revealed that each species within the *O. unilateralis* complex is specific to one ant species, suggesting host-driven speciation (Kobmoo et al., 2012; 2015). Patterns of specificity in other associations between fungi and ants have mostly been investigated in fungus-growing attine ants (Poulsen and Boomsma, 2005; Mikheyev et al., 2010; Mueller et al., 2010) and ant-plant symbioses (Blatrix et al., 2013; Nepel et al., 2016;

* Corresponding author.
** Corresponding author.
E-mail addresses: Rumsais.BLATRIX@cefe.cnrs.fr (R. Blatrix), noppol.kob@biotec.or.th (N. Kobmoo).

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Vasse et al., 2017). Previous studies on *O. unilateralis s.l.* focused on species diversity and taxonomy (Evans et al., 2011; Luangsa-Ard et al., 2011; Kobmoo et al., 2012, 2015), the ecological factors of epidemics on ants (Pontoppidan et al., 2009; Mongkolsamrit et al. 2012) and the mechanisms of ant-behaviour manipulation (de Bekker et al., 2014; 2015) but the proximal mechanisms of the arms-race between *O. unilateralis* species and the ants are yet to be elucidated.

The first step in the infection of insects by entomopathogenic fungi is the germination of spores on the cuticle. Hydrolytic enzymes of entomopathogenic fungi necessary to the degradation of insect cuticle were shown to be virulence factors (Valero-Jiménez et al., 2016). The degradation of cuticle also brings an initial nutritive ration to the growth and virulence of entomopathogenic fungi (Shah et al. 2005). Ants develop social behaviour to protect themselves against pathogens including allogrooming (Ugelvig and Cremer, 2007). Cuticle seems, therefore, to be the place where reciprocal selection leading to the host-pathogen arms race takes place. Ants have numerous exocrine glands that are involved in various activities such as reproduction, social communication and defence against predators and pathogens (Hölldobler and Wilson, 1990; Billen and Morgan, 1998; Morgan, 2009; Yek and Mueller, 2011). At least six exocrine glands, the mandibular, metapleural, pygidial, poison, Dufour's and sternal glands, are known to play a role in defence against pathogens (Hölldobler and Wilson, 1990; Fernández-Marín et al., 2006). Thus, chemical interaction at the cuticle level is a likely mechanism for explaining fungus-ant specificity, but this still remains to be investigated.

The cuticle of ants harbours various aliphatic hydrocarbons that are used for nestmate discrimination (Lahav et al., 1999; Blomquist and Bagnères, 2010). Those compounds, produced in the fat body, are concentrated in the postpharyngeal gland and spread on the cuticle (Soroker and Hefetz, 2000) through grooming activities. Other compounds produced from other exocrine glands are also spread on the cuticle. The secretion from the metapleural gland, in particular, is spread over the body and confers protection against microbes (Yek and Mueller, 2011). More anecdotally, the content of the mandibular gland can reach the cuticle and serve in nestmate discrimination (Sainz-Borgo et al., 2013). The postpharyngeal gland, the metapleural gland and the mandibular gland are the most likely source candidates for the chemical cocktail at the surface of the cuticle and, thus, should be targeted in priority when looking for potential effects of ant chemicals on spore germination of entomopathogenic fungi. Interestingly, most ant species of the tribe Camponotini lack a metapleural gland (Yek and Mueller, 2011) and the question of how other glands of these ants contribute to the protection against generalist or specialist pathogenic fungi has rarely been investigated (Voegtle et al., 2008). The source of chemicals that might potentially inhibit growth of *O. unilateralis s.l.* fungi and other potential entomopathogenic fungi is reduced to the postpharyngeal and mandibular glands. Several ant species that are hosts of these fungi belong to the so-called “exploding ants” of the genus *Colobopsis*, having mandibular glands with a hypertrophied reservoir (Davidson et al., 2012) used in territorial fights against other ants. The explosion of the reservoir (Maschwitz and Maschwitz, 1974), and subsequent release of noxious and sticky compounds from the gland (Jones et al., 2004) lead to the death of the opponent and the ants that try to rescue it. An antifungal role of these compounds is not excluded (Voegtle et al., 2008; Davidson et al., 2009).

The aim of this study was to test the hypothesis that ant-produced chemicals are responsible for the host specificity of *O. unilateralis s.l.* fungi. The two fungal species *Ophiocordyceps camponoti-saundersi* and *Ophiocordyceps polyrhachis-furcata* are specific pathogens of the ant species *Colobopsis saundersi* (an exploding ant) and *Polyrhachis furcata* respectively (Kobmoo et al., 2012). Although the two fungi and their hosts can be found in the same environments, cross-infection has never been detected in the field. Cross infection through direct injection in the laboratory showed that *O. unilateralis* species can kill unspecific ants but never induce behaviour manipulation (de Bekker et al., 2014). We tested the effect of cuticular extracts and extracts of mandibular and postpharyngeal glands of the two host ant species on the germination of spores of each of the two *Ophiocordyceps* species. Under the hypothesis of a specificity driven by ant chemicals, we expected that spore germination would be inhibited by chemicals of the non-host species, but not by chemicals of the host species. At a broader taxonomical scale, as only formicine ants are targeted by *O. unilateralis*, we tested whether the metapleural glands of *Dolichoderus* sp. and *Pheidole* sp., which can be found in the same habitat as both *O. unilateralis* species, could inhibit the germination of this fungus. Finally, we also tested the effect of the ant extracts on the germination of spores of *Beauveria bassiana*, with the expectation that they would have little or no inhibitory effect on this generalist entomopathogenic fungus. We identified the chemical content of the ant-extracts to strengthen our interpretation of the patterns of the spore germination experiments.

2. Materials and methods

2.1. Chemical extracts from ants

Colonies of *P. furcata*, *C. saundersi*, *Pheidole* sp. and *Dolichoderus* sp. were collected in Khao Yai National Park, Thailand. Ants were killed by freezing and kept at −20 °C until use. Glandular extracts were obtained by dissecting ants in distilled water under a stereomicroscope and placing the appropriate gland in solvent (10 glands in 500 μl). To increase the range of compounds extracted, two solvents were used separately: pentane and a mix of dichloromethane + methanol (9:1 by volume). The mandibular gland and the postpharyngeal gland were dissected from *P. furcata* and *C. saundersi*. The metapleural gland was dissected from *Pheidole* sp. and *Dolichoderus* sp. The cuticular extracts of *P. furcata* and *C. saundersi* were obtained by soaking 10 individual ants in 500 μl of solvent for 10 min. The extracts were kept at −20 °C.

2.2. Germination tests with spores of Ophiocordyceps spp.

Specimens of the two focal fungal species, *O. camponoti-saundersi* and *O. polyrhachis-furcata*, at the fruiting stage were collected in Khao Yai National Park. Spore isolation was done within 2–6 h after collecting. Each specimen was fixed to the lid of a Petri dish using vaseline or tape. The lid was placed over the dish filled with potato dextrose agar (PDA) so that released spores fell on the PDA. Plates were kept in a moist chamber at 20 °C for 2–3 d. The presence of spores on the PDA was checked every 6 h. The timing of spore discharge is very narrow (6–12 h) in these species. Consequently, out of 30 specimens of each species collected in the field, only 12 of *O. camponoti-saundersi* and 15 of *O. polyrhachis-furcata* could be used in this experiment.

To test for the effect of extracts on spore germination, Petri dishes were prepared by dropping 50 μl of extract in pentane +50 μl of extract in dichloromethane-methanol on the solid culture medium (PDA), i.e. two glands equivalent, and letting the solvent evaporate for at least 5 min. Extracts naturally spread over the surface of the PDA, forming a disc of *c.* 2 cm in diameter on which 10 single spores from a single fungal specimen were transferred manually under a stereomicroscope. The number of germinated spores was recorded every day for 5 d. Spore germination was tested for each fungus specimen, for each of the following conditions: untreated PDA (negative control), 50 μl of